2016 TAIPEI

HUPO

15th Human Proteome Organization World Congress

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Precision Proteomics for Precision Biology and Medicine

CONGRESS ABSTRACT BOOK

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PLENARY SESSIONS

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PL-01

Precision Medicine: iPOP and hPOP profiling to Manage Health and Disease and Understand Human Variation

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Understanding health and disease requires a detailed analysis of both our DNA and the molecular events that determine human physiology. We performed an integrated Personal Omics Profiling (iPOP) of 100 healthy and prediabetic human subjects over three years including periods of viral infection as well as during controlled weight gain and loss. Our iPOP integrates multiomics information from the host (genomics, epigenomics, transcriptomics, proteomics and metabolomics) and from the gut microbiome. Longitudinal multiomics profiling reveals extensive dynamic biomolecular changes occur during times of perturbation, and the different perturbations have distinct effects on different biological pathways. Overall, our results demonstrate a global and system-wide level of biochemical and cellular changes occur during environment exposures and omics profiling can be used to manage health. In parallel with the iPOP study we have launched the hPOP study, a HUPO/HPP project to understand human variation of omics profiles. Participants at HUPO affiliated meeting will be profile to follow molecular variation across the planet. Preliminary results from 31 individuals profiled at the March USHUPO meeting will be presented.

PL-02

Precision Medicine for Lung Cancer: Current and Beyond

Pan-Chyr Yang

National Taiwan University

Precision medicine is an emerging new era for future health care. It has become feasible because of the recent advances in genome sequencing and panomics technologies as well as the application of large-scale biologic databases to characterize patients identify biomarkers and guide clinical practice. It has significantly improved treatment outcome of human diseases especially in cancers. Here we use lung cancer as an example to show that how we implement precision medicine to improve the patients' treatment outcome. Lung cancer is the leading cause of cancer mortality worldwide. Since the identification of EGFR activating mutation in 2004 and the discovery of specific targeting agents, the treatment of lung cancer has entering a new era of precision therapy. The mutated EGFR may function as an oncogenic driver in more than 50% of Asian and 10-15% of Caucasian lung adenocarcinomas. Patients harboring activating EGFR mutants, most commonly L858R or Ex19Del, usually present good initial responses to EGFR-TKIs, but eventually develop disease progression after a median 12 months. Acquired T790M mutation accounts for 60% of these resistant cases. Other resistant mechanisms include the activation of alternative oncogenic pathways or small cell transformation. There are several strategies to overcome EGFR-TKIs resistance including a switch to chemotherapy; turn off the compensatory oncogenic pathways; dual inhibition with anti-EGFR antibodies and TKIs; development of new generation EGFR TKIs (AZD9291, CO-1686); and knockdown of EGFR expression by siRNA or specific T790M DNAzyme. Promising new approaches include immune checkpoint inhibition by anti-PD-1/anti-PD-L1 antibodies and combination immunotherapy with targeting agents or chemotherapy. Cancer stem cell (CSC) and tumor microenvironment directed therapy is another alternative aiming to overcome drug resistance, recurrence and metastasis. We have developed aptamer based immune checkpoint inhibitors against PD-1, PD-L1 and CTLA4, which showed great potential for development of cancer immunotherapy. We have developed CSC culture and drug screening platform, which allow us to identify compounds against CSC and cancer microenvironment. The recent study by Lung Cancer Mutation Consortium confirmed that multiplexed gene testing is feasible for precision lung cancer therapy. Taiwan established national reference laboratory to provide standardized gene-testing platform for lung cancer patients. Taiwan National Health Insurance reimbursed Gefitnib and Erlotinib for first line therapy of EGFR mutant lung cancer patients since 2011. With the implementation of nation-wide gene testing and precision therapy, the overall 5-year survival for NSCLC has improved from 16% to 32% since 2014. The precision medicine has significantly improved the treatment outcome for NSCLC. We also identified 5 specific susceptibility loci (*TERT*, *TP63*, *HLA Class II*, *POS1-DCBLD1* and *VTI1A*) in never-smoking women in Asia and *YAP1 mutation as a risk for Chinese familial lung cancer*, which may be useful to identify high risk population for LDCT screening of lung cancer patients.

PL-03

The Proteome in Context

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Recent advances in various bottom-up proteomic techniques have resulted in significant advances towards the confident and routine identification and quantification of proteins. Data dependent acquisition (DDA) has resulted in saturation coverage of proteomes of a number of species, and the generation of near complete spectral libraries has paved the way to reliably identify essentially any protein of a number of proteomes by targeted analysis via SRM or SWATH-MS (1). In both – DDA and targeted strategies – each polypeptide is considered as an independent query unit. However, this is not how proteins function in the cell. Most polypeptides carry out their biological function in the context of macromolecular assemblies, including protein complexes, protein-nucleic acid complexes and functional interaction networks.

In this presentation, we will discuss emerging computational and laboratory techniques to determine functional context of proteins in the cell. These include the direct measurement of molecular interfaces in macromolecular complexes by chemical cross-linking MS (XL-MS) (2), the integration of XL-MS data, and structural data generated by cryo-EM single particle analysis to obtain structural models and correlative analyses of proteins precisely quantified by SWATH-MS across large sample cohorts to determine changes in complex stoichiometry (3,4).

We will use selected examples to illustrate the biological significance of measuring or inferring proteins in the context of functional modules

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PL-04

ProteoGenomic Analysis of Cancer: New Opportunities in Cancer Biology and Precision Medicine

Henry Rodriguez

The ability to interrogate cancer at the proteogenomic level (interplay between the genome -DNA, RNA; and proteome - protein) will transform oncology care from one that relies mainly on trial-and-error treatment strategies based on the anatomy of the tumor, to one that is more precisely based on the tumor's molecular profile. Spurred by the U.S. National Cancer Institute, genomics has launched a revolution in precision oncology medicine by identifying targetable mutations in cancers. Unfortunately, there is still key missing biology when trying to reliably predict which patients' tumors respond to any given therapy. However, optimism is because today we know that molecular drivers of cancer are likely derived not just from DNA alterations alone, but from protein expression and activity at the cellular pathway level - proteomics. Understanding this molecular interplay and publicly releasing proteogenomic data sets to create community resources is anticipated to accelerate our understanding of cancer and its treatment. Therefore, a critical next step in the evolution of precision medicine is to continue to study what we are just learning in proteogenomics to identify the gene mutations/alterations and protein pathways in a tumor which drive cancer growth that can be vital to selecting targeted therapies more precisely for cancer patients. This seminar will discuss how genomics, transcriptomics, and proteomics must all be brought together in the quest to understand the etiology of cancer, in addition to highlighting efforts by the U.S. National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium (CPTAC) program in this area of biomedical research.

PL-05

The Proteomic Landscape of Gastric Cancer of the Diffuse Type

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Proteomics research in China has undergone phenomenal growth in the last decade. With recent construction of the Phoenix Center (aka, National Center for Protein Science (Beijing)), proteomics in China has entered a phase of “production”. The Phoenix Center focuses on the task of decoding the proteome with spatiotemporal resolution and supporting protein sciences in the nation and worldwide, providing protein measurement, data analysis and knowledge mining in a centralized fashion. We will describe the current capacity of the Phoenix Center.

We will also describe proteomic and targeted DNA sequencing analyses of 83 gastric cancers (GCs) of the diffuse type. We measured both the cancer and para-cancer tissues from the same patient. We were able to stratify GC patients into 3 major subtypes based on protein profiling alone. These three types have distinct proteome features and clinical outcomes. Type I (PX1) exhibits proteome stability, type II (PX2) dominates with DNA replication, and type III (PX3) hints with immune response. The genome annotation has revealed few mutations in PX1 and higher mutant frequencies in PX3. The three subtypes showed apparent differences in the overall operational survival rate. PX1 processes the best survival, while PX3 has the worst survival. PX2 are more sensitive to chemotherapy than the other subtypes. We were able to narrow down to 4 biomarkers (proteins) that were sufficient to stratify GC patients into the three subtypes, openning the door for clinical applications and interventions based on the three proteome subtypes. We will also describe the altered signaling pathways in GC and the relationship between DNA mutations and protein pathways changes.

PL-06

Glycoproteomics – A Genetic Approach to Deconstruction and Simplification of Protein Glycosylation

Henrik Clausen, Katrine Schjoldager, Zhang Yang, Yoshiki Narimatsu, Hiren Joshi, Shenjun Wang, Catharina Steentoft, Adnan Halim, Eric Bennett, and Sergey Vakhrushev.

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Glycosylation is one of the most abundant and diverse posttranslational modifications of proteins, but arguably also one of the most difficult to study with respect to occurrence, structure, and biological functions in particular at the proteome level. Heterogeneity in occupancy and structures of glycans on proteins is the major obstacle for analytic strategies, and this is also the major obstacle for exploiting biological functions of glycans in biotechnology. Protein glycosylation is a complex process involving many glycosyltransferases and even more accessory enzymes and transporters, and the non-template driven process is directed by multiple factors such as substrate specificities, kinetic properties, and topology. However, the general biosynthetic pathways and roles of most of these 500+ genes are fairly well outlined. We have therefore taken a genetic deconstruction & reconstruction approach to analysis and exploitation of protein glycosylation. We use the gene editing tools (DNA nuclease "scissors" ZFNs, TALENs, CRISPR/Cas9) to simplify[1], dissect[2], and design[3] glycosylation more or less at will. The strategy has enabled detailed mapping of different glycoproteomes as well as discovery of e.g. nucleocytoplasmic O-mannosylation in yeast[4]. Moreover, genetic dissection of glycosylation in cells enables us to explore molecular mechanisms of biological functions and diseases caused by specific glycans at specific sites in proteins. Finally, we are entering an era where custom designed glycosylation of glycoprotein therapeutics is possible. We will present illustrative examples of the power of gene editing in the glycosylation field that is rapidly turning this complex modification into an exercise of "Lego-Toying".

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PL-07

Complementary Methods for Probing Protein Assemblies and Interactions

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Mass Spectrometry based proteomics has played a pivotal role in revealing the plethora of protein interactions that take place inside a cell, wherein proteins form protein assemblies and/or signalling networks. Especially using affinity purification of a tagged proteins followed by mass spectrometric analysis of its binding partners a wealth of data has been gathered revealing the all-embracing protein networks present in cells. Following the charting of all these interactions, a next step will be to now gather more in-depth structural and functional information on these individual protein assemblies. This may come from in-depth high-resolution structural models, as well as detailed information on how they function and dynamically evolve during cellular perturbations. Mass spectrometry may also contribute to this next level of protein interaction analysis although it does require partly different and novel approaches. To contribute to this emerging new area in proteomics, our group is developing new methods using native mass spectrometry and crosslinking mass spectrometry with the aim to bridge the gap between interaction proteomics and structural biology. These new innovations and applications of them in interaction proteomics will be central in this presentation.

In the first part of the talk native mass spectrometry and its applications in probing protein assemblies and interactions will be described, focusing on examples wherein the dynamic assembly of a protein complex involved in the circadian timing in cyanobacteria will be highlighted. Herein, by using a combination of native, HD exchnage and cross-linking mass spectrometry and cryoEM, we were able to define a novel structural model improving our understanding of the circadian rhythm. Additionally, novel developments in MS instrumentation for native MS will be highlighted, especially a new Orbitrap based instrument that offers high-sensitivity and mass resolution, allowing an in-depth detailed analysis of glycoproteins, viruses and even whole intact ribosomes.

The second part of the talk will highlight our recent work on cross-linking mass spectrometry. Cross-linking combined with mass spectrometry (XL-MS) provides another powerful approach to probe the structure and interaction profile of protein assemblies. Up to now XL-MS has been primarily limited to the characterization of purified protein assemblies. We have set out to develop XL-MS methods aimed at probing protein interactions at the proteome level, using complete cell lysates or whole organelles as starting material. We, therefore, combined several novel innovative methods to address some of the hurdles in this field. These innovation include the use of a low energy CID cleavable cross-linker, novel hybrid pepetide fragmentation and acquisition strategies and a dedicated software suite, termed XlinkX. We applied this novel XL-MS strategy to lysates of E. coli lysate and human HeLa cell lines, and to mammalian mitochondria and nuclei. In each of these studies we successfully identified thousands of cross-links. Many of the identified cross-links could be validated by mapping them on available high-resolution structures, but the data also provide information on assemblies for which no high-resolution structures are available, and even reveal new protein interaction networks.

PL-08

Precision Medicine: Proteomics in the Context of Health Assessment and Systems Biology

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Precision medicine requires success in two intertwined aspects: precision therapy and personalized biomarkers. *Precision therapy* is being able to effectively treat the right disease; to have therapies that target for the correct pathological pathways. Our underlying premise is that an individual's baseline proteome reflects their past and present and thus, will dictate their future health and disease. Thus, the crux of precision medicine will be the identification and precise quantification of proteins and their modified forms. One example is the use of proteomics to "fix" drugs targeting protein kinase G. Currently, Sildenafil (Viagra™) a protein kinase G activator has continuously under performed in a numerous heart failure clinical trials. Our work has identified new class of drugs that are impacted by the status of the patient's proteome. Clinical trails of these drugs alone and in combination are to start enrolling in 2017. *Personalized biomarkers* requires diagnosing and risk stratifying an individual's disease status based on accurate assessment their complex health and pathological status. Our studies on 100s of individual's samples with different cardiovascular diseases have provided key insights on the effect of biological and pathological variability. This work has led us to consider the need for continuous patent-centric health screening at the population level. We will share our work on developing technical pipelines for health screening that reduces the barriers around sex, age and economic status, opening the route for *equitable health care*.

KEYNOTE SESSIONS

2016 TAIPEI HUPO

MK-01

immunoMALDI (iMALDI) for Quantifying the Expression and Phosphorylation Level of Cancer Related Proteins for Personalized Medicine

Christoph H. Borchers[1,2], Robert Popp[1], Adriana Aguilar-Mahecha[2], Oliver Pötz[3], Mark Basik[2], Gerald Batist[2]

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Introduction and Objectives

Aberrations of cell signaling pathways play a critical role in cancer development. Novel therapeutic agents target members of these pathways, but only work in a minority of patients. Our objective, therefore, was to develop immuno-Matrix Assisted Laser Desorption/Ionization (iMALDI) assays to measure PI3K/AKT pathway activity by accurately and precisely quantitating key phosphopeptides from the proteins AKT1 (P31749) and AKT2 (P31751) in cancer tissues, for use as predictive biomarkers for targeted therapies. Our phosphatase-based phosphopeptide quantitation (PPQ) approach was used for accurate quantitation of these phosphorylated peptides.

Methods

After tryptic digestion of tissue lysate (10 µg protein/replicate), a stable isotope-labeled standard (SIS) peptide corresponding to the target peptide was added as an internal standard. The solution was split into two aliquots, one of which was treated with alkaline phosphatase (the PPQ approach). The non-phosphorylated target peptides from both aliquots were then captured by anti-target peptide antibodies coupled to magnetic Protein G Dynabeads. The beads were washed and spotted directly onto a MALDI target. Addition of the acidic HCCA-MALDI matrix eluted the captured peptides from the beads, and a Bruker Microflex LRF MALDI-TOF instrument was used for the absolute quantitation of non-phosphorylated target peptide in both aliquots. This allowed determination of the degree of phosphorylation (stoichiometry) of the target phosphopeptides in the sample, by difference.

Results and Discussion

Target peptides were quantified from colon cancer (SW480, HCT116) and breast cancer (MDA-231) cell lines, as well as fresh-frozen and formalin-fixed paraffin-embedded (FFPE) breast cancer, and HCT116 mouse xenograft colon cancer tissues. Per sample, ~50 µg protein per sample are required (10 µg protein per

MK-01

capture). Target peptide levels ranged from ~ 0.1-1 fmol/µg protein lysate with phosphorylation levels of 0-50%. The lower limit of detection was 100 amol of peptide, with a linear range of 0.5-10 fmol of peptide on the MALDI plate. CVs for iMALDI assays were consistently <10%. All liquid handling steps were automated and performed on an Agilent Bravo liquid handling robot.

Conclusions

Automated iMALDI assays were developed for quantifying phosphorylated and non-phosphorylated AKT1 and AKT2 in cancer patient samples.

Keywords

AKT, Cancer, iMALDI (immunoMALDI), Phosphorylation, Mass Spectrometry, Personalized Medicine

MK-02

Verification of Oral Cancer Biomarkers and Their Translation to Clinical Settings

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Introduction and Objectives

Oral squamous cell carcinoma (OSCC) represents a serious and growing problem in many parts of the globe, including Taiwan. Most cases of OSCC develop from visible oral potentially malignant disorders (OPMDs), which exhibit heterogeneous subtypes with different transforming potentials. Such complexity and uncertainty complicate the early detection of OSCC during routine visual oral cancer screenings. Thus, we urgently need new approaches that will enable the early detection of OSCC, such as the development of saliva protein biomarkers. Although >1400 published studies have searched for biomarkers for head/neck cancers, including OSCC, few reported biomarkers have moved into clinical practice.

Methods

In discovery phase, we analyzed the differentially expressed proteome between microdissected OSCC tumor cells and their adjacent nontumor epithelia by a 16O/18O-based quantitative proteomics approach, and identified proteins that are secreted/released by OSCC cell lines and also highly expressed in OSCC tissue specimens. In verification phase, we identified and prioritized OSCC biomarkers reported in the literature (between 1995 and 2012) and/or through in-house studies. We then developed quantitative assays for these targets using multiple reaction monitoring-mass spectrometry (MRM-MS) technology, assayed these targets in cases and controls (from Taiwan's Oral Cancer Screening Program), and used statistical analysis to identify a panel of proteins that can effectively discriminate OSCC from healthy controls/OPMD subjects. The cilinical utility of this identified protein panel will be futher assessed in saliva samples propectively collected in the same screening program in validation phase.

Results and Discussion

We identified hundreds of proteins as potential OSCC biomarkers from the literature and/or through in-house studies. Forty-nine proteins that have high potential to be detected in saliva were selected for MRM-MS assay development and quantified in

saliva samples collected from ~500 subjects. Statistical analyses of the quantification data are in progress.

Conclusions

Verification of reported candidate OSCC biomarkers in saliva samples by targeted mass spectrometry will facilitate the development of marker panel(s) useful in real clinical settings.

Keywords

Saliva Protein Biomarkers, OSCC, OPMD, MRM-MS

MK-03

Comprehensive Analysis of Human Proteomes with Similar Depth as RNA-Seq

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Introduction and Objectives

Truly comprehensive proteome analysis is the Holy Grail in proteomics for enabling routine systems biology analyses and biomarker discoveries. Despite decades of tremendous developments in the proteomics technologies, complete proteome characterization of human cells have remained a formidable task. This is mainly due to the high dynamic range of a human cell proteome spanning at least seven orders of magnitude. To overcome this challenge and routinely perform comprehensive analyses of human proteomes novel technologies are needed.

Methods

Here we developed multi-shot proteomics, an optimized approach for comprehensive proteome analysis with high sample capacity based on offline peptide high pH reversed-phase chromatography collecting high numbers of fractions in combination with short LC-MS/MS gradients and high peptide sequencing speed on a Q-Exactive HF mass spectrometer.

Results and Discussion

Our technology has been streamlined for one day mass spectrometric measurements of human proteomes from cells and tissues. Our optimized multi-shot proteomics workflow is based on high-resolution offline high-pH reversed-phase peptide fractionation with fast sampling. Each peptide fraction is analyzed by rapid peptide sequencing using orbitrap tandem MS with short online chromatographic gradients allowing just one day per replicate proteome analysis. This strategy enables deep analysis of the HeLa proteome of close to 600,000 unique peptides covering more than 12,000 protein-coding genes comparable in coverage to next-generation RNA-Seq data. Searching our dataset for major post-translational modifications (PTMs) identified 10,500 phosphorylation sites and determined their stoichiometry without the need for specific enrichment methods. We further demonstrate the general applicability and clinical potential of this proteomics strategy by comprehensively quantifying global proteome expression in several different human cancer cell lines and tissue samples from patient biopsies.

Conclusions

One day comprehensive analysis of human proteomes with similar depth as next-generation RNA-Seq technologies and simultaneous deep coverage of major post-translational modifications without the need for specific enrichment strategies.

MK-03

Keywords
Human proteome
HeLa
Orbitrap
Deep sequencing
High pH reversed-phase chromatography
PTM
Phosphorylation
LC-MS/MS

MK-04

Comprehensive DIA with High Precursor Selectivity: How Can We Have Our Cake and Eat It Too?

Michael J. MacCoss[1], Jarrett Egertson[1], Austin Keller[2], Sonia Ting[1], Brian Searle[1], Brendan MacLean[1], Gennifer Merrihew[1], Lindsay Pino[1], and Richard Johnson[1]

[1]University of Washington, Department of Genome Sciences, [2]University of Washington, Department of Chemistry

Introduction and Objectives

Proteomics technology has improved dramatically over the last decade. The technology developments have largely been directed around instrument hardware, where instruments have been developed that scan faster, are more sensitive, and have greater mass measurement accuracy. However, the basic workflow has remained largely unchanged -- mass spectrometers are directed toward the acquisition of tandem mass spectra on the most abundant molecular species eluting from a chromatography column. More recently, efforts have been focused on the acquisition of mass spectrometry data on target peptides of interest. With improvements in instrument hardware and instrument control software, the practical experimental difference between a targeted and discovery proteomics is beginning to become blurred. These analyses are a significant change from the traditional proteomics workflow and have required the development of novel computational strategies to analyze, visualize, and interpret these data. We will present work illustrating our efforts in the development of targeted proteomics and provide a vision for challenges that still need to be overcome before these analyses become routine and replace more traditional discovery proteomics methodology.

Results and Discussion

We know that precursor selectivity is arguably the most important factor in defining the selectivity, sensitivity, and dynamic range for targeted peptide quantification. However, the latest advancements in data independent acquisition has sacrificed precursor selectivity for comprehensiveness. In this presentation, we will demonstrate that it is possible to be selective, sensitive, and comprehensive at the same time. We have developed new methods for acquiring tandem mass spectrometry data and demultiplexing them to improve the precursor selectivity using principles of compressed sensing.

Conclusions

Comprehensive data independent acquisition using narrow precursor isolation windows on a chromatographic time-scale.

Keywords

Quantitation, data independent acquisition, dynamic range, informatics

MK-05

Spatially-Resolved Proteomic Mapping in Living Cells via Enzyme-Mediated Proximity Labeling

Alice Ting

Stanford University, USA

(Abstract not available as of September 9, 2016)

MK-06

Drug Resistance Assessed by Multi-Proteomics Approaches

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Introduction and Objectives

Constitutive activity of kinases is known to be crucial for a tumor to maintain its malignant phenotype, a phenomenon which is often referred to as oncogene addiction. Small molecule inhibitors are therefore attractive therapeutics as they target these kinases that are critical for tumor cell survival and proliferation. Several small molecule inhibitors have been proven successful in the clinic, but despite initial success most cancers eventually develop resistance against these drugs. Resistance to drugs, both intrinsic and acquired is a major problem and is believed to be the major cause of failure of drug treatment.

Methods

We used different cancer cell lines and drug treatments and applied a combination of mass-spectrometry based proteomics, chemoproteomics, phosphoproteomics and metabolomics in an effort to globally asses the molecular consequences of small molecule inhibitor treatment and resistance. We analyzed the changes in proteome and phosphoproteome in a temporal manner, ranging from hours to weeks after drug treatment. Novel, potential pharmacologically actionable resistance targets were validated by chemical inhibition and/or functional knock down. The effect of inhibition/knock down was assessed at a molecular level using targeted proteomics.

Results and Discussion

We obtained deep insight into cellular reprogramming upon resistance. Our results indicate that immediately after drug treatment a large proportion of cells rewire signaling in order to evade drug treatment. Importantly, the approach readily confirms and extends previously described mechanism of resistance. In addition our data suggest a great heterogeneity and context specific functionality of resistance drivers. Based on data from other studies and from our analysis, there might be great merit in searching for unifying features of resistance rather than solely relying on the identification of potentially interchangeable molecular events which might converge on the same molecule, pathway or phenotype. In addition, both proteome and phoshoproteome data confirm an increased addiction to anaerobic glycolysis. In contrast to previous, expression based mechanisms, this study uncovers a phosphorylation mediated reprogramming of metabolic enzyme activity which increases the sensitivity of the resistant cells to glycolysis inhibition. As glucose addiction can occur via multiple different routes, this phenotype might potentially represent a common and targetable convergence point.

Conclusion

Our findings demonstrate how multi-proteomics contribute to a better understanding of the molecular pathways underlying drug resistance. In addition we show that multiple resistance mechanisms converge on the same pathway, which could potentially be used

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as an universal entry point for targeting resistance.

Keywords: phosphoprotoemics, kinome, resistance, cancer, multi-omics analyses, signaling

MK-07

Single Cell Metabolomics and Applications

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Small molecules, such as metabolites and lipids in a live single cell are now rapidly detected by our method, called “Live Single Cell Mass Spectrometry (Live-MS)”. The content of the cell is sucked by a metal-coated glass micro-needle tip (nanospray tip) under a microscope and fed directly to a mass spectrometer inlet by nano spray ionization after distally adding the ionization organic solvent (Fig.1). Detected thousands of molecular peaks are analyzed by t-test or PCA (principal component analysis) to find site or stage specific metabolites which are projected in a metabolic map. Applications to plant cells, mammalian cells, tiny amount of biological fluids and body fluids for diagnosis are reported for future rapid and direct understandings of biological and medical molecular mechanisms.

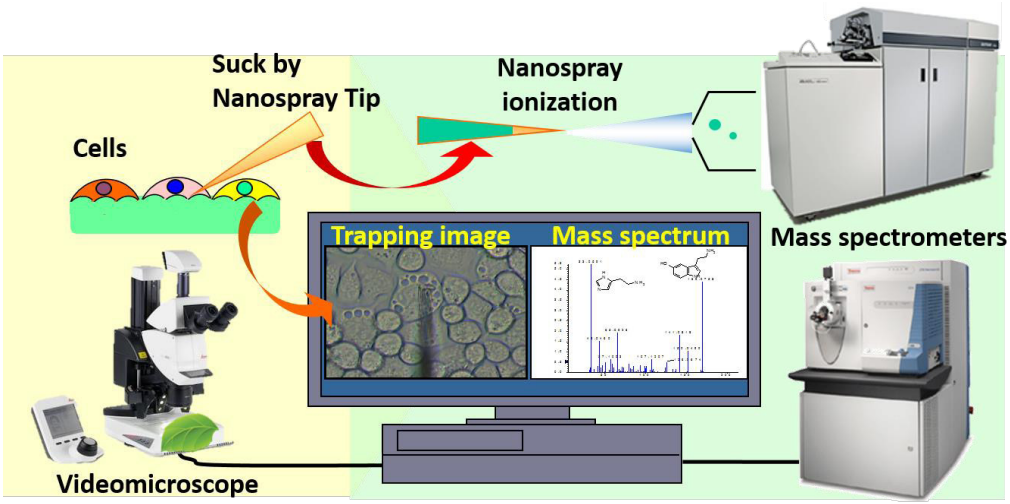

Fig.1 Scheme of the Live Single Cell Mass Spectrometry

MK-08

The Role of Gut Microbiota, Bile Acids, Intestinal Farnesoid X Receptor Signaling, and Ceramides in Metabolic Disease

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The gut microbiota is associated with metabolic diseases including obesity, insulin resistance and non-alcoholic fatty liver disease (NAFLD), as demonstrated by correlative studies and by transplant of microbiota from obese humans and mice into germ-free mice. Modification of the microbiota by treatment of high-fat diet (HFD)-fed mice with tempol or antibiotics resulted in decreased adverse metabolic phenotypes. This was due to lower levels of the genera *Lactobacillus* and decreased bile salt hydrolase (BSH) activity. The decreased BSH resulted in increased levels of tauro-β-muricholic acid (T-β-MCA), a substrate of BSH and a potent farnesoid X receptor (FXR) antagonist. Mice lacking expression of FXR in the intestine were resistant to HFD-induced obesity, insulin resistance and NAFLD thus confirming that intestinal FXR is involved in the potentiation of metabolic disease. A potent intestinal FXR antagonist glycine-muricholic acid (Gly-MCA) that is resistant to BSH, was developed that when administered to HFD-treated mice, mimics the effect of the altered microbiota on HFD-induced metabolic disease. Gly-MCA had similar effects on genetically obese leptin-deficient mice. The decreased in adverse metabolic phenotype by tempol, antibiotics and Gly-MCA was due to decreased serum ceramides. Mice lacking FXR in intestine also have lower serum ceramides, are metabolic fit and resistant to HFD-induced metabolic disease, and this is reversed by injection of C16:0 ceramide. In mouse ileum, due to the presence of endogenous FXR agonists produced in the liver, FXR target genes involved in ceramide synthesis are activated and when Gly-MCA is administered, they are repressed, which likely accounts for the decrease in serum ceramides. These studies reveal that ceramides produced in the ileum under control of FXR, influence metabolic diseases. Mechanistically, mice under intestinal FXR antagonism by Gly-MCA or lacking intestinal FXR have increased beige adipose depots that mediate weight loss through increased energy expenditure and heat production, similar to brown adipose tissue. This is due in part to the lowering of serum ceramides through modulation of FXR signaling in the ileum. Induction of beiging in cultured epididymally-derived white adipocyte cultures was inhibited by treatment with ceramides. Increased adipose beiging after intestinal FXR inhibition may account for the increased energy expenditure and weight loss in obese mice treated with Gly-MCA. Although it was not determined, decreased insulin resistance in Gly-MCA treated mice could also be due to the effects of ceramides that impair pancreas beta-cell insulin production. Increased ceramides also cause an elevation in fatty acid synthesis in liver which accounts for the NAFLD in in obese mice where FXR is constitutively activated. Decreasing ceramide levels through inhibition of intestinal FXR signaling is a feasible means to reduce metabolic diseases.

MK-09

Neuroproteomic Profiling of Proteins and Autoantibody Repertoires in Plasma and CSF

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Introduction and Objectives

The Human Protein Atlas (HPA, www.proteinatlas.org) currently contains more than 25.000 validated antibodies targeting 17.000 proteins corresponding to approximately 86% of the encoded human proteins. The publicly available portal contains several million high-resolution images generated by immunohistochemistry on tissue microarrays and by confocal microscopy for subcellular localization. In total are 48 000 array validated antibodies and 42 000 mass spectrometry validated antigens accessible within HPA for systematic biomarker discovery efforts utilizing various protein array-based platforms.

Proteomic profiling of serum, plasma and cerebrospinal fluid are currently performed on antigens arrays for analysis of autoimmunity repertoires and on antibody arrays for generation of multiplex protein profiles. Through a combination of planar and bead-based microarray formats, assays are set up both for broad screening studies as well as targeted analysis for verification and validation of initial findings.

Methods

We have assembled two sets each of 380 HPA antibodies as well as the corresponding 380 HPA protein fragments coupled to colorcoded magnetic beads, representing 280 brain-enriched proteins and 220 neurodisease associated proteins. The former category is selected based on RNAseq data (Uhlen et al Science 2015) and the latter is selected based on various literature and previous inhouse profiling.

These affinity reagents and the inhouse established suspension bead array technogy for highly multiplex protein and autoantibody profiling constitute the core in an extensive neuroproteomic profiling effort.

In total have so far more than 3000 samples from various cohorts been profiled, ie serum, plasma and cerebrospinal fluid (CSF) from patients within the areas of multiple sclerosis, ALS, Alzheimer's disease and Parkinson's disease as well schizophrenia and obsessive compulsive disorder (OCD).

Results and Discussion

In one part of the study were the 280 brain-enriched proteins profilied in CSF from patients with Alzheimer's disease (AD), Parkinson's disease (PD) and dementia with Lewy bodies (DLB). In total, 441 human samples of ventricular CSF collected post

mortem and lumbar CSF collected ante mortem were analyzed using 380 antibodies in a suspension bead array setup, utilizing a direct labelling approach.

Among several proteins that displayed differentiated profiles between sample groups, we focus here on two synaptic proteins, neuromodulin (GAP43) and neurogranin (NRGN). They were both found at elevated levels in CSF from AD patients in two independent cohorts, providing disease-associated profiles in addition to verifying and strengthening previously observed patterns. Increased levels were also observed for patients for whom the AD diagnosis was not established at the time of sampling.

Conclusions

These findings indicate that analyzing the brain-enriched proteins in CSF is of particular interest to increase the understanding of the CSF proteome and its relation to neurodegenerative disorders. In addition, this study lends to support the notion that measurements of these synaptic proteins could potentially be of great relevance in future diagnostic tests for AD.

Keywords

Neuroproteomics, affinity proteomics, autoantibody profiling, protein profiling, protein microarrays, CSF, plasma, serum, Alzheimer's disease

MK-10

Proteomic Approaches to Understanding Age-Related Macular Degeneration

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Age-related macular degeneration (AMD) is the leading cause of visual loss among adults aged 65 or older in developed countries. Genetic background is estimated to account for ~70% of the risk of AMD. The three genes associated with the highest AMD risk are complement factor H (*CFH*) on chromosome 1q32 and age-related maculopathy susceptibility protein 2 (*ARMS2*) and serine protease HTRA1 (*HTRA1*) on chromosome 10q26. Two major variants in *CFH*, tyrosine 402 to histidine (Y402H) and isoleucine 62 to valine (I62V), a variant in *ARMS2*, alanine 69 to serine (A69S), and rs11200638 in the promoter region of HTRA1 are strongly associated with AMD risk. Although systemic complement is widely suspected to play a role in AMD pathogenesis, the relationship of circulating CFH variants with AMD has not been characterized. The roles of ARMS2 and HTRA1 in AMD are not well understood. Specific Aim 1 is to characterize the relationship between circulating CFH variants and AMD. Specific Aim 2 is to understand the role of ARMS2 and HTRA1 in the pathogenesis of AMD. For aim 1, we developed a selected reaction monitoring (SRM) assay to measure the plasma concentrations of CFH variants Y402, H402, I62, and V62. We used tryptic peptides unique to each protein were identified using PeptideCutter, NCBI BLAST, and UniProt/BLAST searches and further refinement through Skyline. Seven proteotypic peptides were selected for CFH and its four variants. All peptides were optimized and validated using liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a triple quadrupole mass spectrometer (5500 QTrap, Sciex). Plasma concentrations of CFH variants were measured using the SRM assay in 344 adults. Plasma CFH concentrations (mean, SE in µg/mL) by inferred genotype were: YY402, II62 (170.1, 31.4), YY402, VV62 (188.8, 38.5), HH402, VV62 (144.0, 37.0), HY402, VV62 (164.2, 42.3), YY402, IV62 (194.8, 36.8), HY402, IV62 (181.3, 44.7). There were no individuals with HH402, II62 or HH402, IV62 genotype. For aim 2, we undertook protein-protein interaction studies of ARMS2 and HTRA1 in human retinal pigment epithelium (RPE) derived from induced pluripotent stem (iPS) cells, in iPS-RPE in which CRISPR/cas9 was used to insert A69S, and in ARPE-19 cells. Immunoaffinity purification-MS

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approaches were used to identify protein interactions with HTRA1 and ARMS2. Our SRM assay will facilitate the study of the role of systemic CFH variants and AMD risk in large epidemiological studies. Protein-protein interaction studies will allow new insights into the role of ARMS2 and HTRA1 in AMD pathogenesis.

MK-11

A Systems View of the Spatial-Temporal Dynamics of Host Organelle Morphology and Composition during Viral Infection

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Introduction and Objectives

Viruses and host cells have established complex, dynamic interactions that function either in promoting virus replication and dissemination or in host defense against invading pathogens. Thus, viral infection triggers striking transformations in intracellular proteomes, including the reorganization of organelles. A relevant example is the infection with human cytomegalovirus (HCMV), a beta-herpesvirus that infects a large majority of the adult population worldwide, leading to life-threatening diseases in immunocompromised individuals. HCMV triggers an extensive reorganization of organelle structures for energy production, intracellular trafficking, and generation of a virion assembly compartment. However, the viral proteins that target distinct organelles or the specific organelle proteins recruited for these morphological changes remain in large part unknown.

Methods

Here, we report the first global, spatial and temporal, proteomic study of subcellular organelles throughout the progression of a viral infection. We monitored organelle shape, motility, density, and composition at different stages of HCMV infection. First, using time-lapse microscopy and fluorescent confocal microscopy, we monitored changes in organelle morphology during infection. We observed the signature mitochondria fission, rearrangement of the Golgi apparatus around the viral assembly complex, increased lysosome size, and increased density of the endoplasmic reticulum. Next, organelle densities were examined by ultracentrifugation, while changes in organelle composition during infection were assessed using quantitative mass spectrometry. A combination of label-free and isobaric labeling (using tandem mass tags) approaches provided spatial and temporal information of organelle composition. Dimensional reduction algorithms and supervised machine learning allowed the confident assignment of proteins to organelles throughout the time course of infection.

Results and Discussion

This study represents the first spatial and temporal analysis of organelles in virally infected cells, providing information about the subcellular localization of host and viral proteins throughout HCMV infection. Importantly, we discovered which viral proteins target distinct organelles during infection. A previously uncharacterized HCMV viral protein is shown to have a temporally dynamic localization to multiple organelles and to be required for efficient virus production. These results are put in the context of our findings of host antiviral factors that are mitochondrial residents. Integration of the organelle dataset with virus-host protein interaction studies provided insights into mechanisms of virus suppression of host defense responses.

Conclusion

Altogether, our study highlights mass spectrometry-based proteomics as a critical

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component for understanding virus biology and resulting pathologies.

Keywords: HCMV infection; organelles; label-free; TMT quantification; viral infection; mitochondria

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IMMUNOTHERAPY OF CANCER: AN OVERVIEW AND NEW RESULTS

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Introduction and Objectives

This lecture will describe how the immune system works to keep us free of cancer most of the time and how cancer cells can eventually learn to escape the immune system. Also described will be a number of scientific breakthroughs that have occurred in the last 6 years that clearly suggest that the immune system can be re-educated and upregulated to cure even late stage cancer. Science magazine labeled cancer immunotherapy as the breakthrough event of the year in 2013 and tremendous progress has been made since then. This lecture will try to capture some this excitement and also pinpoint some of the contributions that the Hunt group has made to the field over the past 23 yrs.

More Power and More Depth: New Tools for Proteomics Data Processing

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Introduction and Objectives

Mass spectrometry based proteomics has become the method of choice to identify and characterize large amounts of proteins in a sample. Over the past ten years, proteomics has benefitted from drastically improved instrumentation and ever more sophisticated experimental protocols to dig ever deeper into the proteome, providing greater coverage and increasing depth.However, like in any high-throughput analytical field, the advances in proteomics have also come at the price of vastly increased data acquisition. These large amounts of data in turn require management and processing solutions that can scale with the data.

Methods

In order to provide such solutions, we've built the SearchGUI (Vaudel et al., 2011) and PeptideShaker (Vaudel et al., 2015) tools for the identification of tandem mass spectra. And we very recently added two tools for quantification as well: the Reporter tool for isobaric labelling, and the modest feature finder (moFF) tool for MS1 quantification. A special-purpose tool is the pride-asap annotation pipeline (Hulstaert et al., 2013), that allows us to automatically extract the correct search parameters for public data sets deposited in PRIDE/ProteomeXchange (Vizcaíno et al., 2014).

Results and Discussion

These tools are all scalable in the sense that they make maximal use of the available compute cores in the system they are running on, but to achieve true scalability, our software needs to be able to scale across different computers and should therefore work on a compute cluster or a grid (Verheggen et al., 2014). We therefore built a user-friendly grid engine called Pladipus (Verheggen et al., 2016) where each of these tools, amongst many others, can be plugged in. With the Pladipus grid engine, any interested researcher can now transform their heterogeneous collection of desktop computers (Windows, Mac, Linux, or any combination thereof) and servers into a dynamic, ad-hoc cluster environment that can automatically process the large amounts of data that are so typically encountered in proteomics experiments.We will also briefly discuss two additional tools that show great promise. The first of these is the fragmentation spectrum predictor MS2PIP that will output reliable CID and HCD intensities for each fragment ion peak based on an input peptide sequence (Degroeve et al., 2015). The second is the Percolator-based (Käll et al., 2007) ReScore algorithm that builds on MS2PIP predictions to aid in the reliable peptide identification of complex samples. We demonstrate the performance of ReScore on a set of proteomes that were derived from different Mycobacterium tuberculosis lineages with altered virulence (Peters et al., 2016).

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Conclusion

Powerful and scalable software tools are an essential part of proteomics experiments, and it is therefore imperative that the field continues to evolve and improve its algorithms and tools.

Keywords: bioinformaticsidentiificationquantification

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Exploring the Diversity in the Human Proteome

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Introduction and Objectives

Coverage of the diversity and sequence space in the human proteome remains low, with most proteins covered by only a few peptides, most of which reported only in their unmodified forms. Since full coverage of the human proteome cannot be achieved by any single lab nor by any single search tool, we show how integrated data and algorithms can be used to efficiently explore large search spaces and to aggregate worldwide contributions in a reusable community-wide platform.

Methods

We propose to expand coverage of the human proteome in three ways. First, public data should be readily searchable with a variety of algorithms such as spectral library search and proteogenomics. Second, spectral alignment algorithms reveal numerous unexpected post-translational modifications and unidentified connections between datasets. Third, the resulting knowledge should be aggregated in an open platform and easily accessible to verification by the whole community.

Results and Discussion

Building on the growing availability of public mass spectrometry (MS) data, we implemented large scale searches of over 15 terabytes of data from a range of human samples analyzed with various types of instruments, thus more than doubling the volume of annotated human data and increasing the number of peptide spectrum matches by several fold. In addition to a broad baseline of standard searches, we discuss how stratified search spaces substantially improve peptide identification and show how MassIVE search algorithms can reveal thousands of novel proteogenomics events via reusable workflows that are readily applicable to new public and private data.

Beyond identification of common, mostly-unmodified peptides, we show how spectral alignment algorithms can improve the confidence of peptide identifications by several orders of magnitude and use these to reveal dozens of unexpected putative modifications supported by multiple highly-correlated spectra. These further show that protein regions can be observed in over 50 different variants with various combinations of post-translational modifications and cleavage events, thus suggesting that current coverage of proteome diversity (at ~1.3 variants per protein region) is far below what should be expected.

Finally, we present an open platform for community sharing of MS data and knowledge. Building on large-scale automated reanalysis and distributed computing

workflows designed to integrate public and private data, we show how thousands of users from over 100 countries have embraced open knowledge to analyze billions of spectra from millions of samples, thereby enabling the concept of 'living data' and improving identification rates by over 20-fold.

Conclusions

Transforming MS big data into 'living data' by automated integration of advanced algorithms and community contributions.

Keywords

Spectral libraries and alignment, big data algorithms, post-translational modifications, community knowledge base, living data.

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Modulation of Multiple PTMs Upon Brief Cellular Stimulation

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Introduction and Objectives

Post- or co-translational modifications (PTMs) of proteins refer to the reversible or irreversible addition of chemical modifying groups to proteins after or during their synthesis. Proteins has often multiple PTMs decorating the primary amino acid sequence and often these affect each other to produce a given function or activity of a protein. PTMomics can be defined as the global study of PTMs and is a relatively new research area aiming at understanding the function and consequences of PTMs of proteins in a cell. The success of PTMomics relies on the ability to efficiently purify the relevant PTMs and subsequently identify and characterize the nature and function of the PTM using for example mass spectrometric analysis. Previously, we have developed a number of successful methods for enrichment of PTMs

Methods

Cells (HeLa or human neurons) were stimulated up to 20 min with EGF or sAPPa and after harvesting the cells the membrane proteins were enriched by Na2CO3 treatment and ultracentrifugation. After trypsin digestion and multiplex labeling the various PTM peptides were enriched using our comprehensive workflow which is a further development of our TiSH method. After separation, peptides were fractionated by HILIC or stage tip based High pH RP. All the samples were subsequently analysed by nLC MS/MS using a Q-exactive HF instrument. Several bioinformatics programs were used for subsequent pathway analysis

Results and Discussion

For the brief EGF stimulation we have found significant regulation in phosphorylation, sialylation and reversible Cys modification of proteins involved in EGFR signaling. Beside regulation of phosphorylation observed after EGF stimulation a substantial modulation of surface sialylation and reversible cysteine modifications in numerous proteins was observed. Several surface proteins with altered sialylation, involved in cell adhesion (integrins), cell-cell communication and migration, and degradation or internalization of surface receptors (ephrin B2) was observed. Several protein tyrosine phosphatases showed a reduction of the catalytic Cys site in the conserved phosphatase HC(X)5R motif indicating an activation of de-phosphorylation. Furthermore, we observe regulation of Cys sites in a large number of enzymes that are associated with other dynamic PTMs supporting the idea of reversible Cys modification having substantial cross-talks with other PTMs. In the sAPPa stimulation of human neurons we observed a substantial modulation of sialylation and phosphorylation within the first 20 min of stimulation. Several pathways were found regulated incl. MAPK, Akt and cAMP mediated signaling. Most of the changes observed were directly linked to the neuroprotective function of sAPPa including synaptic plasticity regulation, axonal guidance, neural survival and differentiation

Conclusion

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The results presented here show that our comprehensive and very sensitive methods ofr assesment of PTMs are useful in studying signalling pathways in biological systems

Keywords: PTM crostalking

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Nutrient Regulation of Signaling & Transcription by O-GlcNAcylation: Fundamental Roles in Diabetes

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Introduction and Objectives

O-linked N-acetylglucosamine (O-GlcNAc) is an abundant protein modification that serves as a nutrient sensor to modulate nearly all cell functions. Recent studies showing that O-GlcNAcylation is not only a major regulator of signaling and transcription, but also is a major molecular mechanism underlying glucose toxicity in diabetes will be presented.

Methods

Identifying and mapping O-GlcNAcylation sites requires enrichment of glycopeptides to prevent ion-suppression and ETD fragmentation to prevent cleavage of the labile glycosidic bond as occurs in CID. O-GlcNAc peptides are enriched using enzymatic tagging to add an azido sugar (eg. GalNAz) and enriched using Click-chemistry to attach affinity tags.

Results and Discussion

O-GlcNAc cycles on and off thousands of nucleocytoplasmic proteins and has extensive crosstalk with protein phosphorylation. O-GlcNAc occurs on nearly all proteins involved in transcription, where it serves to regulate gene expression in response to nutrients. O-GlcNAc not only regulates assembly of the pre-initiation complex and the activity of RNA polymerase II, but also the sugar regulates the cycling of the TATA-binding (TBP) protein on and off DNA during the transcription cycle. O-GlcNAcylation of TBP is required for its ability to bend DNA, to mark sites of transcription initiation. In adult mice, targeted, inducible, deletion of the O-GlcNAc Transferase in excitatory neurons results in a morbidly obese mouse, which cannot stop eating. Thus, O-GlcNAcylation not only serves as a nutrient sensor in all cells, but also is directly involved in appetite regulation (satiety) in the brain. In neurons, O-GlcNAcylation also plays an important role in the trafficking of AMPA receptors and in the development of functional synaptic spines. More than one-half of all human protein kinases are modified by O-GlcNAc and all kinases that have been tested are indeed regulated by the sugar. For example, abnormal O-GlcNAcylation of CAMKII contributes directly to diabetic cardiomyopathy and to arrhythmias associated with diabetes. Over eighty-eight mitochondrial proteins are O-GlcNAcylated, and O-GlcNAcylation not only regulates mitochondrial functions, but also O-GlcNAc cycling is grossly abnormal in cardiac mitochondria from diabetic animals. These findings in mitochondria provide a molecular basis for mitochondrial dysfunctions in hyperglycemia associated with diabetes.

Conclusions

The fundamental involvement of O-GlcNAcylation in signaling and transcription explains why when O-GlcNAc is elevated for extended periods of time, as it is in diabetes, it contributes directly to diabetic complications and is a major mechanism of glucose toxicity. Supported by NIH P01HL107153, R01DK61671 and N01-HV-00240. *Dr. Hart receives a share of royalty received by the university on sales of the CTD 110.6 antibody, which are managed by JHU.*

Keywords

O-GlcNAc, transcription, signaling, ETD MS/MS, diabetes, glucose toxicity

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Proteomics 2.0: Recent Advances in Top Down Proteomics

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Introduction and Objectives

Over the past decade, top down proteomics has morphed from a technique capable of identifying a handful of proteins to one capable of identifying thousands of unique molecular species. However, the thorough characterization of highly modified proteoforms on a LC time scale remains a grand challenge to current generation instrumentation and ion fragmentation methods.

Methods

An Orbitrap Fusion Lumos mass spectrometer was equipped with an ultra violet laser aligned with the axis of the ion traps through a laser window custom machined into the vacuum flange. Triggering of the laser was integrated into the instrument control software to enable data dependent acquisition in a high-throughput mode.

Results and Discussion

Recently, our laboratory has developed several metrics of performance of Top Down MS/MS, including for protein identification (Q-values) and proteoform characterization (the C-score). These metrics allow us to quantify the information content of high-resolution MS/MS spectra and thus compare different fragmentation modalities. As an initial case study, yeast whole cell extracts were subjected to GELFrEE fractionation followed by reversed phase chromatography, with data dependent acquisition of MS/MS spectra using HCD, ETD and UVPD.

Conclusion

Analyzing these data enable a clear look into the utility of UVPD for both protein identification and proteoform characterization by Top Down run in discovery mode via high throughput methods on an Orbitrap Fusion Lumos mass spectrometer.

Keywords: Top Down ProteomicsUltra Violet PhotodissociationMass Spectrometry

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Capturing the spatial proteome in action

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Introduction and Objectives

Proteins adopt multiple functions controlled by their subcellular location, binding partners and post transcriptional and post translational modification status, significantly increasing the functionality of the proteome over what is encoded by the genome. The processes governing these features are highly dynamic. The ability to chart changes in the dynamic proteome upon perturbation such as drug treatment or cell stress is of paramount importance to our understanding of cellular mechanisms. Methods designed to map these changes are very reliant on methods which result in reproducible data which offer excellent subcellular resolution.

Methods

Here, we utilise the hyperLOPIT method (Christoforou Nat. Comm 2016) to gain insight into the steady state location of proteins in human mouse and yeast cells with high subcellular resolution, and reproducibility. We use a combination of different subcellular fractionation methods based on detergent solubilisation, differential and equilibrium density centrifugation to fractionate cells into distinct subcellular fractions. We map the distribution of proteins through fractions using quantitative proteomics approaches, and apply bespoke machine learning tools to further analyse data and classify proteins into distinct subcellular niches (Breckels PLoS Comp Biol. 2016, Gatto Bioinformatics, 2014.).

Results and Discussion

Firstly we reveal that high quality of data that can be achieved using different spatial proteomics approaches. We demonstrate that choice of marker proteins, and analytical workflows can impact the number of false discoveries incurred and thus conclusions which can be drawn. Secondly, in all examples, we demonstrate that over half of the proteome is located in multiple places giving insight into spatially dependent functionality of proteins. Furthermore, for the human and yeast examples, comparison with high content data obtained by microscopy methods shows some overlap between spatial assignments, but highlights inherent issues of immune reagent quality and mis-localisation artefacts which may result from fluorescent fusion proteins.Finally, the level of resolution allowed us to capture protein re-localisation in response to nitrogen starvation of yeast using the hyperLOPIT method in a dynamic format. We demonstrate re-localisation involves proteins mainly mediated by the TOR signalling pathway, which are involved in chromatin remodelling and licencing of DNA replication. These included a member of the vacuole-associated TORC-activating EGO complex and a member of the EGO-inhibitory complex SEACIT, as well as members of the SWR1 and INO80 chromatin remodelling complexes and the DNA replication licencing MCM complex.

Conclusion

Spatial proteomics methods are capable of visualising high resolution data which is

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complementary to fluorescent microscopy methods. Different approaches yield data with differing levels of resolution and care needs to be taken when analysing data to ensure biologically meaningful datasets.

Keywords: Spatial proteomics

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Proteomic and Metabolic Health Phenotypes in Dietary Clinical Interventions

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Introduction and Objectives

Obesity has become one of the major global epidemics. The pan-European nutritional intervention program “DiOGenes” was designed to advance understanding of how obesity can be prevented and treated from a dietary perspective. We investigated the differential plasma proteome profiles, generated before and after weight loss and maintenance, of obese and overweight individuals undergoing nutritional intervention. This study aimed at discovering proteins associated to phenotypic changes during the dietary intervention and, in particular, exploring proteins in relation to changes in body mass index, fat mass, insulin resistance, and insulin sensitivity.

Methods

Relative protein quantification was obtained in plasma samples at baseline and after weight loss (8 weeks)/maintenance (6 months) phase using isobaric tagging and MS/MS. A Welch t-test was used to determine proteins differentially present after intervention, correcting for multiple testing. The relationships of the differentially abundant proteins with clinical variables were explored using univariate linear models, considering collection center, gender and age as confounding factors. Results were validated with permutation tests and bootstrapping.

Results and Discussion

In total, 473 subjects were measured at baseline and at the end of the intervention; 183 proteins were quantified in more than 70% of the plasma samples; and 39 proteins were found to be longitudinally differential without confounding effects (24 proteins showed increased levels and 16 displayed decreased levels). Overall, 12 differential proteins with mean fold change outside of the 10% fold change threshold were pinpointed. Of those, 10 proteins have already been, and most of them extensively, reported in the literature in relation to obesity and weight changes.

Conclusion

We have performed a unique proteomic analysis of a large cohort of non-diabetic overweight and obese individuals who participated in a nutritional intervention comprising both a weight loss and a weight maintenance period. Our clinical MS-based proteomic workflow enabled consistent measurements of 183 proteins in plasma across patients and time points and was shown to be informative and robust. Most of our significant findings in terms of differential protein profiles in plasma samples collected at baseline and at the end of the intervention are supported by previous literature reports. Observed modulations should be ultimately confirmed in an independent cohort. As an extension of the observational study presented here, the proteome profiles have ultimately allowed us

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to build predictive models of weight loss for personalized nutritional intervention.

Keywords: Biomarker; Proteomics; Mass spectrometry; Obesity; Diabetes; Large-scale study

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Characterization of Protein Dynamics in the Myocardium using Multidimensional Proteomics

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Introduction and Objectives

Advances in proteomics technologies have created new opportunities to understand complex diseases from a systems perspective. It is increasingly recognized that protein functions are the complex outputs orchestrated by multiple molecular parameters including the abundance of protein species, their modifications, localization, interactions, and others. The continual development of analytical methods to characterize these proteome parameters is imperative in advancing our understanding of disease mechanisms and aiding in the discovery of protein biomarkers. In this study, we developed a technological platform to elucidate protein turnover kinetics, an independent proteome parameter that contributes to the regulation of protein homeostasis in the myocardium.

Methods

Using metabolic labeling, high-resolution mass spectrometry and mathematical modeling, we created a technological platform to measure the individual protein expressions and turnover dynamics in a mouse model of cardiac hypertrophy. In parallel, we translated this methodology to obtain the turnover rates of blood proteins from 10 healthy human subjects.

Results and Discussion

Application of this platform led to findings including (i) the recognition that cardiac mitochondrial proteins have diverse half-lives spanning multiple orders of magnitude, suggesting a finer and independent level of regulation in parallel to mitophagy; (ii) the discovery of differential protein turnover dynamics in metabolic pathways and the identification of novel markers of cardiac hypertrophy; and (iii) measuring for the first time the half-life of ~500 human blood proteins.

Conclusion

Taken together, our results suggest that regulations of protein functions in the cardiovascular system are more complex than previously thought, where individual proteins and pathways may alter synchronously in turnover/degradation in response to stress, without necessarily changing in abundance.

Keywords: Protein turnover

TK-01

Charting the Landscape of Temporal EGF-Stimulated EGFR Interaction Network

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Introduction and Objectives

Transmembrane receptors typically contain at least one, often multiple transmembrane domains that anchor the receptor to the plasma membrane. This property presents a challenge for isolating membrane-bound protein complexes, as solubilizing the membrane with detergent could potentially disrupt protein-protein interactions, particularly those weak and transient interactions. Formaldehyde-mediated crosslinking has been a method of choice for preserving protein-protein interactions, as covalent bonds formed between interacting proteins make them resistant to disruption by detergent extraction and subsequent stringent wash conditions to remove non-specifically bound proteins.

Epidermal growth factor receptor (EGFR, also so known as ERBB1 or HER1), is one of the most extensively characterized transmembrane proteins. EGFR is a member of ERBB receptor tyrosine kinase (RTK) family and plays pivotal roles in many biological processes, including embryonic development and tissue homeostasis, through promoting cell proliferation, differentiation, and survival. Unstimulated, EGFR exists as a monomer; upon ligand stimulation, it undergoes conformational change that facilitates its dimerization, leading to its activation through autophosphorylation. as well as of the activation of a plethora of its substrates. Multiple signaling pathways activated by EGFR, including PI3K/AKT/mTOR, RAS/MAPK, JAK/STAT3, and JNK pathways, are critical for cell growth and survival,.

The EGFR-PPI network has been extensively studied and as a result, more than 150 proteins have been reported in the literature as being physically associated with EGFR. It is well established that activated EGFR undergoes internalization, endocytosis and endocytic trafficking through multiple intracellular vesicle compartments that eventually leads to its degradation or recycling to the plasma membrane. The dynamic nature of the signaling and the involvement of multiple intracellular compartments make it challenging to quantitatively map the spatial-temporal network. Furthermore, how the trafficking proteins regulate the final destinations of EGFR and its stability are not well understood.

Methods

Here we develop a workflow for the isolation and identification of signal-induced dynamic membrane protein interactions using EGFR as an example. We report a time-resolved, signal-dependent EGFR proteome. We employed a formaldehyde-mediated cross-linking strategy to immuno-precipitate endogenous

TK-01

EGFR-associated complexes upon EGF treatment, and analyzed the associated proteins by quantitative mass spectrometry.

Results and Discussion

We show that our cross-linking condition has greatly enriched proteins that are involved in endocytosis and trafficking, allowing us to follow EGFR to various vesicle compartments with great spatial-temporal resolution.

Keywords

EGFR, Quantitative mass spectrometry, PPI network

TK-02

Multilayer Proteomics Approaches to Understanding Common Human Diseases

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Introduction and Objectives

Alzheimer's disease (AD), the most common form of dementia, is the sixth-leading cause of death in the US with a care cost of $226 billion in 2015. Despite extensive investigation of AD pathogenesis and tremendous investment in developing therapeutics for AD, no cure is available. Deposition of protein aggregates is a common feature of AD pathology. Identification of the aggregated proteins, together with genetic studies, can provide crucial insights into the molecular pathogenesis of AD. The resulting amyloid cascade and tau hypotheses dominate AD studies. AD etiology, however, remains to be fully illustrated.

Methods

We seek to develop novel mass spectrometry-based proteomics technologies, and systems biology approaches to investigate AD etiology. A range of methods are used in this study, including (i) 10-plex TMT-LC/LC-MS/MS proteomics analysis of the AD mouse models; (ii) a streamlined method for profiling aggregated proteome in clinical human brain specimens; (iii) antibody-based Western blotting and immunohistochemistry for validating newly discovered U1-70K pathology in AD; (iv) RNAseq analysis of AD brain samples; (v) integrated MS methods for mapping aggregated U1-70K protein cleavage site; (vi) developing a primary neuronal model to examine U1-70K fragment toxicity; (vii) generating a transgenic mouse model to recapitulate AD symptoms; (viii) molecular, cellular, electrophysiological, and behavior analyses of the new AD model.

Results and Discussion

We performed a comprehensive study of the human brain aggregated proteome, identifying 36 proteins accumulated in AD, including U1-70K and other U1 small nuclear ribonucleoprotein (U1 snRNP) spliceosome components. Multiple U1 snRNP subunits form cytoplasmic tangle-like structures in AD but not in other neurodegenerative disorders, indicating that U1 snRNP pathology is AD specific and cannot be induced by tauopathy alone. Deep RNA sequencing revealed global dysregulation of RNA processing in AD brain. Importantly, U1-70K aggregation was found in mild cognitive impairment and early Braak stages of AD, indicating that U1 snRNP alteration occurs early in AD development. In addition, aggregation of protein proteolytic fragments is common in neurodegeneration. In the AD samples, U1-70K is internally cleaved, resulting in a N40K fragment, also detected in the aggregated proteome. Together, these results raise an exciting hypothesis that U1 snRNP and RNA dysfunction can mediate neurodegeneration in the AD pathogenesis. To test this hypothesis, we generated a transgenic mouse model to express the U1-70K fragment (i.e. N40K) and fully characterized this new AD model.

Conclusion

The proteomics-based analysis of diseased brain tissues enables the discovery of a novel pathological pathway of RNA dysregulation in AD.

TK-02

Keywords: Clinical tissue proteomics; AD pathology; novel mouse AD model; RNAseq and deep proteomics

TK-03

Extracellular Glycosylation: How to See the Forest Despite All the Trees

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Introduction and Objectives

There is growing evidence about the biological significance of extracellular glycosylation, and obtaining site-specific information about the modification of proteins is essential. Recent technical advances have enabled the first large scale analyses of intact glycopeptides. Existing search engines have been transformed and new bioinformatic tools have been created to aid glycopeptide identifications from the huge datasets generated. We have been on the forefront of this research, and I want to present our success stories along with the obstacles we confronted, and the solutions we developed so far.

Methods

An extensive study on GlcNAcylation of synaptosomal proteins revealed that wheat germ agglutinin (WGA) efficiently and indiscriminately binds glycopeptides, thus, yielding the largest glycopeptide dataset analyzed up to date. Since then this enrichment protocol has been used for general glycopeptide enrichment from different sources, such as mouse liver, arabidopsis flowers and human serum. Electron-transfer dissociation was essential for the characterization of these mixtures, but we believe that obtaining HCD data is also a must. Two different software: Byonic and Protein Prospector have been used in the data interpretation process. However, despite all the improvements in these search engines manual interpretation of the data was (and is) still necessary.

Results and Discussion

The careful analysis of the intact glycopeptide datasets yielded i) site-specific N- and O-glycosylation information for hundreds of glycoproteins; ii) revealed a surprisingly high presence of truncated N-glycan structures; iii) showed N-glycosylation in non-consensus motifs; iv) revealed O-glycosylation of Tyr residues; v) identified a new substrate for O-mannosylation; vi) we detected acetyl-sialic acids in liver N- and O-glycans. Tissue- and cell-organelle specific N- and O-glycosylation information was obtained comparing data derived from mouse liver and mouse synaptosome. Manual data evaluation also revealed glycan and peptide side reactions, peak-picking problems and other issues that represent roadblocks for efficient streamlined glycopeptide assignments.

Conclusion

During the analysis of these large intact glycopeptide datasets we have developed an iterative approach that provides reliable glycan composition information without prior knowledge about the oligosaccharides present. In addition, based on the extensive manual data evaluation we also have identified data analysis approaches whose automated implementation may make the glycopeptide identifications more reliable.

Keywords: glycopeptide, HCD, ETD, mass spectrometry, lectin-affinity chromatography, N-linked, O-linked

TK-04

Methods and Software Tools for the Comphrehensive Analyses of Glycoproteins

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Introduction and Objectives

Protein glycosylation is one of the most common and important protein modifications. It has long been known to play fundamental roles in many biological processes, as well as in the pathological progression of many diseases. Glycoproteins constitute the major biochemical class of current therapeutic targets and clinical biomarkers. Protein glycosylation is also the most structurally complicated and diverse type of protein modification. The complexity of glycosylation heterogeneity poses great challenges for structural and functional studies designed to identify glycoproteins and their specific glycosylation forms in association with different biological functions and diseases. In the last decade, mass spectrometry-based glycoproteomics and glycomics have become emerging technologies for the large-scale characterization of glycoproteins.

Methods

We developed a novel chemoenzymatic method for the comprehensive characterization of glycoproteins. The glycans and glycosite-containing peptides were isolated and analyzed by mass spectrometry (MS), and the identified glycan and glycosite information was used to identify the intact glycopeptides for site-specific glycosylation analysis of glycoproteins using our recently developed software tools GPQuest by selection of N- and O-linked glycan-containing peptides and spectral library matching.

Results and Discussion

When applied the methods and softare tools for the analysis of a standard glycoproteins or glycoproteins from cells, NGAG method allowed us to quantitatively analyze glycoproteins, glycans, glycosite-containing peptides (glycosites), and intact glycopeptides.

Conclusions

This method provides a foundation for the large-scale and comprehensive characterization of glycoproteins.

Keywords

Glycoproteins, glycans, glycosylation sites, site-specific glycosylation, mass spectrometry

TK-05

Systems-Wide Analysis of Properties and Functions of Lysine Acetylation and Ubiquitylation

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Introduction and Objectives

Lysine acetylation and ubiquitylation are evolutionarily conserved, frequently occurring posttranslational modifications (PTMs), which are involved in regulation of diverse cellular functions.

Methods

We use quantitative proteomics to investigate the scope and dynamics of acetylation and ubiquitylation in human cells.

Results and Discussion

We applied quantitative mass spectrometry to investigate the dynamics of acetylation in response to most commonly used lysine deacetylase inhibitors. In addition to confirming the specificities of many deacetylase inhibitors, we discovered unexpected acetylation patterns for some inhibitors, and identified their acetylation targets affected in vivo. I will also present our recent work on proteome-wide analysis of ubiquitylation in response to B cell receptor (BCR) and tumor necrosis factor alpha (TNF-a) receptor signaling. Our quantitative proteomic analyses of BCR-induced ubiquitylation identified an important function of linear ubiquitylation in BCR signaling. Our TNF-a receptor signaling analyses identified SPATA2 as a novel component of the TNF-a receptor signaling complex. We showed that SPATA2 interacts with the deubiquitylase CYLD, and recruits it to the receptor. Downregulation of SPATA2 augments transcriptional activation of NF-κB and inhibits TNF-α-induced necroptosis, pointing to an important function of SPATA2 in modulating the outcomes of TNF-α signaling.

Conclusion

Together, our proteomic investigations provide important insights in to the regulation and function of acetylation and ubiquitylation in human cells.

Keywords:

Acetylation, ubiquitination, ubiquitylation, mass spectrometry, SILAC, proteomic, PTM, posttranslational modifications, cell signaling

TK-06

Discovery and Characterization of a Family of Lysine Acylation Pathways

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Our recent studies detected and comprehensively validated eight types of new short-chain, lysine acylation histone marks: propionylation, butyrylation, crotonylation, malonylation, succinylation, glutarylation, lysine 3-hydroxybutyrylation, and 2-hydroxyisobutyrylation. We identified about 350 new histone marks, which more than doubles the number of previously described histone marks that were discovered over the past few decades. We used proteomic approaches to characterize the pathways by identifying key regulatory elements (substrates, enzymes, and binders), and study their functions. These pathways are mechanistically and physiologically distinguished from widely studied histone lysine acetylation pathway, and contribute to cellular dysfunctions associated with multiple inborn metabolic diseases.

In this presentation, we will report the identification and verification of a β-hydroxybutyrate-derived protein modification, lysine β-hydroxybutyrylation (Kbhb), as a new type of histone mark. Histone Kbhb marks are dramatically induced in response to elevated β-hydroxybutyrate levels in cultured cells, and in livers from mice subjected to prolonged fasting or streptozotocin-induced diabetic ketoacidosis. In total, we identified 44 histone Kbhb sites, a figure comparable to the known number of histone acetylation sites. By ChIP-seq and RNA-seq analysis, we demonstrate that histone Kbhb is a mark enriched in active gene promoters, and that the increased H3K9bhb levels that occur during starvation are associated with genes up-regulated in starvation-responsive metabolic pathways. Histone β-hydroxybutyrylation thus represents a new epigenetic regulatory mark that couples metabolism to gene expression, offering a new avenue to study chromatin regulation and the diverse functions of β-hydroxybutyrate in the context of important human pathophysiological states, including diabetes, epilepsy, and neoplasia.

Peptide and Glycan Mass Spectrometry Imaging as Diagnostic Tool in Cancer Research

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Introduction and Objectives

Imaging Mass Spectrometry (IMS) is typically used to determine the distribution of proteins in fresh frozen tissue. Tryptic Peptide and Glycan Imaging has some advantages over imaging of intact proteins. These include peptide level analysis provides the possibility for identification by matching accurate m/z and *in situ* MS/MS to high quality LC-MS/MS data obtained through digestion of relevant laser dissected tissue. Formalin-fixed paraffin embedded (FFPE) tissue can be analysed after antigen retrieval. A novel method for investigating tissue-specific N-linked glycans was recently developed by our group on formalin-fixed paraffin-embedded (FFPE) murine kidney. We have used those methods to potentially make diagnostic decisions for patients with endometrial and ovarian cancer.

Methods

Here we present the latest developments within our group, including up-to-date methods for analysis of FFPE tissue (e.g. tryptic peptide and PNGase F Glycan MALDI-IMS), a method for linking LC-MS/MS data of peptides to MALDI-IMS data using internal calibrants as well as the generation of the first data for a MALDI-IMS patient and disease specific tryptic peptide database and the use of tissue micro arrays. We present that IMS spatially profiles glycoforms in tissue-specific regions, while through liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) the corresponding glycol compositions are structurally characterized. These methods are applied to endometrial and ovarian cancer FFPE tissues.

Results and Discussion

Metastasis is a crucial step of malignant progression and remains the primary cause of death from solid cancers. In endometrial cancer lymph node metastasis is a crucial factor in the choice of treatment and prognosis of patients. As it is impossible to accurately predict lymphatic metastasis in individual patients, a large number of women who would be cured by local treatment alone, undergo radical surgery

including lymph node dissections. Peptide Imaging Mass Spectrometry is used in combination with laser capture dissection and LC-MS/MS to distinguish if patients have metastasis by analysing their primary tumour using FFPE tissue from large patient cohorts. Ovarian cancer is the most fatal gynaecological malignancy in adult women with a five-year overall survival rate of 30%. Patients presented with advanced metastatic disease, requiring tumour debulking surgery and chemotherapy 80% of patients who receive chemotherapy suffer disease relapse, 20% of patients who receive chemotherapy do not respond to treatment at all. We have used MSI and LC-MS/MS on FFPE tissue of chemotherapy responder and non-responders to distinguish them in order to tailor their treatment

Conclusions

We show the potential of tryptic peptide and glycan imaging on FFPE tissue as a diagnostic tool for metastasis of endometrial cancer and for chemotherapy response in ovarian cancer to give surgeons and oncologists a decision making tool to personalize the treatment of their patients.

Keywords

Mass spectrometry Imaging, Endometrial Cancer, Ovarian Cancer, Glycan Imaging

Molecular Imaging of the Brain by Mass Spectrometry

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Introduction and Objectives

Current neuroimaging techniques have limited abilities to directly identify and quantify chemical signaling molecules from brain tissue sections. Small-molecule neurotransmitters are important chemical messengers that mediate signaling between neuronal cells in the brain. Fluctuations in their concentrations are associated with numerous normal neuronal functions including sleep and aging, but they are also associated with pathological changes in neurological disorders such as Parkinson's disease (PD). With neurological processes involving multiple neurotransmitters and neuromodulators, it is important to have the ability to directly map multiple signaling molecules simultaneously in a single analysis.

Methods

Experiments were conducted using rodent (mouse, rat) brains (vehicle controls; Parkinson's disease 6-OHDA lesion model; and neuroactive drugs; 14 µm thick, sagittal or coronal tissue sections). Without modification, most neurotransmitters are not directly detectable by MALDI-MS due to their poor ionization efficiency and the overlapping signals of isobaric compounds. Therefore we developed a method for their in situ chemical derivatization. Our strategy uses pyrylium salts such as 2,4-diphenyl-pyranylium tetrafluoroborate (DPP-TFB), which react selectively with primary amines to produce N-alkyl- or N-aryl-pyridinium derivatives. DPP-TBF was applied onto the tissues using an automatic sprayer (TM-Sprayer, HTX Technologies) to produce related easy-to-ionize pyridinium derivatives. MALDI-MSI experiments were performed using Tof or FTICR (Ultraflextreme or solariX, Bruker Daltonics) mass spectrometers. Data processing, normalization and visualization were performed by in-house developed msIQuant software.

Results and Discussion

By utilizing a molecular-specific approach we demonstrated that multiple neurotransmitters and their metabolites (e.g., dopamine, dihydroxyphenylacetic acid, 3-methoxytyramine, homovanillic acid, serotonin, glutamate, glutamine, aspartate, γ-aminobutyric acid, adenosine), as well as neuropeptides, neuroactive drugs and drug metabolites can be imaged in situ directly in brain tissue sections. The number of identified molecular targets increased by the use of both positive and negative ionization MS modes. Chemical derivatization by charge-tagging the primary amines of molecules significantly increased the sensitivity, enabling the detection of low

abundant neurotransmitters and other neuroactive substances previously undetectable by MSI.

Conclusion

The methods presented here represent powerful tools for quantitative imaging of neurotransmitters, including biogenic amines, amino acids, and acetylcholine in histological brain tissue sections. The imaging approach of neurochemicals have a great potential in many diverse applications in fields such as neuroscience, pharmacology, drug discovery and development, and medicine.

Keywords:

Brain, neurotransmitter, neuropeptide, imaging, Parkinson's disease

TK-09

Systematic Analysis of One Carbon Metabolism by SRM. Implications in the Progression of Chronic Liver Disorders

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Introduction and Objectives

iver disorders are the fifth leading cause of death with a growing incidence in western countries. Despite great efforts and progress have been made in recent years improving our understanding of the molecular mechanisms underlying the progression of chronic liver diseases, there is still an urgent need for novel biomarkers and targets that may promote the development of more efficient therapeutic options. Metabolic remodelling is a common trait of all liver ailments from steatosis to HCC, where cancer cells adapt to their proliferative phenotype, which is known as Warburg effect. One of the essential pathways to preserve the quiescent and differentiated state of hepatocytes is one carbon metabolism. However, despite many studies are available reporting alterations on individual enzymes, no study has focusing on the complete pathway have been yet conducted to understand in detail the implications of one carbon metabolism in the molecular pathogenesis of chronic liver disease. As a general aim, we propose the comprehensive analysis of one carbon metabolism and connected pathways to define its alterations in different stages of chronic liver disease: steatosis, non-alcoholic steatohepatitis, liver fibrosis, cirrhosis and HCC.

Methods

To this end, one carbon metabolism related enzymes were monitored by selected reaction monitoring (SRM) in the liver of murine models of pathological conditions resembling the pathogenesis of liver injury, including tumors from two KO murine models defective on MAT1A and MDR2.

Results and Discussion

In a first approach, SRM methods have been developed for the systematic detection and relative quantification of 11 one carbon metabolism enzymes. The performance of the analytical methods has been assessed both, in cell lines and wild type and tumoral murine liver. In addition to its clear adaptation to the tumoral liver, one carbon metabolism displays remarkable differences in different tumors, suggesting mechanisms that might explain different sensitivity to chemotherapeutic agents. Connection of one carbon metabolism homeostasis with Gly and Thr metabolism as well as the protein methylation status, are issues under study.

Conclusion

Systematic measurement of one carbon metabolism enzymes provides means to evaluate the malignancy of a liver lesion and might be indicative of the susceptibility of liver tumors to chemotherapy.

Keywords: HPP (Chr16), one carbon metabolism, NAFLD, liver fibrosis, hepatocellular carcinoma, proteomics, protein methylation

TK-10

Drug-Drug Interaction: Monitoring CYP450 enzymes and Transporters in Mice and Men

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Introduction and Objectives

Toxicoproteomics focuses on toxicological mechanisms, drug-induced organ injury and the indirect detection of such events in body fluids (safety biomarkers). Within the liver endo- and exogenous substances - natural compounds, drugs, toxins, and chemicals – are metabolized. Proteins which activate, conjugate, and transport these substances are classified as phase I (CYP 450 system), phase II (UGTs, NATs, SUTs, and GSTs) and phase III (transporter) proteins. Gene transcription of those proteins can be triggered and induced by drugs or chemicals itself while binding to soluble nuclear receptors. This leads to faster transformation and elimination rates of the inducing substance, thereby also lowering the bioavailability of other drugs.For the evaluation of potential drug-drug interaction effects it is recommended by regulatory authorities to test the effects of drug candidates and chemicals on primary human hepatocytes by mRNA analyses. Here is a demand for fast and sensitive protein analyses methods which would allow more accurate quantification of the induction potential of drugs and chemicals.

Methods

Mass spectrometry-based immunoassays are used here to address toxicological issues. The antibodies used in these assays, comprise epitopes of four amino acid length (TXP-antibodies). Targeting conserved motifs in peptides from the same protein from different species enables the application of these antibodies for the analysis of samples derived from mice, rats, dogs, monkeys and men. The implementation of isotopically labeled reference standards during the capturing process combined with a mass spectrometry-based readout allows fast and flexible development of quantitative and highly specific assays.

Results and Discussion

To address the growing interest in pharmacologically and toxicologically relevant events like drug-drug interaction processes, we have developed fast, reliable, sensitive, and quantitative assays to monitor cytochrome P450 enzymes and transporters. The usage of TXP antibodies allowed the development of >100 MS-based immunoassays targeting these proteins in humans and animal models. We applied these assays for the analyses of induction studies in animals, primary human hepatocytes, and clinical human samples. The analyses of relevant drug metabolizing enzymes and transporters corroborated the translational character of animal studies and the relevance of protein analyses versus mRNA-profiling.

Conclusion

Overall, we used targeted proteomics methods in particular MS-based immunoassays to quantify drug-matabolizing enzymes and transporters in samples derived from induction

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studies in animals, in primary hepatocytes, cell cultures, and patient samples. The methods used here are suitable for the analyses and quantification of the expression of these molecular entities in in vitro and in vivo-induction studies.

Keywords: Targeted Proteomics, Immunoaffinity MS, Cytochrome P450, SISCAPA, TXP antibodies, Affinity Proteomics, Transporter, Drug-Drug Interaction

TK-11

A Protein Array Approach to Identify Novel mCpG Readers that Bind Methylated *cis*-Regulatory Elements to Activate Transcription

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Introduction and Objectives

In higher eukaryotes, DNA methylation in the *cis*-regulatory elements has always been viewed as the hallmark of gene silencing, and altered DNA methylation status has been found to often associate with human diseases and cancers. However, recent analyses of the DNA methylomes and global gene expression profiles have revealed that the promoters of as many as 20% highly expressed genes are heavily methylated in tumors, suggesting that, at least in some cases, DNA methylation positively correlates with gene expression. Using human protein arrays as a screening platform, we recently discovered that many human transcription factors (TFs) could interact with methylated DNA in vitro and shown that one of the four Yamanaka factor, KLF4, recognized a new motif only when it was methylated, and that an Arg458-to-Ala (R458A) mutation completely abolished this activity but showed no impact on interaction with its canonical unmethylated motif. These emerging evidences suggest that TFs can act as a new class of methylated DNA readers. However, whether such mCpG-dependent binding activity plays a significant physiological role in cells remains elusive. Here, we took advantage of the R458A in KLF4 and demonstrated that mCpG-dependent DNA binding activity yielded strong phenotypes in primary brain tumor cells via direct activation of genes responsible for cell morphology, cell adhesion and migration. More importantly, recruitment of KLF4 to inactive chromatins leads to a global chromatin remodeling.

Methods

We used a protein microarray-based approach to systematically survey the entire human TF family with 154 DNA motif sequences that each carries at least one methylated CpG site and found numerous purified TFs with methylated CpG (mCpG)-dependent DNA-binding activities.

Results and Discussion

Interestingly, we found that 17 TFs exhibit specific binding activity to methylated and unmethylated DNA motifs of distinct sequences. To elucidate the underlying mechanism, we focused on Kruppel-like factor 4 (KLF4), and decoupled its mCpG- and CpG-binding activities via site-directed mutagenesis. Furthermore, KLF4 binds specific methylated or unmethylated motifs in human embryonic stem cells in vivo. We took advantage of the R458A in KLF4 and demonstrated that mCpG-dependent DNA binding activity yielded strong phenotypes in primary brain tumor cells via direct

activation of genes responsible for cell morphology, cell adhesion and migration. More importantly, recruitment of KLF4 to inactive chromatins leads to a global chromatin remodeling.

Conclusions

Our study suggests that mCpG-dependent TF binding activity is a widespread phenomenon and provides a new framework to understand the role and mechanism of TFs in epigenetic regulation of gene transcription. It also provides a new paradigm of DNA methylation-mediated gene activation and chromatin remodeling and a general framework to dissect the biological functions of DNA methylation readers and effectors.

Keywords

Human protein arrays – Fabricated by spotting down indivudally purified human proteins on glass slides at high density

DNA methylation – A covalent modification by a methyl group at the 5-position of the cytosine in double-stranded DNA in eukaryotes

TK-12

Validation of Antibodies to Study the Human Proteome

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To begin to address the problem of antibody specificity, selectivity, and reproducibility in biomedical research, an ad hoc International Working Group for Antibody Validation (IWGAV) has been formed to delineate the scientific issues related to the use of antibodies with a focus on protein analysis in research applications. The primary objective is to formulate best practice approaches for validating antibody specificity for commonly used applications. These recommendations will be further developed in accordance with feedback received from the research community. These guide-lines will be followed in the classification of all the protein coding genes in humans using a combination of genomics, transcriptomics, proteomics and antibody-based profiling (1). We have used this data to study the global protein expression patterns in human cells, tissues and organs as well as a discovery tool to find potential biomarkers and drug targets for disease (2,3,4). As part of this effort, we have developed bacterial cell factories to generate 55,000 human protein fragments called PRESTs that has been used primarily as antigens for antibody generation and secondly as standards in mass spectrometry based targeted proteomics (5,6). The experience from these efforts will be discussed.

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Advances in Glycoproteomics Facilitate the Discovery of a New Class of Functionally Important Cancer and Inflammation-Centric Human Glycoproteins

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Introduction and Objectives

The human glycoproteome remains severely understudied due to the analytical challenges associated with the system-wide site-specific analysis of intact glycopeptides defined as glycoproteomics. We here demonstrate how recent advances in *N*-linked glycoproteomics have paved the way for novel structural insights into glycoimmunology and disease-related glycobiology.

Methods

With equal emphasis on recent technological advances in LC-MS/MS-based *N*-glycoproteomics and on examples of biological applications in infection/inflammation and neutrophil biology, this talk will showcase the potential of the rapidly maturing discipline of glycoproteomics for studying the structural features of the *N*-glycoproteome and their regulation.

Results and Discussion

Recent developments in glycoproteomics technologies and the beneficial interplay with modern glycomics and glycogene-centric proteomics will first be presented (Parker et al., *Mol Cell Proteomics*, 15(1):141-53, 2016 and Thaysen-Andersen et al., *Mol Cell Proteomics*, 15(6):1773-90, 2016). These developments were crucial for the recent discovery of a class of previously overlooked truncated human *N*-glycoproteins, called paucimannosidic proteins, in neutrophils in the innate immune system (Thaysen-Andersen et al., *J Biol Chem*, 290(14):8789-802, 2015 and Loke et al., *Biomolecules*, 5(3):1832-54, 2015). The paucimannosidic proteins, which display relative simple monosaccharide compositions i.e. $GlcNAc_2Man_{1-3}Fuc_{0-1}$, were also shown to be enriched in inflamed tumour-microenvironments (Sethi et al., *Glycobiology*, 25(10):1064-78, 2015). Orthogonal evidence in glycobiology supports and mechanistically explains the immune-centric expression and biosynthesis of paucimannosidic proteins in human immune cells and provide clues regarding their functions including the involvement in cell-cell communication and pathogen defence within the innate immune system (Loke et al., *Mol Aspects Med*, *In press*, 2016).

Conclusions

Although analytical challenges still remain for the system-wide analysis of intact

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glycopeptides, glycoproteomics is now sufficiently mature to utilise these powerful tools to probe the extreme molecular complexity displayed by the *N*-glycoproteome. This opens new exciting opportunities to increase our understanding of the functional roles of protein *N*-glycosylation in health and disease.

Keywords
Glycoproteomics, *N*-glycopeptide, Innate immunity, Neutrophils, Glycobiology.

TK-14

Accurate Mass- and Glycan Composition-Based Assignment of Glycosylation Site-Specific Glycomes of Complex Glycoprotein Mixture

HIROYUKI KAJI[1]; Azusa Tomioka[1]; Erika Noro[1]; Mika Fujita[1]; Masako Sukegawa[1]; Chiaki Okatani[1]; Toshihide Shikanai[1]; Hisashi Narimatsu[1]

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Introduction and Objectives

Protein glycosylation is a post-translational modification and has important roles for expression and regulation of activity of the proteins to be glycosylated. Until recently, glycoproteomic analysis means large-scale identification of glycopeptides which are enriched by affinity-based chromatography or chemical capture via glycans, followed by mass analyses after removal of the glycan moiety from the glycopeptides with PNGase or endoglycosidase. This approach provides a large number of glycosylation sites of the glycosylated proteins, but cannot reveal glycan structural information on each site. To uncover the roles of glycans, especially those of specific glycan motifs such as Lewis antigens, branching, LacdiNAc, and polylactosamine, it is important to reveal what glycans are attached to which sites and how they are changed in association with diseases and stimuli. Therefore, we developed a method to assign site-specific glycomes at proteomic scale.

Methods

Glycopeptide samples were collected by HILIC from the protease digests of isolated glycoproteins and protein mixtures and treated with acid to remove sialic acids. An aliquot of the desialylated glycopeptides was analyzed by the IGOT-LC/MS method to identify the core peptides contained. Released glycans were assigned by MALDI-TOF MS after permethylation. Another aliquot was analyzed by LC/MS using a high accuracy LTQ-Orbitrap mass spectrometer to obtain the accurate masses and retention times of the glycopeptides. According to the differences of the masses and retention times between signals, glycopeptide signals were selected as clusters using software we developed. By matching the masses of the core peptides and glycopeptides, combinations of the core peptides and glycan compositions were assigned.

Results and Discussion

We applied this method to the analyses of glycopeptides prepared from isolated glycoproteins, cultured cells, and mouse tissues and efficiently obtained the large-scale site-specific glycome information. There were many cases that each site of the same proteins has different glycan structures. By comparison of the site-specific glycomes of kidney glycopeptides of wild type and Fut9 (Lewis x-synthesizing enzyme) knockout mice, many Lewis x-carrying glycoproteins could be identified (Noro E. et al. 2015, J. Proteome Res.)

Conclusion

This method will serve as a useful tool for comprehensive elucidation of site-specific glycan heterogeneity. The complementally use of this multistep dissociation (MSn)-independent manner with MSn-based methods will improve the sensitivity of

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glycopeptide assignment significantly.AcknowledgementWe are grateful for Prof. Kiyoko F. Kinoshita (Soka Univ) and Dr. Issaku Yamada (Noguchi Inst.) for cooperation on the software development.

Keywords: Glycoproteomics, site-specific glycome, glycan heterogeneity

TK-15

Positional Proteomics Technologies to Functionally Annotate Tissue Proteomes in Pathology: Mechanistic Analysis Linear Ubiquitination by LUBAC in Immunodeficiency Disease by TAILS

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Introduction and Objectives

A mutation in the lymphocyte human *MALT1* gene led to deficiency in MALT1 protein and loss of paracaspase proteolytic activity and molecular scaffolding key for CARMA1–BCL10–MALT1 (CBM) complex formation. The CBM transduces signaling from lymphocyte antigen receptors. MALT1 cleaves and removes negative checkpoint proteins to amplify lymphocyte responses in NF-κB activation, but has proven intractable to conventional proteomics analysis. Thus, the discovery of this mutation in the only known living patient with this combined immunodeficiency disease provided a unique opportunity to identify new MALT1 substrates through *positional proteomics* using TAILS, a N-terminomics technique.

Methods

We compared B cells from the MALT1$^{mut/mut}$ patient with healthy MALT1$^{+/mut}$ family members and normal individuals using 10-plex Tandem Mass Tag TAILS with MS3 synchronous peak selection quantification, with and without antigen receptor stimulation.

Results and Discussion

We identified >47,000 peptides from 5,278 unique proteins and 7,498 unique N-termini identified at FDR <0.01. From the MALT1 cleaved neo-N terminal peptide (prime side) and the natural N terminus of HOIL1 identified by TAILS, and the nonprime side of the HOIL1 cleavage site identified by preTAILS shotgun proteomics, we identified HOIL1 of the linear ubiquitin chain assembly complex (LUBAC) as a novel MALT1 substrate. Upon B and T cell receptor stimulation HOIL1 cleavage resulted in disassembly of LUBAC and loss of linear ubiquitination in T and B cells, including of the targets NEMO and RIP1. HOIL1 cleavage, dampened LUBAC-mediated NF-κB activation at later times and prevented reactivation of NF-κB signaling—deficiency of which in the patient is consistent with the clinical phenotype.

TK-15

Conclusions

This is the unique example of positional proteomics to mechanistically decipher a human disease. Our unexpected findings show that MALT1 is both a positive and negative regulator of the human canonical NF-κB pathway—first promoting activation via stabilizing protein interactions in CBM assembly—then triggering HOIL1-dependent negative-feedback to terminate NF-κB signaling and so prevent reactivation and chronic inflammation.

Keywords

TAILS, terminomics, NF-κB, LUBAC, HOIL1, HOIP, SHARPIN, linear ubiquitin, BCR, TCR, B cells, T cells, immunity

TK-16

Ubiquitin Proteomics – Revelations of a Master Manipulator

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The ubiquitin molecule comprises a significant fraction of total cellular protein and regulates a wide range of processes. Research studies using mass spectrometry have demonstrated that the majority of proteins within a eukaryotic cell can be covalently modified by ubiquitin. These post-translational modifications control protein stability, interactions, activity, and localization. Inducible ubiquitination is a common hallmark of many cellular stresses, xenobiotics, and genetic perturbations, and can provide insights into acute remodeling of proteins and pathways within cells. With these vastly heterogenous functions, designing experiments to elucidate the roles of ubiquitin, ubiquitin regulatory enzymes, and ubiquitin receptor proteins within specific pathways involves careful balancing of cell biology, protein biochemistry and analytical mass spectrometry. Particularly valuable, immunoaffinity enrichment coupled with mass spectrometry provides a means to directly assay inducible ubiquitination events on a broad range of substrates. This talk will focus on investigation of substrate specific ubiquitination in the context of small molecule drug development.

Keywords: ubiquitinmass spectrometrydegradationE3 ligasedeubiquitination

TK-17

The Human Cell Atlas

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Introduction and Objectives

Compartmentalization of biological processes is a fundamental principle of eukaryotic cells that enables multiple processes to occur in parallel. The Cell Atlas aims to systematically localize the human proteome using an antibody-based approach as part of the Human Protein Atlas project.

Methods

Data generation include automated sample preparation, high-resolution confocal microscopy and computational image analysis. The integrative approach includes strict validation criteria using gene silencing, paired antibodies and fluorescently tagged proteins.

Results and Discussion

In total, over 13000 human proteins have been localized to 25 organelles and cellular structures. The high spatial resolution allows identification of novel protein components of fine cellular structures such as the cytokinetic bridge, nuclear bodies, and rods and rings. Interestingly, as much as 40% of all proteins localize to multiple compartments and 15% show cell cycle dependent expression.

Conclusion

Here we discuss the importance of spatial proteomics for cell biology, including the citizen science effort “Project Discovery”, and present the content of the Cell Atlas as well as the path ahead to define the spatiotemporal organization of the human proteome.

Keywords: Human Protein Atlas, Cell Atlas, Spatial proteomics, Immunofluorescence, Antibody

TK-18

Analysis Of Tumor Heterogeneity in Three Dimensions by Imaging Mass Cytometry

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Introduction and Objectives

Cancer is a tissue disease. Heterogeneous cancer cells and normal stromal and immune cells form a dynamic ecosystem that evolves to support tumor expansion and ultimately tumor spread. The complexity of this dynamic system is the main obstacle in our attempts to treat and heal the disease. The study of the tumor ecosystem and its cell-to-cell communications is thus essential to enable an understanding of tumor biology, to define new biomarkers to improve patient care, and ultimately to identify new therapeutic routs and targets. To study and understand the workings of the tumor ecosystem, highly multiplexed image information of tumor tissues is essential. Such multiplexed images will reveal which cell types are present in a tumor, their functional state, and which cell-cell interactions are present.

Methods

To enable multiplexed tissue imaging, we developed imaging mass cytometry (IMC). IMC is a novel imaging modality that uses metal isotopes coupled to antibodies as reporters. The antibodes are bound to epitopes of a tissue. A laser ablation system coupled to a CyTOF mass cytometer is then used to visualize the isotope/antibody distribution. IMC currently allows to measure over 50 antibodies simultaneously on tissues with subcellular resolution. In the near future we expect that over 100 markers can be visualized.

Results and Discussion

We applied IMC for the analysis of hundreds of breast cancer samples in a quantitative manner. To extract biological meaningful data and potential biomarkers from this dataset, we developed a novel computational pipeline geared for the interactive and automated analysis of large scale, highly multiplexed tissues image datasets. Our analysis reveals a surprising level of inter and intra-tumor heterogeneity and identify new diversity within known human breast cancer subtypes as well as a variety of stromal cell types that interact with them. Furthermore, we identified cell-cell interaction motifs in the tumor microenvironment correlating with clinical outcomes of the analyzed patients.

Conclusions

Our results show that IMC provides targeted, high-dimensional analysis of cell type, cell state and cell-to-cell interactions within the TME at subcellular resolution. Spatial relationships of complex cell states of cellular assemblies can be used as biomarkers.

TK-18

We envision that IMC will enable a systems biology approach to understand and diagnose disease and to guide treatment.

Keywords
Single cell proteomics, highly multiplexed tissue imaging, mass cytometry.

TK-19

Recent Progress and New Projects for the HUPO Proteomics Standards Initiative

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Introduction and Objectives

The HUPO Proteomics Standards Initiative (PSI) defines community standards for data representation in proteomics to facilitate data comparison, exchange, and verification. We contribute to data management and integration in all other HUPO initiatives, and have published a modular set of standards for proteomics data representation.

Methods

The PSI hosts workshops, develops formats and guidelines, and fosters synergistic activities with other groups. The PSI hosts a 3-day workshop every spring (~April) to gather input from many participants around the world to provide input for ongoing projects; the 2017 workshop will be held in Beijing, China. There are currently multiple formats and guidelines that are either being finalized or undergoing updates to accommodate new technological innovations in the field of proteomics.

Results and Discussion

The PSI Enhanced FASTA Format (PEFF) is being designed to encode protein sequences along with extensive annotations such as PTMs and sequence variations for the next generation of sequence search engines that will automatically take all such variations annotated in the public databases into account. The proBAM and proBED formats are being designed as a standardized mechanism for encoding the output of proteomics analysis in the BAM and BED formats popular in the genomics field, so that the proteomics results may be more easily viewed with genomics data viewers and integrated with genomics data. The mzIdentML format is being updated to version 1.2 in order to support advanced features such as cross-linking data. The PSI-MI molecular interactions format is being updated to 3.0 in order to support advanced features such as conditional constraints on when interactions may take place.

Conclusion

The HUPO PSI continues to develop formats and guidelines that support public repositories and other modes of data sharing, and enable new methods of data analysis and integration. The past successes and future directions of the PSI will be presented in order to foster discussion about existing formats and new opportunities.

Keywords: Standards, formats, mzML, mzIdentML, PEFF, molecular interactions, controlled vocabularies, bioinformatics

TK-20

The Golgi Localized UDP-GlcNAc transporter is required for the Maturation of Complex N-glycans in Plants

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Introduction and Objectives

Glycosylation reactions require activated glycosyl donors in the form of nucleotide sugars to drive processes such as post-translational modifications and polysaccharide biosynthesis. Most plant cell wall polysaccharides are synthesized in the Golgi apparatus from cytosolic-derived nucleotide sugars, which are actively transferred into the Golgi lumen by nucleotide sugar transporters (NSTs). We have established a yeast proteoliposome transport assay coupled to LC-MS specifically designed to screen substrates of plant encoded NSTs. Using this approach we recently identified a plant UDP-GlcNAc transporter responsible for the delivery of substrate for the maturation of N-glycans.

Methods

To ascertain the biochemical phenotype of UDP-GlcNAc transporter loss-of-function mutants, we have been using HILIC to enrich glycopeptides from plant samples then identifying and characterizing glycopeptides using complementary fragmentation approaches. This involves the generation of an ETD spectrum based on the presence of an oxonium ion derived from the glycan (e.g. HexNAc, 204.09 m/z) in the HCD spectra. This approach has enabled us to profile thousands of glycopeptides from our samples.

Results and Discussion

The analysis of glycopeptides from the loss-of-function UDP-GlcNAc transporter mutant indicated that peptides containing complex glycans only comprised about 7% of the population compared to wild-type plants which contained about 33% of the population. Significantly, over 50% of the glycopeptide population of the loss-of-function mutant contained high mannose structures, namely HexNAC(2)Hex(5), which represents the structure prior to the addition of the first GlcNAc in the Golgi lumen. In order to confirm that these observation were consistent with our expectations for a reduction in lumenal UDP-GlcNAc, we also analyzed the Arabidopsis N-acetylglucosaminyltransferase mutant (gnt1) which adds the first lumenal GlcNAc to N-glycans. Similar to the loss-of-function UDP-GlcNAc transporter samples, the gnt1 mutants were enriched in high mannose N-glycan structures, supporting the in vivo role for the biochemically characterized UDP-GlcNAc transporter.

Conclusion

Our findings indicate that the reference plant Arabidopsis contains a single UDP-GlcNAc transporter responsible for the maturation of complex N-glycans in the Golgi lumen.

Keywords: UDP-GlcNAc, membrane transport, glycoproteomics, Golgi apparatus, N-glycans, Arabidopsis

WK-01

Kinase-Centric Pharmacoproteomics for Molecular-Targeting Drug Discovery

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Introduction and Objectives

Rapid progress has been made in mass spectrometry-based phosphoproteomics with specific enrichment methods of phosphopeptides, and the current technology allows to identify thousands of phosphopeptides from complex biological samples. Further improvement will be achieved by increasing the resolution both in m/z and retention time in LCMS separation. We have employed meter-long monolithic silica C18 columns to improve LC separation, identifying more than 10,000 unique phosphopeptides from 0. 1 mg of HeLa lysates with sequential elution of singly phosphorylated peptides followed by multiply phosphorylated peptides from a single titanium dioxide microcolumn. Together with 10-plex tandem mass tags, this approach allows high throughput quantitative phosphoproteome analysis of human samples for routine use. For most of the phosphosites, however, their biological functions have not been well characterized. So, kinase-centric approaches rather than phosphoproteomics approaches would be preferable to link the MS results to drug discovery. Here I would like to present kinome profiling approaches with molecular-targeting drugs.

Methods

Two LC-MS systems with Sciex TripleTOF 5600 or Thermo Q-Exactive have been used through this study. Peak lists were created from the recorded product ion spectra. Peptides and proteins were identified by means of automated database searching using Mascot (Matrix Science, London) against UniprotKB. A part of data were deposited to a public proteome repository named jPOST.

Results and Discussion

In order to predict the sequence preference of phosphorylation by kinase, public database for protein-protein interaction including kinase was used. Using the reported kinase-substrate relationship as the reference, it was found that the success rate for the prediction was approximately 60%. Next, we experimentally obtained the kinase-substrate relationship using 3 recombinant kinases and the dephosphorylated lysate. Thousands of the substrates were identified for each kinase and the phosphorylation motifs were extracted. Using position weight matrix and fold increase, we generated a new measure to predict the kinase substrate, named phosphorylation sequence preference (PSP) score. The PSP score was used to design an artificial substrate peptide for each kinase with specificity and sensitivity. Finally, a novel approach called motif-targeting quantitation method was developed for human phosphorylation stoichiometry typing and high sensitivity quantitation. By using this approach, we successfully quantify the phosphorylation changes of kinases belonging to MAPK pathway, induced by MEK inhibitor.

Conclusion

WK-01

Our kinase -centric approaches based on phosphoproteomics have been successfully applied to cell signaling studies for drug discovery.

Keywords: Kinase, phosphoproteomics, in vitro kinase, molecular-targeting drug

Quantitative Proteomics to Support the Discovery and Development of Targeted Drugs

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Introduction and Objectives

Mass spectrometry-based proteomics has emerged as a powerful technology platform to address many key aspects in drug discovery and development. While chemical proteomics technologies enable target deconvolution of bioactive compounds and proteome-wide drug selectivity studies, global protein expression and modification analyses support unbiased drug mode-of-action and target engagement studies as well as the discovery of target and biomarker candidates.

Methods

To efficiently support drug discovery programs, flexible proteomics solutions are required enabling comprehensive, quantitative analysis of any type biological sample material. Furthermore, appropriate experimental designs and statistics are mandatory to identify significant regulation events or specific targets. We have developed quantitative mass spectrometry strategies to both identify and quantify drug interactions with their cellular targets, and systematically compared different isotope labeling approaches and label-free quantification to facilitate large-scale quantitative proteomics in primary cells, tissues and body fluids.

Results and Discussion

Our chemical proteomics strategy enabled both cellular selectivity profiling and target deconvolution upon cell-based phenotypic screening, as demonstrated for diverse small molecule compounds. To advance cellular mode-of-action analysis of targeted drugs, we compared SILAC and mTRAQ labeling when quantifying phosphoproteome regulation upon inhibitor treatment. Our data established mTRAQ quantification as an alternative in case SILAC labeling is not practicable, for example when analyzing non-dividing cells or tissue samples. We further combined quantitative phosphoproteomics with tightly controlled pharmacological inhibition of the mitotic kinase Mps1. We identified 19 significant phosphorylation changes among more than 10,000 quantified sites, highlighting specific Mps1 functions at the kinetochore-microtubule interface. Finally, we systematically evaluated label-free and SILAC quantification for high-coverage proteome expression analysis. Our results benchmarked and emphasized the utility of label-free proteome quantification, which we have applied to study molecular pathologic changes in animal brain tissue or intratumoral heterogeneity in colon cancer patients.

Conclusion

These examples illustrate how advances in quantitative proteomics translate into dedicated applications to support the discovery and evaluation of drugs, drug targets and investigational biomarkers.

Keywords: Drug discovery, chemical proteomics, phosphoproteomics, protein kinases, tissue proteomics

WK-03

A Novel Data-Independent Acquisition (DIA) Mass Spectrometry Approach Integrated With RNA-Seq for Deep Proteogenomic Profiling

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We have recently initiated integrated analysis of mRNAs with high resolution mass spectrometry for proteogenomic analysis of individual cell types. The availability of corresponding RNA-seq data provides a unique opportunity to search a database of peptides encoded only by mRNAs expressed in the individual cell types – i.e. intra-exonic as well as junctional (encoded by exon-exon junctions) peptides. This strategy also allowed us to directly interrogate the non-synonymous SNPs that were translated into proteins. However, one problem of current data-dependent approaches is that the more abundant peptides are readily identified while those that are less abundant (e.g. regulatory proteins such as transcription factors, certain alternative protein isoforms, proteins with alternative start sites, peptides from translation of microORFs) are not sampled. Data-independent acquisition (DIA) strategies provide an opportunity to sample peptides that would otherwise not be selected for fragmentation. However, the current implementation of such DIA approaches relies on very large m/z windows, which precludes identification of low abundance peptides. We have developed a novel pipeline to carry out DIA analysis on an Orbitrap Fusion Lumos mass spectrometer using very small m/z windows for fragmentation. We analyzed primary human umbilical vein endothelial cell (HUVEC) lysates by scanning sequentially through 150 Th windows in each LC-MS/MS run using the advanced quadrupole mass filter followed by fragmentation of peptide ions in the HCD cell in small m/z windows and ultimate detection of fragment ions in the Orbitrap mass analyzer. Several runs were carried out to cover the entire (350-1,450 m/z) mass range on an Orbitrap Fusion Lumos mass spectrometer. Our strategy allowed us to “enrich” several low abundance proteins and peptides, including post-translationally identified peptides, which would otherwise be missed by conventional strategies. The expression of these low abundance proteins was corroborated with the expression of transcripts in RNA-seq data. Overall, this approach can be coupled to any upstream fractionation/enrichment method for a more comprehensive characterization of the proteome.

WK-04

A Proteogenomic Analysis of Early Onset Gastric Cancer

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Introduction and Objectives

EOGCs are characterized by female predominance, diffuse histology, and aggressive clinical course. Molecular characterization is a high priority for EOGC due to its aggressive clinical course and to infrequent endoscopic surveillance in young groups.

Methods

Here, we performed an integrated proteogenomic analysis of early onset gastric cancers (EOGCs) using exome and mRNA sequencing and global proteome, phosphoproteome, and glycoproteome profiling.

Results and Discussion

Single amino acid variants (SAAVs) identified by exome sequencing and supported by proteomic and mRNA transcriptomic data enabled effective prioritization of candidate driver genes that are functional at the protein level. Protein abundance changes between paired tumor and adjacent normal tissues were poorly predicted by mRNA abundance changes, indicating that protein measurements enabled a more reliable evaluation of cancer-related alterations of cellular processes. Integrated proteogenomic analysis identified three subtypes of EOGCs (subtypes 1-3), with subtypes 1 and 3 not distinguishable by mRNA transcriptomic data only; subtypes 1 and 3 associated with PI3K-AKT signaling by integrating mRNA transcriptomic, proteomic, phosphoproteomic, and glycoproteomic data; and phosphoproteome data revealing that subtype 3 is uniquely associated with RhoA signaling. Interestingly, somatic SAAVs of RhoA were enriched in subtype 3.

Conclusion

Our integrated proteogenomic analysis provided a more complete and better refined molecular characterization of EOGCs than genomic analysis alone, thus affording a paradigm for enhanced understanding of cancer biology and a roadmap for patient stratification as it relates to this disease.

Keywords: Early Onset Gastric Cancer, Proteogenomics, Exome-seq, mRNA-seq, phosphoproteome, network analysis

Proteomic-Scale Approaches for Identifying Reversible and Irreversible Cysteine Redox PTM in Myocardial Ischemia / Reperfusion

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Introduction and Objectives

Redox post-translational modifications (PTM) are emerging as important regulatory mechanisms in signaling and pathogenesis. Cysteine (Cys) is the most redox active amino acid and is a target for these PTM, some of which are biologically reversible (e.g. disulfides, S-nitrosylation, sulfenic acid) while others (sulfinic [Cys-SO2H] and sulfonic [Cys-SO3H] acids) are considered irreversible. Blockage of coronary flow leading to myocardial ischemia can result in reversible contractile dysfunction or irreversible cardiomyocyte apoptosis and necrosis depending on the duration of hypoxia. While reperfusion is critical to myocyte survival, it comes with the burden of an influx of reactive oxygen species (ROS) that may be generated via uncoupling of mitochondrial electron transport, elevated myocardial succinate, reduced activities of antioxidants, conversion of xanthine dehydrogenase to oxidase and immune cell infiltration. Elevated ROS contribute to contractile dysfunction via protein oxidation and unfolding during ischemia / reperfusion (I/R) injury, however the molecular targets of ROS remain poorly understood.

Methods

We have developed enrichment methods to examine reversible and irreversible Cys redox PTM on a proteome-wide scale. Rapid and specific alkylation of free Cys, followed by thiol-based reduction and resin capture by thiol-disulfide exchange chemistry was applied to isolate reversibly modified Cys-containing peptides. We next developed an enrichment method to isolate Cys-SO2H/SO3H-containing peptides from complex tissue lysates. The method is based on electrostatic repulsion of Cys-SO2H/SO3H-containing peptides from cationic resins (i.e. ‘negative’ selection) followed by ‘positive’ selection using hydrophilic interaction liquid chromatography (HILIC) prior to analysis by tandem mass spectrometry.

Results and Discussion

The method was applied to a protein lysate generated from rat myocardial tissue and 6559 reversibly redox modified Cys-containing peptides from 2694 proteins were identified by tandem mass spectrometry (MS/MS). We next identified 181 Cys-SO2H/SO3 H sites from rat myocardial tissue subjected to physiologically relevant concentrations of H2O2 (<100 µM) or to I/R injury via Langendorff perfusion. I/R significantly increased Cys-SO₂H/SO3H-modified peptides from proteins involved in energy utilization and contractility, as well as those involved in oxidative damage and repair. Finally, we have combined these methods to enable a multiplexed quantitative analysis of reversible/irreversible Cys redox PTM in response to I/R and in the presence of a broad-spectrum thiol-reactive antioxidant (N-2-mercaptopropionyl glycine, MPG). We quantified >1350 Cys sites that are reversibly and/or irreversibly oxidized by I/R, including many sites that are protected by MPG.

WK-05

Conclusion

This technique allows for the quantitative profiling of reversible/irreversible Cys PTMs in response to oxidant / antioxidant stimulus, and their delineation within the context of protein abundance, during I/R injury and cardioprotection.

Keywords: Cysteine, Redox, Mass Spectrometry

ROSics: Principle of ROS in Oxidative Modifications and Structural Regulations

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Introduction and Objectives

Reactive oxygen species (ROS) are key molecules regulating various cellular processes. However, what the cellular targets of ROS are and how their functions are regulated are not well understood. Cysteine (Cys) residues are major targets of ROS, the least abundant amino acid (1~2%) of all amino acid residues, are unique in that they play key roles in maintaining stability of protein structure, participating in active sites of enzymes, regulation of protein function and in metal binding, among others.

Methods

This study will discuss the newly developed proteomic technologies to identify the Cys-containing ROS target proteins and their redox sensitivity, as well as the sites and species of their PTMs.

Results and Discussion

Cutting edge proteomics tools by employing peptide sequencing with UPLC-ESI-MS/MS adopting our MS and informatics tools including SEMSA, MODi and DBond, have also revealed that Cys residues are modified in numerous ways, not recognized until recently: thiosulfinate and thiosulfonate similar to disulfide, oxidation to sulfenic, sulfinic, sulfonic acids and thiosulfonic acid, transformation to dehydroalanine (DHA) and serine etc.. In order to identify ROS target proteins containing redox sensitive Cys residues, we have developed the chemical probe (NPSB-B) labeling specifically redox sensitive Cys residues and found that the redox sensitivities of Cys residues are regulated by the modified environments. To confirm the possible structure-activity relationship of redox sensitive proteins, we have employed hydrogen-deuterium exchange mass spectrometry (HDX-MS), which have revealed stepwise protein oxidation of Nm23-H1/NDPKA and calcium binding proteins in response to oxidative stresses.

Conclusion

We suggest the new name “ROSics” for the science of describing the principles of mode of action of ROS at molecular levels. [Supported by Global Research Laboratory Program of NRF]

Keywords: Reactive Oxygen Species, Cysteine oxidation, Proteomics, Chemical probe for redox sensitive cysteine, Hydrogen-deuterium exchange MS, Stepwise oxidation, ROSics

Decoding Ligand Receptor Interactions

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Introduction and Objectives

Ligand-induced changes in cell surface receptors result in physiological responses, which constitute the biological activity of various ligands such as proteins, peptides, pharmaceutical drugs, toxins or whole pathogens. However, traditional approaches for the ligand-based identification of corresponding receptors are usually limited to non-transient, high affinity interactions and highly artificial experimental set-ups. Therefore, many signaling molecules remain orphan ligands without a known primary molecular target – invaluable information in understanding the respective mechanisms of signal transduction, drug action or disease. Previously, we have developed the cell surface capturing (CSC) technology for the unbiased identification and quantification of cell surface N-glycoproteomes by mass spectrometry (MS). This demonstrated the powerful applicability of chemical reagents in the tagging of cell surface glycoproteins at carbohydrate groups and the subsequent purification of the corresponding peptides for MS analysis.

Methods

Based on these results we now synthesized a set of trifunctional cross-linkers for the ligand-based tagging of glycoprotein receptors on living cells and the purification of receptor-derived peptides for MS analysis. Through quantitative comparison to a sample generated with an unspecific control probe, this ligand-based receptor capturing (LRC) approach allows for the highly specific and sensitive detection of ligand interactions with their corresponding receptors under near-physiological conditions.

Results and Discussion

Experiments with ligands ranging from peptide hormones to clinical antibodies demonstrate the potential of this approach to specifically identify one or more target receptors for a given ligand with great statistical power. Advanced discovery-driven applications reveal potential receptors and receptor panels for ligands ranging from protein domains to intact viruses.

Conclusions

Together, I will present a short summary of our recent biomedical research to understand the surfaceome as a cellular signaling gateway and a chemoproteomic technology for the unbiased detection of ligand-receptor interactions on living cells.

Keywords

Ligand, Receptor, Dynamic Protein-Protein Interactions, Surfaceome

WK-08

Targeting Epidermal Growth Factor Receptor (EGFR) in Lung Cancer: Towards Interactome-Informed Medicine

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The Epidermal Growth Factor Receptor (EGFR) is a receptor tyrosine kinase (RTK) that, once activated upon ligand-binding, leads to receptor dimerization, recruitment of protein complexes and the triggering of multiple signaling cascades. EGFR is frequently overexpressed or mutated in various cancers leading to aberrant signaling and ultimately tumor growth. Hence, identification of protein interactors that specifically bind to mutated EGFR can help identify novel targets for drug discovery.

During my talk, I will report on our newly developed cell based high-throughput proteomics screening technology, called the Mammalian Membrane Two-Hybrid (MaMTH) assay. In addition, I will discuss how our recent application of MaMTH to human EGFR identified several novel interactors of oncogenic EGFR (L858R) capable of promoting persistent activation of aberrant signaling in non-small cell lung cancer (NSCLC) cells. Lastly, I will also demonstrate how MaMTH can efficiently be used as a drug discovery assay for identification of inhibitory compounds that change the phosphorylation status of full-length EGFR proteins in the context of living cells and in the low nanomolar range, an advance which may open up a whole new approach to drug development and lead to more effective treatments for lung cancer patients.

Food and Nutrition Proteomics: Focus on Food Allergens and Allergies

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Introduction and Objectives

Biology and Disease- Human Proteome Project has very recently (2016) approved the new initiative called Food and Nutrition Proteomics. In this light, we activated different study group that address to answer to several arguments, from diet including food safety and quality till to interaction with microbiome. One of the main goal of this action is to promote proteomics inside the study of food allergen- allergies. « Quod aliis cibus est aliis fuat acre venenum. »(Tito Lucrezio Caro, De rerum natura, Book IV (from v. 637) ' What it is food for someone is poison for others'. The definition of allergy includes immunological response, and, in the case of food allergy reaction, could be confirmed by immunological methods such as the detection of IgE antibodies in serum. Food allergies are provoking by food proteins, usually in very fast way, when natural 'tolerance' is compromised. We know a lot of food allergens, but the more common come from peanuts and nuts, flour, fish and shellfish. Also cow milk proteins, wheat and eggs proteins often trigger allergies among children, that very often disappear after puberty.

Methods

to characterize the allergen foods proteome and, specifically, IgE-reactive proteins as well as to compare the IgE response in define food allergic individuals.

Results and Discussion

Overview about food allergen quantification and quantification; unravel immunoglobulin E epitope mapping, new procedures to improve early detection and to avoid allergic reaction, also through the control of food processing.

Conclusion

Proteomics could be a valuable approach to detect food allergens in food, able also to understand the pathogenesis of this severe disease, that it is not depend from gene; in fact it is unclear why some children develop food allergy. Last but not the least, definition of allergens, risk assessment, accuracy of diagnoses, together with interaction of food with microbiota is presented as state of the art of proteomics in this field.

Keywords: food allergies, food allergens, food safety, microbiota

WK-10

The Human Immuno-Peptidome Project: A New Initiative of B/D-HPP Program

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Introduction and Objectives

T lymphocytes recognize antigens in the form of short peptides presented by major histocompatibility complex (MHC; HLA in humans) proteins on the surface of antigen presenting cells. The repertoire of MHC-associated peptides is referred to as the immunopeptidome and its robust characterization is of great importance for understanding the immune system as well as to improve prophylactic and therapeutic approaches against cancer, infectious diseases, autoimmunity and allergies. Here, we introduce a new B/D-HPP initiative known as the 'Human Immuno-Peptidome Project' (HIPP). The long-term objective of this collaboration is to make the robust analysis of immunopeptidomes accessible to any immunologist by the generation of suitable protocols and informational resources, including an immunopeptidome spectral- and database for different HLA types. To this end, the first immediate goal of HIPP is to provide a platform enabling every participant to access new technologies and knowledge for the identification and quantification of HLA-associated peptides. The second goal is to collect, organize, store and share immunopeptidomic data generated by the HIPP participants. Until recently, HIPP participants have demonstrated the feasibility of an international effort to build standardized HLA allele-specific peptide assay libraries toward highly reproducible MS-based measurements of HLA peptidomes across many samples. To organize, store and share immunopeptidomic data, the SysteMHC Atlas project has recently been initiated and is currently under development. The SyteMHC Atlas project will provide a publically accessible standardized database of HLA peptide assay libraries. This information will be stored by class and allele in the Atlas, which will also provide links to the well-established Immune Epitope Database (IEDB) and the SWATHAtlas database. This novel interoperable infrastructure is being developed toward a community-driven global mapping of the human imunopeptidome and is expected to enable navigation of robust quantitative digital maps of immunopeptidomes. Collectively, we anticipate that HIPP will provide key insights to the immunology community through standardized software platforms and reproducible MS-based methodologies. If successful in longer term, HIPP might contribute importantly to the development of next-generation immunotherapies against various immune-related diseases, including food allergies.

Keywords: Immunopeptidome, mass spectrometry, HIPP, SystMHC Atlas

Genetically Encoded Protein Photocrosslinker for Comparative Proteomics

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Introduction and Objectives

Globally profiling and comparing the condition-specific client pools from two different proteins are still challenging, mainly due to the lack of strategies to capture and side-by-side compare the native client pools without the interference from their distinct "bait" proteins. Here we developed a genetically encoded releasable photocrosslinker and adapted it to perform proteome-wide comparison of the client specificity of two acid-activated chaperones.

Methods

The photocrosslinker was site-specifically incorporated into the target proteins via genetic code expansion technology and the pure client pools were obtained after *in vivo* photocrosslinking, affinity-based purification and *in situ* oxidative cleavage. Two-dimensional difference gel electrophoresis (2D-DIGE) was performed to compare the client pools and the protein spots were analyzed by LC-MSMS.

Results and Discussion

We demonstrate that this releasable photocrosslinker not only covalently traps prey proteins under living conditions but also allows for the subsequent separation of bait and prey proteins via H_2O_2-mediated oxidative cleavage. Our comparative proteomic strategy which combines the releasable photocrosslinker with 2D-DIGE enables us to conduct the unbiased side-by-side comparison of the entire client pools from two acid-activated chaperones in *E. coli*. Our results also reveal the distinct pH-regulated client specificities between them *in vivo*.

Conclusions

We developed a genetically encoded photocrosslinker which can be adapted to comparative proteomic strategy and enabled the unbiased side-by-side comparison of the entire client pools from two chaperones in *E.coli*.

Keywords

Genetic code expansion, comparative proteomics, photocrosslinking

WK-12

Thermal Proteome Profiling for Drug Discovery

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Introduction and Objectives

The thermal stability of proteins can be used to assess ligand binding in living cells. We have earlier generalized this concept by determining the thermal profiles of more than 7000 proteins in human cells by means of quantitative mass spectrometry using isobaric mass tags, TMT10. Monitoring the effects of small-molecule ligands on the profiles delineated more than 50 targets for the kinase inhibitor staurosporine. Unexpected novel targets were identified and it was shown that changes in pathway activity could be detected by monitoring protein stability. Recently we improved the thermal proteome profiling, TPP, technology which enabled identification of novel and unexpected targets of histone deacetylase inhibitors.

Methods

We further developed the TPP approach by multiplexing temperatures and drug concentrations using TMT10. the resulting methodology 2D TPP is more sensitive because the target proteins have to exhibit dose dependent behaviour in there thermal stability changes and because both untreated and treated conditions are compared in the same experiment.

Results and Discussion

We used 2D TPP to profile a marketed histone deacetylase inhibitor and found an unexpected target that would not have been detectable with the previous version of the methodology. Results on target validation as well as discussion of potential side effects and repurposing strategies will be presented. Data elucidating the mechanism of inhibition will be discussed.

Conclusion

Improved thermal proteome profiling technology enables identification of an unexpected target of a marketed histone deacetylase drug.

Keywords: Thermal proteome profiling, HDAC, drug discovery

Precision Analysis of Proteome by HPLC-ESI-MS

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Introduction and Objectives

Precision analysis of proteome has been bring much of attentions recently for HPLC-ESI-MS based proteomics, which let us to analyze target proteins or proteome precisely and accurately. However, because there are a quite lot physiochemical processes involved in the HPLC, ESI, MS and their interface regions, the prediction of an interested peptide in terms of retention time (RT) with its M/z pattern in MS or MS/MS spectra is rather difficult. In the other hand, it has been acknowledged that there are about total of 20,000 protein-encoding-genes in human species with nearly 11,000 of them are frequently expressed in one kind of cells. In addition, the common expressed proteins in many type of cells are very similar, such as house-keeping proteins. We thus thought the precision analysis of proteome would help us to create a repeatable RT-M/z intensity image for any given type of cells or tissues, which could be verified and shared by inter- or intra-labs in the world.

Methods

Human heart sample was used. Proteins extraction and pretreatment were followed with the procedure as applied in a common routine analysis. All experiments performed on a Thermo Q-Exactive Obtrap mass spectrometer with a Waters UHPLC.

Results and Discussion

We optimize the HPLC gradient elution time first in order to produce a minimum relative errors in the RT prediction which can be calculated nicely by a number of RT prediction strategy available now. It is understandable that the shorter gradient time would yield a large error in the RT prediction since the HPLC does not have enough time to build up an equilibrium between mobile phase with varied-concentration and stationary phase; but the longer gradient time could worse peak width and cause a tedious workflow. We have developed a theoretical equation to describe the gradient elution process kinetically, which was verified with experimental evidences. With this gradient equation, the relative errors in the RT prediction can be greatly alleviated.

We reduce the size of total amount peptides then to rise-up those meaningful and observable peptides in a RT-M/z intensity image. In the intensity prediction for a given peptide, there could be a mess of peptides in the RT-M/z intensity image and the data analysis could be very complicated. We have considered the processes of peptide dissociation in HPLC, and ionization in ESI. We have computed these peptide dissociation and ionization and obtained peptide MS intensity eventually. By

cutting-off those peptides with weak intensity, we are able to create a peptide data-set which are nicely confirmed in experiment, with much of diminished number of peptides. We find our predicted RT-M/z intensity image could be matched with the experimental one, and would led to a repeatable measurement with the same pattern in the RT-M/z intensity image for a given type of cell.

Conclusions

The accurate predictions for peptide gradient elution and MS intensity are the key steps in precision analysis of proteome.

Keywords

Prediction, Gradient elution, Peptide MS intensity, Precision analysis

WK-14

Big Data Infrastructure for Chinese Human Proteome Project

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Chinese Human Proteome Project (CNHPP) is a Chinese government-funded effort to delineate protein profiles of major human organs/tissues at normal and disease states with proteomics-centric pan-omics approaches. Big data infrastructure is to be built to provide a wide-range of project-wide data services for data processing, management, integration, annotation, interpretation, as well as search and browsing. By working closely with HUPO-PSI initiatives, the infrastructure is also designed to include components interfacing with public community in data sharing and data standardization. A prototype of such infrastructure, Firmiana @ Phoenix Center, will be discussed, and its unique features in addressing big data challenges will be highlighted.

WK-15

Employing Neuroproteomics to Understand the Molecular Basis of Schizophrenia

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Introduction and Objectives

In the post-genomic era, proteomics has emerged as a powerful tool to unravel biomarker candidates and to understand human diseases from the molecular point of view. Within a decade, our group mapped the proteome of 5 post-mortem brain regions and the cerebrospinal fluid from schizophrenia patients and controls to help deciphering schizophrenia's pathobiology. These results led us to following up leads on energy metabolism and oligodendrocytes dysfunction.

Methods

Following the obtained results in schizophrenia post-mortem brains, we investigated *in vivo* and *in vitro* pre-clinical models to schizophrenia, including cerebral organoids obtained out of iPSCs from patients. We also analyzed post-mortem brains from patients suffering from depression using state-of-the-art mass spectrometry-based proteomics.

Results and Discussion

Energy associated differences recurrently found in schizophrenia brains were associated to the dysfunction of oligodendrocytes and myelin-associated proteins. Interestingly, MBP and MOG found differently in brain tissue and in the cerebrospinal fluid from a separate sample cohort of first-onset patients.

On the basis of impaired glutamatergic transmission, MK-801-treated oligodendrocytes and neurons shown differences similar to what we observed in schizophrenia human brains, including nuclear proteins, which are associated to the regulation og gene expression.

Conclusions

Proteomics findings may provide an integrated picture of schizophrenia´s pathobiology and the identification of energy metabolism dysfunction in oligodendrocytes reinforces their involvement in schizophrenia´s origin. These data can be useful to the development of innovative therapeutic strategies.

Keywords

Schizophrenia, depression, psychiatry, neuroproteomics, brain.

Unravelling the Multifaceted Pathogenesis of Amyotrophic Lateral Sclerosis

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Introduction and Objectives

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease of the motor system characterised by progressive paralysis and death within five years. Many factors have been implicated in the cellular damage. If, on one side, glial cells are considered to play important roles in neurodegeneration, on the other side it is accepted that a severe mitochondrial dysfunction leads to an unavoidable neuronal death. Despite this, there is no certainty on the intimate mechanisms sustaining the progressive loss of motor neurons. Hydrogen sulphide (H_2S) is an essential body product, mainly produced in the brain by astrocytes and microglia through the cystathionine-β-synthase (CBS), a cytoplasmatic enzyme that accumulates in mitochondria under oxygen sensitive conditions. It has already been demonstrated an involvement of H2S in ALS patients and in the familial ALS mouse model $SOD1^{G93A}$ [1]. Therefore, the aim of this study is to further unravel the complexity of H2S metabolism and the molecular mechanisms through which H_2S could contribute to the ALS-related neurodegeneration

Methods

We chose as representative biological model SOD1G93A transgenic mice, an established fALS animal model that recapitulates the human form of the disease. Proteomics investigations were performed on spinal cord tissues derived from $SOD1^{G93A}$ mice treated and untreated with hydroxylamine hemihydrochloride (AOAA), a CBS inhibitor, at different developmental/disease stages. Differential protein expression of total protein extracts and mitochondrial enriched fractions were evaluated with a shotgun proteomics DIA based on nLC-MSE. Clinical data were used to validate the current findings.

Results and Discussion

We looked putative dysregulated biological processes linked to H2S metabolism between the cytosolic and mitochondrial compartments. In particular, our data highlighted an alteration in subunits of cytochrome complexes and in proteins of

mitochondrial quality control. (MQC), involved in recognition and correction of the mitochondrial proteome, We focused on key proteins that are affected directly by H2S, such as GADPH, GSH and SOD1 that could be relevant for ALS. Our data show that the increased H_2S amount in ALS could further distress an already compromised mitochondrial function.

Conclusions

H_2S toxic effects seem to associate with phenotype development in ALS. Our study introduces H_2S as a new player to the cohort of pro-inflammatory/degenerative factors that could be involved in the aetiology of ALS, contributing to further distress an already compromised mitochondrial function and vulnerable motorneurons.

References:

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WK-17

New Proteomic Strategy to Dissect Signaling Pathways in Plant Stress Response

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Phosphorylation, as one of critical regulation mechanisms in plants, remains underrepresented compared to massive phosphoproteomics data in other organisms such as mammalian systems. We focus on the SNF1-related protein kinase 2 (SnRK2), an important regulator which links abscisic acid (ABA) signaling with plant stress responses under osmotic stresses such as drought and high salinity. With the core components of ABA signaling that have been identified, our groups have attempted to identify the direct substrates of SnRK2 activated in response to ABA stimulus. Identification the downstream substrates of SnRK2 not only facilitates dissecting the signaling transduction of ABA-dependent pathways but also shedding light on improving the plant survival under osmotic stresses. Here, we report recent development of stable isotope labeled protein kinase assay linked-phosphoproteomics (siKALIP) by using phosphoproteomes as substrate candidates. The subsequent ^{18}O-ATP labeled kinase reaction significantly simplified phosphoproteome complexity for LC-MS analyses while achieving better sensitivity and specificity. This approach was applied to identify SnRK2 substrates in response to ABA activation. A total of 778 ^{18}O-phosphopeptides representing 614 phosphoproteins were identified as the candidate ABA responsive substrates of SnRK2. Besides the reported basophilic phosphorylation motifs of SnRK2 were enriched from the candidate substrates, we have also found a new phosphorylation motif in which the glycine residue at -1 position of the phosphorylation sites is significantly enriched on SnRK2 substrates. The substrate interaction networks reveal that a variety of biological processes are activated through SnRK2 under ABA stimulus, suggesting the diverse functions of SnRK2 in regulating plant stress responses.

WK-18

Application of Mass Spectrometric Technology for Quantifying Changes in Plasma Membrane Receptor-Mediated Signaling in *Arabidopsis Thaliana*

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Abstract

In the past decade, almost all advances in identifying receptor proteins that bind and mediate the effects of hormones (e.g., ABA, auxin, GA, ethylene, cytokinin, etc.) in higher plants has come out of genetic studies using *Arabidopsis thaliana*. My laboratory has recently achieved success with a biochemical approach. This involved the development and application of mass spectrometric-based technologies to identify a new plasma membrane receptor kinase-peptide hormone cognate pair that regulates cell expansion (1). This discovery began with a careful identification and quantification of changes in autophosphorylation of a receptor kinase induced in planta by the brief treatment of plant cells with the peptide hormone, RALF (**R**apid **Al**kalinizing **F**actor). Reverse genetic studies, as well as direct binding experiments with the ligand and its receptor produced in heterologous systems, demonstrates that Feronia and RALF are representatives of a large family of receptor kinases and peptide hormones that regulate the rate of cell expansion. RALF and Feronia act, at least in part, via causing changes in regulatory phosphorylation of the plasma membrane proton pump (2) but other rapid changes at the plasma membrane and in the cytoplasm are also induced. More generally, Feronia and RALF represent the nexus for several signaling pathways that control cell expansion.

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WK-19

Social Implementation Vision of All-in-One Urine Test for Health Checkups

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Introduction and Objectives

Regular health checkups may be done once a year by examining blood and urine and by using many devices such as EKG, X-ray and echo. Recently hospitals or health checkup centers have introduced extensive, luxurious health checkup courses ranging from general to specialized for cancer, brain, vascular system, women's health screening, etc. These health checkups are useful to detect early cancers or diseases. However, the checkups will take time and cost much.

Methods

Therefore, in 2013 we started a project, "All-in-One Urine Test", which makes it possible to examine health conditions and detect early diseases only by urine examination. For this project, urine samples are collected from many healthy volunteers and patients with variety of diseases in a urine bank and used for biomarker discovery by mass spectrometry (MS). Urine proteins and native peptides are targeted to analize comprehensively (proteomics and peptidomics) and their qulitative and quantitative profiles are compaired among urine samples from volunteers and patients to establish biomarkers for each early disease. Protein and peptide preparation from urine for quantitative analysis is the first hurdle needs to be established. For quantitative comparision, we employed non-label quantitative MS, such as SWATH and normalized spectrum index (SIN) in the "All-in-One Urine Test" project.

Results and Discussion

By these analysis, about 1,500 protein groups are quantitatively detected in a individual urine sample. Identification and quantitation of native urine peptides have several difficulties in preparation and identification of peptides. To solve these difficulties, we are testing methods to prepare peptides efficiently and creating a new urine paptide database by collecting information from annotated protein database and literatures.

Conclusion

The progress of the "All-in-One Urine Test" project will be introduced in this talk and the project will contribute to human health and medication in the near future.

Keywords: proteomics, peptidomics, urine, biomarker

WK-20

Affinity Proteomics with Plasma Biobanks - From Discovery to Validation

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Introduction and Objectives

Affinity-based assays offer a valuable approach to analyze larger numbers of patient samples collected in biobanks. Current advances are driven by new technologies, growing libraries of binding reagents as well as the need to increase the knowledge about the performance of an antibody in a given assay and sample context. Alongside this, the number well-characterized and categorized patient biobanks is expanding, in particular for more accessible specimen such as serum or plasma. There are several examples of current affinity proteomics efforts to profile plasma across different diseases, but strategies need to go beyond discovery, thus include multiplexed immunoassays as well as other techniques antibody and target validation.

Methods

We have used 384-plexed bead based assays and the unique antibody resource of the Human Protein Atlas (www.proteinatlas.org) to perform biomarker discovery in body fluids. In these assays, proteins are capture from biotinylated plasma samples that have been randomized in and across 384-well plates with the support of liquid handling systems. Signatures from interesting candidate antibodies can be validated by several antibodies towards a common target, epitope mapping, dual capture assays, we all as immuno-capture mass spectrometry (shotgun or targeted). Lastly, we perform multiplexed screenings for antibody pairs to develop sandwich ELISA assays for target validation with independent study sets.

Results and Discussion

For a systematic exploration of proteins in plasma, antibody-based assays have been applied to profile 1,000s of patient samples. Across different studies, biobanks were the primary source to preferentially provide 300+ samples per group with detailed information about specimen and subjects. Both of the latter are needed to design the study across several assay plates and to reduce unwanted bias. In data sets with > 384 samples, batch effects are addressed by multidimensional normalizations and to prepare the data for statistical analyses. To confirm an observed antibody-phenotype association, commercially available assay can be used if available. For novel targets, the combination of different validation methods appears optimal when considering specificity, sensitivity, low reagent and sample consumption. In the end, we aim to develop sandwich ELISA assays for replication purposes, as demonstrated for reduced plasma levels of CNDP1 in males with prostate cancer related lymph node metastasis (Qundos et al, 2014) and cachexia in gastrointestinal cancer (Arner et al, 2015).

Conclusion

With continuing optimization of assays and technologies, schemes for antibody and target

validation, affinity proteomics assays can harvest the valuable information from plasma biobanks and contribute with candidates for clinical translation.

Keywords: antibodies, multiplexed immunoassays, target validation, antibody validation, study design

POSTERS

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MP01-01

Acid-based SCX fractionation for in-depth proteome and phosphoproteome analysis

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Introduction and Objectives

StageTips have advantages on their high sample recovery rate, throughput and cost as a device for sample purification, enrichment and fractionation. However, StageTip-based fractionation for large-scale phosphoproteome analysis has not been succeeded yet. Elution methods of strong cation exchange (SCX) chromatography have been based on two principles using salt or pH gradient. In this study, we report the first observation that peptides are eluted by acid gradient. A degree of peptide separation on C18-SCX StageTip was greatly improved by our acid-based elution method compared with a salt-based elution method. This development enabled us to identify over 25000 phosphorylation sites from two mg of protein within a day.

Methods

Tryptic digests of Hela-S3 cells were fractionated into 7 fractions on C18-SCX StageTips. TFA concentration was increased from 0.5% (first fraction) to 3% (fourth fraction). We used TFA and ammonium acetate as eluent in the last three fractions. Peptides were analyzed by Q Exactive instrument and MS data were processed by MaxQuant. Search results were filtered to a maximum FDR of 0.01 for proteins and peptides. For phosphoproteome analysis, IMAC enrichment of phosphopeptides was performed using tryptic digests of DLD-1 cells and SNU-19 cells. Enriched phosphopeptides were fractionated and analyzed by LC-MS/MS. Class I phosphorylation sites (localization probability, $p > 0.75$) and fully localized phosphopeptides were counted as identified sites/peptides.

Results and Discussion

For total proteome analysis, we quantified 87160 non redundant peptides from 7 fractions fractionated on C18-SCX StageTips by TFA-based stepwise gradient. The basicity of the identified peptides in each fraction as represented by the number of basic residues (Lys, Arg and His) showed clear dependency on the order of the fractions. For phosphoproteome analysis, we compared acid-based fractionation with traditional salt-based fractionation. Acid -based fractionation showed superior separation whereas salt-based fractionation showed “smear” separation. Acid -based fractionation showed 34% increase in the number of quantified phosphopeptides compared with salt-based fractionation. We also found the basicity of the phosphopeptide is dependent of the order of acid-based fractions, whereas it is not strongly dependent of the order of salt-based fractions. For large-scale phosphoproteome analysis, we identified ~24,000 distinct phosphopeptides in 21 hours (3 hours *7 fractions) MS measurements from two mg of proteins. Furthermore, over 28,000 phosphopeptides were identified in replicate experiments. In terms of reproducibility, the correlation coefficients of peptides’ intensity

MP01-01

were over 0.89 in all fractions of replicate experiments.

Conclusion

Our fractionation method is very simple, flexible in scale, low-cost (less than $0.1/sample) and does not need any special equipment and techniques. Furthermore our method has a potential to be applied to other fields using SCX chromatography.

Keywords: Stagetip fraction

MP01-02

A new workflow for deep proteome profiling of the human sperm

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Introduction and Objectives

Sperm are highly specialized cells that acquire specific morphological traits during the process of spermatogenesis, which include undergoing meiosis, cell polarization, the formation of a flagellum, and formation of a sperm head which includes highly compacted DNA and an acrosome. The compact structure renders sperm resistant to lysis comparing to most somatic cells. Although normally millions of cells are produced daily, patients with alterations to testicular function may present very few sperm in the ejaculate. Therefore, improved protocols to allow high recovery and protein coverage from low number of sperm are important for the application of proteomics in fertility studies. The goal of this study was to establish a new protocol for proteome profiling using less than two million sperm.

Methods

Institutional Review Board approval was obtained from the Sao Paulo Federal University Research Ethics Committee and semen samples were obtained after written informed consent from the donors. Following semen liquefaction, samples were centrifuged at 800 g for 30 min to isolate sperm. These were then submitted to density gradient centrifugation at 1,300 g for 30 minutes to remove contaminating round cells. Isolated sperm were then lysed with SDS and deoxycholate buffer and total protein content was assessed using the modified tryptophan fluorescence method. Proteins were extracted using Trizol and digested using a modified version of enhanced filter assisted aided sample preparation (eFASP). Tryptic digest were analysed by labelfree shotgun proteomics in a system composed by nanoUPLC (nanoAcquity, Waters) coupled to a quadrupole-orbitrap instrument (Q-Exactive, Thermo) operating at data-dependent acquisition method. MS raw files were analyzed by Maxquant software and peak lists were searched against the human SwissProt Uniprot by Andromeda search engine.

Results and Discussion

While the protein concentration is routinely measured by the BCA or Bradford assays, they do not provide sensitivity to work with a small number of sperm cells. Using tryptophan fluorescence, low protein amounts from 0.5e5 to 2e6 cells were precisely determined. Trizol method coupled to a solubilization buffer with SDS and deoxycholate showed efficient cell lysis and good protein recoveries. The application of eFASP allowed complete removal of detergents and high peptide recoveries (19,432 identified peptides) resulting in 2,727 identified proteins in a single unfractionated 90 minute analysis. The number of proteins can be expanded either by protein level fractionation by C4 chromatography or by peptide level fractionation by pH 10 C18.

Conclusion

MP01-02

Assessing the sperm proteome has been one of the most promising tool to understand specific alterations which contribute to the determination of male infertility. In this study we established a method to obtain high recovery of proteins on research projects with samples from patients with very few sperm in the ejaculate.

Keywords: sperm proteomicsfertilityprotein extraction from sperm

MP01-03

Comprehensive Analysis of Human Urinary Proteome through Ampholine@PM Fractionation Strategy

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Introduction and Objectives

Urine is one of the most desirable body fluids in clinical proteomics for the diagnosis and classification of diseases, as it can be obtained non-invasively in a large amount with more convenience than tissues or cell lines. Urinary proteome analysis could provide a privileged source of information regarding kidney, urogenital tract and systemic physiology.

Methods

Up to now, various analytical approaches have been constructed to provide the deep insight of urine proteome by multiple protein separation methods and mass spectrometry (MS) technologies. This study reports a comprehensive analysis of urine proteome through ampholine immobilized polymer microsphere (ampholine@PM) fractionation strategy.

Results and Discussion

With ampholine@PM treatment, 2,728 non-redundant urinary proteins, corresponding to 16,173 unique peptides were identified, while only 954 proteins, corresponding to 3,927 peptides were identified in the crude urine sample. The number of proteins and peptides increased by 312% and 186%. The non-redundant proteins identified in this study could serve as a comprehensive reference for future urinary proteome in-depth research. This dataset included 691 diseases-related proteins that were represented on the Online Mendelian Inheritance in Man database (OMIM database). The number of low abundant proteins (less than 100 ng/mL) increased from 25 to 45 after treatment. In addition to the urine proteome, N-glycoproteome analysis was also performed after ampholine@PM fractionation followed by N-glycopeptides enrichment. With more than 1.19 times of N-glycoproteins (439 vs 200) identified, the fractionation efficiency of the ampholine@PM for urine sample was proved sufficiently, which offered a beneficial tool for urine proteome post translational modification analysis.

Conclusion

In this work, a large urine protein dataset including 2728 unique proteins was reported based on ampholine@PM fractionation strategy. The number of N-glycoproteins and the ratio of membrane proteins identified in urine proteome also get improved after ampholine@PM fractionation. Among the identified proteins, 691 proteins were reported to be disease related. Forty five proteins were discovered with low abundance. This study could provide a solid support for biomarker development in various diseases research.

MP01-03

Keywords: Human urinary proteome analysis; Ampholine@PM; Fractionation strategy; Low abundant proteins

MP01-04

Systematic comparison of sample complexity reducing methods in amniotic fluid proteomics

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Introduction and Objectives

Discovery and validation of amniotic fluid biomarkers is of immense value for pregnancy related complications, neonatology and obstetrics. High throughput shotgun proteomics analysis can provide closer insights into the biomarker discovery but is substantially limited by sample complexity, common to most biological materials such as blood plasma, serum, cerebrospinal fluid, urine or amniotic fluid. Not only is amniotic fluid highly complex with regard to the number of contained proteins, but these are present at an extremely wide range of concentrations. Detection and identification of low abundant proteins in such a complex mixture is a major challenge in biomarker discovery.

Methods

The most commonly used approaches for lowering sample complexity are different fractionation methods allowing thorough separation of complex mixtures based on chromatographic or electrophoretic principles or removal of highly abundant proteins by immunodepletion techniques. Although proteomic studies dealing with amniotic fluid use fractionation and/or depletion methods to our best knowledge there is no work describing benefits of usage one of them or their combination. The aim of this work is to provide a systematic overview of the real contribution of these methods in amniotic fluid proteomics.

Results and Discussion

Using nanoLC MS/MS (UltiMate 3000 RSLCnano system coupled to QExactive Plus (Thermo Scientific)) we analyzed amniotic fluid samples with different approaches for reduction of sample complexity prior to analysis. We used immunoaffinity chromatography removing 14 high abundant proteins and multidimensional fractionation using high pH separation.

Conclusion

The most effective method giving the highest number of protein identifications was combination of depletion and fractionation technique. The usage of both techniques increase the total number of protein identifications almost five times compared to untreated sample. Meanwhile the depletion alone may increase the number of identifications twice, the fractionation itself has rather small effect in increasing number of identifications compared to the untreated sample of amniotic fluid.Acknowledgments:This project was supported by Grant of Czech Ministry of Youth, Sports and Education : SGS2016004 and by Grant of Ministry of Health of the Czech Republic MH CZ - DRO (UHHK, 00179906)

Keywords: proteomics, amniotic fluid, sample complexity

MP01-05

Improved throughput of DIA quantitation using microflowLC

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Introduction and Objectives

Data independent acquisition (DIA) methods are currently used by many proteomics labs for large scale quantitative analysis on 1000s of proteins in complex matrices. Utilizing microflow LC has shown to increases robustness and throughput, with very good protein quantitation numbers. In this poster, we describe further improvements to protein quantitation coverage with SWATH® acquisition through optimizing sample load, longer columns and LC gradients.

Methods

Tryptic digested proteins from yeast and human cell lysates were analyzed on a NanoLC™ 425 System (SCIEX) using microflow LC (5 µL/min) on a 15 and 30 cm C18 column using 43 and 103 minutes gradients with total run time of 60min vs 120min and different sample loads. SWATH acquisition was performed on a TripleTOF® 6600 system with a DuoSpray™ Source and 25 µm I.D. electrode (SCIEX) with five replicate analysis for quantification at a <1% FDR rate with < 20% CV utilizing SWATH® 2.0 .

Results and Discussion

During quantitation by microflow SWATH acquisition using a single 15 cm column, 43 minute gradient and a 6µg sample load, ~29000 unique peptides were quantitated (<1%FDR) from a HEK cell lysate (tryptic digest), resulting in quantitation of ~4500 proteins with a CV< 20%. Increasing the gradient length to 103 minutes resulted in 63% more peptides and 29% more proteins (6 µg) and increasing sample load from 6-10µg resulted in 20% more peptides and 10% more proteins. A significant improvement was seen when loading 10 or 20 µg of samples on a 103 minutes gradient with 76-79% more peptides and 38-42% more proteins. However, when combining the two columns with same gradient length and sample load, we observed no improvement of quantifiable peptides/proteins. Multiple sample complexity was tested to determine optimal acquisition strategies for the various sample types. This work highlights an additional microflow SWATH acquisition strategy when greater coverage is a higher priority over sample throughput, while maintaining the microflow robustness.

Conclusions

The use of longer microLC gradient and increased sample load significantly improves the number of proteins quantified using high throughput microflow SWATH acquisition.

Keywords: DIA SWATH microLC protein quantitation

MP01-06

Development of immobilized metal-ion affinity (IMA) monolith incorporated microfluidic device for plasma proteomics

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Introduction and Objectives

Proteomic analysis of valuable clinical samples such as plasma is of prime importance for diagnosis and therapy. However, high and middle abundant proteins mask the detection of low abundant proteins that are potential biomarkers. Fractionation of plasma is quite challenging due to the various difficulties like removal of non-targeted proteins, protein-protein interactions within the sample etc. Currently, various pre-fractionation strategies like reverse phase, affinity chromatography, ion exchange etc. are widely used. However, multiplexing these into a single device is quite challenging due to their requirement of different binding and elution conditions. Immobilized metal-ion affinity (IMA), a pseudo affinity approach, has the efficiency to differentiate structural variations in proteins purely depending on surface exposed Histidine topography. In IMA, under identical adsorption conditions, the four chelated-transition metal-ions (Co (II), Zn (II), Ni (II), and Cu (II)) display different demands in terms of Histidine surface topography of the protein. This enables the opportunity to multiplex these metal-ions into a single microfluidic system. The objective of this project is to develop a conjoined IMA microfluidic device as a new pre-fractionation approach to fractionate proteins from human plasma.

Methods

An organic monolith is prepared by homogenous polymerization of monomer unit (2-Hydroxyethylmethacrylate), cross-linker units (N,N'-Methylenebisacrylamide & N, N'-Diallyltartaramide) and an initiator in a suitable porogen. Material modifications to achieve separation of proteins are made to enhance the surface property by 2-step approach. Monoliths (0.12 g) are suspended in individual model protein solution (HSA, IgG & Transferrin) to check non-specific and specific adsorption.

Results and Discussion

We have successfully developed a methacrylate (HEMA-co-MBAAm-co-DATD) monolith in 35 µL of glass capillary (~1 mm i.d.). The prepared monolith displayed highly interconnected porous structure (SEM analysis). Surface modification of the monolith with IMA chemistry was confirmed with FT-IR analysis. Both static & dynamic protein adsorption studies on Cu (II)-IDA monolith displayed the following binding order: IgG > Transferrin > HSA, which is in agreement with existing literature on other matrixes. Preliminary data using artificial protein mixtures will be demonstrated. Future work, involves development of a microfluidic device retaining the above mentioned chelated transition metal-ions in a single system and its evaluation for plasma pre-fractionation.

Conclusion

1. The polymer monolith possesses highly interconnected porous structure.2. The chelating agent i.e. Iminodiacetic acid (IDA) was immobilized on the monolith in two steps3. Interaction of the 3 model proteins (IgG, Transferrin, and HSA) to Cu (II)-IDA

MP01-06

monolith was in accordance with their surface exposed histidine topography

Keywords: Immobilized metal-ion affinity chromatography, Monolith, Proteomics, Microfluidics

MP01-07

High throughput proteomic analysis using different OFFGEL fractionation panels

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Introduction and Objectives

Protein fractionation is a required step for efficient protein identification especially low abundant proteins. Among the promising fractionation methods, OFFGel fractionations became a favored method either on protein or peptide level. However, no clear comparison or study was done to recommend when to use each of these fractionation levels.

Methods

OFFGEL fractionation of mouse kidney protein lysate and its tryptic peptide digest has been examined in this study for better understanding the differences between protein and peptide fractionation methods and attaining maximum recruitment of this modern methodology for in-depth proteomic analysis. With the same initial protein/peptide load for both fractionation methods

Results and Discussion

protein OFFGEL fractionation showed a preponderance in terms of protein identification, fractionation efficiency, and focusing resolution, while peptide OFFGEL was better in recovery, number of peptide matches, and protein coverage. This result suggests that the protein fractionation method is more suitable for shotgun analysis while peptide fractionation suits well quantitative peptide analysis [isobaric tags for relative and absolute quantitation (iTRAQ) or tandem mass tags (TMT)]. Taken together, utilization of the advantages of both fractionation approaches could be attained by coupling both methods to be applied on complex biological tissue.

Conclusion

typical result is shown in this article by identification of 8262 confident proteins of whole mouse kidney under stringent condition. We therefore consider OFFGEL fractionation as an effective and efficient addition to both label-free and quantitative label proteomics workflow.

Keywords: OFFGEL fractionation, proteomics, mouse

MP01-08

Automated pull-down of extracellular vesicles (EVs) on KingFisher™ using Dynabeads™ magnetic beads - standardizing EV capture and analysis

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Introduction and Objectives

Exploring the biology of extracellular vesicles (EVs) and their potential use in clinical applications has over the last years gained considerable interest. In the biomarker and "liquid biopsy" space EVs are regarded as complementary or even superior to both cell-free DNA (cfDNA) and circulating tumor cells (CTCs). So far the main focus has been on the nucleic acid (NA) cargo of EVs namely the micro- and mRNA. However, the protein cargo of EVs is highly complex, involved in numerous biological functions and ripe for further in-depth analysis. In addition, EVs show great potentials for therapeutic applications such as drug delivery vehicles, immune modulating elements and the "active" substance in cell-based regenerative medicine. Here we present an automated system for isolation of EVs for further downstream protein analysis.

Methods

Cell culture (SW480 or Jurkat) exosomes were used as EV source DynabeadsTM magnetic beads were used for EV pull-down and analysis KingFisherTM Flex with the BindIT 3.3.1 software was used for automation Downstream analysis included standard electrophoresis and Western Blotting using the Bolt® System and flow analysis using the BD LSR Fortessa™

Results and Discussion

Scripts in BindIT 3.3.1 for KingFisherTM (Flex) has been designed for capture of EVs using magnetic beads (DynabeadsTM) coupled with antibodies against CD9 or CD81, tetraspanins, present on several groups of EVs, including exosomes. Successful isolation of EVs was demonstrated using Western Blotting and flow analysis targeting the tetraspanin not used for isolation, i.e. CD9 when pull-down was done with CD81 and visa verse. Factors affecting pull-down efficiency were explored, including magnetic bead- and EV-concentration, incubation time and –temperature. Factors affecting purity such as number of washing steps, -volume and mixing speed have also been explored and optimized. Increasing pull-down efficiency was mainly dependent on choice of the pull-down antibody clone, bead concentration and incubation time. Optimal pull-down was demonstrated within 3-5 hours of incubation in up to 1 ml sample volume using 40-50 µg of DynabeadsTM. For more generic pull-down up to three antibodies were coupled to the magnetic beads to target EV sub-populations carrying either of the most common tetraspanins (CD9, CD63 and CD81) and used for EV pull-down. Alternatively, a physical interaction approach was used enabling optimal EV-capture within 10 minutes incubation and release of the isolated EVs from the magnetic beads by another 10 minutes incubation in release buffer. The overall %CV for the EV pull-down and analysis was below 5 %.

MP01-08

Conclusion

Standardisation of EV isolation and characterization has for several years been a highly demanded need expressed by the international society of extracellular vesicles (ISEV). A highly reproducible method for EV pull-down and analysis was developed using KingFisherTM Flex in combination with DynabeadsTM magnetic beads.

Keywords: AutomationExtracellular vesicles

MP01-09

A Charge-Suppressing Strategy for Probing Protein Methylation

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Introduction and Objectives

Methylation of arginine and lysine (RK) residues play essential roles in epigenetics and the regulation of gene expression. However, research in this area is often hindered by the lack of effective tools for probing the protein methylation.

Methods

The protein extract is first digested into peptides using trypsin. The tryptic peptide mixture is reacted with malondialdehyde (MDA) and ortho-phthalaldehyde (OPA) sequentially to suppress the positive charges of side chains primary amine groups of un-methylated RK residues and free N-terminal amines of peptides. These two reactions block most of the positive charge-carrying functional groups in the digested peptides, mainly leaving behind methylated peptides with a positive charge at neutral to basic pH. The methylated peptides are then readily enriched by charge-based separation techniques, such as strong cation-exchange chromatography. Un-methylated peptides co-enriched by non-specific binding would be hard to ionize due to a lack of charges and are therefore less likely to be identified by mass spectrometry.

Results and Discussion

Test on methylated BSA digest shows significant enrichment effect on methylated peptides by the optimized workflow. We identified 742 methylation events from two enrichment analyses of the same 500 µg HEK293 total lysate as starting material. Of these sites, 399, 240 and 103 were observed on lysine, arginine, and histidine, respectively. The number of methylation events is conservative considering that we used 1% site-specific FDR filtering in MaxQuant and removed all C-terminal trimethylation events. Protein surface accessibility analysis showed a significant enrichment of methylated RK residues on the protein surface (p=6.3e-47, against all amino acids of methylated proteins as the background). 23% of our 742 identified methylation events are present in PhosphositePlus The UniProt database has 60 methylation events overlapped with our methylation list, including the recapitulation of known histone methylation events. Motif analysis of the flanking sequences surrounding the methylated amino acids revealed a RGG-rich motif for arginine methylation consistent with the literature. Pfam protein domain analysis was also very consistent with previous work10, with significant enrichment of the RNA recognition motif RRM_1.

Conclusion

In this study, we have reported the first chemistry-based strategy for the high-throughput discovery of lysine and arginine methylation events. The approach is simple, inexpensive, and does not rely on specific antibodies to perform charge-based methylated peptide enrichment and identification by MS. This generic and non-sequence-biased methodology has the potential to be used for large-scale methylome profiling and

MP01-09

methyltransferase/demethylase discovery, which are important in cancer research

Keywords: protein methylation; PTM; methylation enrichment

MP01-10

Identifying Novel Cancer Antigens using Immunoproteomics

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Introduction and Objectives

The Blackburn lab has developed a highly sensitive and selective native cancer-antigen (CT100+) microarray, used to detect autoantibodies in the blood samples of cancer patients. Although technically advanced, the microarray has biological limitations as we observed a random antibody response in 10-20% of patients. Our aim is therefore to use immunoproteomics to identify TSAs which can reproducibly be used for cancer diagnosis and prognosis.

Methods

We have an archive of 67 cancer and paired normal tissues, with corresponding autologous blood plasma samples, from patients with colorectal cancer (CRC) (Groote Schuur Hospital, South Africa) for identifying novel TSAs. For each patient, we have a set of clinical information which can be used to identify cancer antigens associated with disease pathogenesis. To identify novel cancer antigens, we have developed an immuno-pulldown assay, in which Protein A and Protein G magnetic microbeads are used to selectively capture antibodies from the patient blood plasma. The Ig-bound microbeads were incubated with native cancer and control tissue lysates to capture antigens. After performing an on-bead tryptic digestion, the peptides were eluted and proteins were identified by mass spectrometry, using a Q-Exactive mass analyser.

Results and Discussion

Using the MaxQuant proteomics software, we were able to identify a total of 1276 proteins, of which 360 were unique to the cancer samples. Eight of the 360 cancer-unique proteins were matched to the Tantigen database, a data source and analysis platform for cancer vaccine target discovery. Furthermore, 3 of the 8 proteins identified have been reported to induce antibody responses in cancer patients.

Conclusion

In conclusion, we have developed an immuno-pulldown assay that captures and identifies proteins which are unique to cancer tissues, of which several have shown to induce a T- and B-cell response in cancer patients. Although we are able to identify protein candidates of interest, we observe several limitations with the assay, for which we are currently troubleshooting. Once the method is optimised, the newly identified TSAs will be fabricated on to the CT100+ microarray, and validated with patient sera to confirm the presence of cancer-specific antibody response.

Keywords: Cancer antigens, Immunoproteomics

MP01-11

High pH Reversed-Phase Sample Fractionation for Phosphoproteomic and Glycoproteomic Workflows

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Introduction and Objectives

While phosphorylation and glycosylation of proteins are very common post-translational modifications (PTMs), only a very small percentage of peptides from a complex digest have these modifications. This necessitates enrichment of phosphopeptides and glycopeptides comprehensive proteomic studies. Off-line fractionation of complex peptide mixtures using a high pH reversed-phase approach followed by low pH LC-MS improves protein identification numbers and provides better site-specific modification information. In this work, we investigated whether upfront, large scale fractionation followed by enrichment of each fraction results in higher identification numbers of phosphopeptides and glycopeptides compared to the reversed approach of PTM enrichment followed by fractionation.

Methods

Protein extracts from HeLa lysates and K562 were digested sequentially with Lys-C and trypsin. Protein and peptide concentrations were determined using BCA assay and the Pierce™ Colourmetric Peptide Quantitation kits, respectively. Phosphopeptide enrichment was performed using Fe-NTA resin, and glycopeptides were enriched using HILIC resins. Pierce High pH Reversed-Phase Fractionation Kits were used to fractionate phosphopeptide and glycopeptides into eight fractions by increasing acetonitrile step-gradient elution. A scaled-up version of the high pH-reversed phase spin-column with load capacity of up to 20mg of digest material was used in pre-fractionation experiments. Samples were analyzed using a Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer.

Results and Discussion

In our preliminary experiments, we observed that enriched phosphopeptides and glycopeptides were not retained well hydrophobic resins used for high pH reversed-phase fractionation. While it is well known that phosphorylation and glycosylation modifications render peptides more hydrophilic, we were surprised by the poor retention of enriched peptides under low pH ion-pairing (0.1% TFA) loading conditions compared to unenriched samples. We hypothesized that the presence of sample matrix (i.e. non-modified peptides) may have an effect on retention of phosphopeptides and glycopeptides. To test this, we developed a large-capacity spin column, which can be loaded with 10-20 mg of complex digest for off-line fractionation. Unenriched HeLa lysate digests fractionated using the large column formant compared to the small column format show similar fractionation profiles with improved fractionational resolution. We are presently assessing the fractional profiles and peptide recovery from both the preenrichment-fractionation and prefractionation-enrichment strategies to determine which approach results in the most phosphopetide and glycopeptides identifications.

MP01-11

Conclusion

Comparison of high pH reversed phase fractionation followed by PTM enrichment or vise versa revealed differences in fractional resolution, PTM enrichment efficiency and modified peptide identifications.

Keywords: high pH fractionation, phosphopeptide, glycopeptide, enrichment, Fe-NTA

MO02-01

MS1 based quantification optimization on DIA methods on a quadrupole-Orbitrap mass spectrometer

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Introduction and Objectives

Targeted analysis of DIA is a powerful mass spectrometric approach for comprehensive, reproducible and precise proteome quantitation. It provides valuable insight into biological processes or enables the discovery of novel biomarkers. Today, identification of the majority of the expressed proteins can be achieved. With this achievements in the identification, the reproducibility and quantitative accuracy and precision have become increasingly important. Here, we optimize data-independent acquisition (DIA) on a Thermo Scientific™ Q Exactive™ HF mass spectrometer for quantification on the MS1 level. DIA data can be processed in a targeted mode based on MS2 fragment information. MS1 scans have potentially a higher sensitivity since the peptide precursor is not fragmented in multiple fragments. Novel methods using high resolution full range and segmented MS1 enable high precision quantification on MS1. Here we perform quantification of over 60,000 peptide precursors on MS1 level and benchmark MS1 based interference correction implemented in Spectronaut.

Methods

Protein samples for HeLa were prepared using the FASP protocol. Biognosys' iRT kit was spiked into the samples before injection. The samples were acquired on a Q Exactive HF mass spectrometer. Shotgun runs were performed and searched using MaxQuant software. Spectral libraries were generated with Biognosys' Spectronaut. DIA methods were acquired with varying gradients lengths. Targeted analysis of DIA runs was performed using Spectronaut.

Results and Discussion

The performance of a DIA methods is influenced by the number of DIA windows, the scan resolution, the gradient length and the sampling of the chromatographic peaks. Additionally, the MS1 scan of the DIA method has an important influence on the performance. To optimize quantification on MS1 we evaluated the following conditions. First, the sampling of the chromatographic peaks was addressed, therefore, DIA methods with on average three, six, nine and twelve data points per peak (peak width at 13.5% height) were generated for a 90 min method. The CV of triplicate acquisitions showed, that six data points per peak was optimal for MS1 quantification. Next, the influence of the gradient length was performed. DIA runs with gradients of 30, 60, 90, 120 and 240 min were acquired. The identifications increased with the length of the gradient, but the reproducibility and quantification on MS1 were optimal at 90min. Finally, the MS1 scan was set to 120k or 240k and the MS2 resolution was set to 30k or 60k keeping the data points per peak constant at six.

Conclusion

MO02-01

The combination of 240k MS1 with 30k MS2 resolution resulted in in optimal quantification for the 90 min gradient.The EASY-Spray/Q Exactive HF instrument setup combined with Spectronaut is a robust and powerful setup for high precision quantitative protein profiling.

Keywords: DIAQ Exactive HFSpectronautHigh-throughput Quantitation

MO02-02

Digging deeper into large SWATH MS1 windows using Gas Phase Fractionation SWATH-MS

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Introduction and Objectives

SWATH-MS as a data-independent acquisition method is gaining much attention for cell biology and biomarker discovery projects. SWATH-MS has advanced from using 32x25 m/z windows to cover the 400-1200 m/z MS1 range, to utilize variable windows (vW) width dependent upon peptide precursor density in a given MS1 m/z range. This resulted in deeper proteome coverage, especially due to improvements in the tryptic peptide rich region between 600-800 m/z. Despite deploying vW SWATH for analysis of plasma, which is dominated by numerous highly concentrated proteins, it remains challenging to reliably quantitate more than 250 proteins. Therefore, it commonly only utilizes a small proportion of large plasma protein assay libraries available in online repositories. To address this limitation, we explore gas phase fractionation (GPF) SWATH to narrow MS1 windows and examine the impact on quantitative proteome profiling.

Methods

Tryptic digests of non-depleted human plasma and colorectal cancer cell line SW480 were analyzed by GPF SWATH and vW SWATH using 60 min gradients on a 6600 triple TOF (SCIEX). 400–600 m/z with 4 Da fixed windows, 600–800 m/z with 4 Da windows, and 800-1200 m/z with 8 Da windows were chosen for GPF SWATH. The vW SWATH method used 100 windows from 400–1200 m/z. A high pH fractionation assay library was used for SW480 and an online repository assay library was used for plasma.

Results and Discussion

GPF SWATH was optimized with SW480 lysates. Information extraction from 400–1200 vW SWATH analysis using an assay library with 5700 proteins with peptides >0.99 confidence revealed quantitative information for 2600 protein with 9800 peptides. Using the same library to extract information from GPF SWATH analysis resulted in ~3500 proteins (30% increase) and ~15500 peptides (60% increase) with only 2.3% of proteins and 3.6% of peptides unique to vW SWATH. Striking improvements were seen for the 400–600 m/z range with 2-fold increase in peptide extraction compared to vW SWATH and 1.5-fold increases for 600–800 m/z and 800–1200 m/z.For plasma, similar trends were observed. Using an online repository assay library with more than 1500 known secreted proteins, vW SWATH extracted quantitative information for 300 proteins and 1500 peptides. Improvements with GPF SWATH were less dramatic than in the SW480 lysate, but proteins with quantitative information increased by 25%, while peptides increased by 20%. Interestingly, proteins uniquely present in GPF SWATH are not classical acute-phase reactants, but rather less abundant cellular proteins such as S100 family proteins, shed membrane proteins and extracellular matrix proteins.

Conclusion

GPF SWATH of SW480 lysate and human plasma reveals improvements in protein

MO02-02

quantitation compared to vW SWATH. In plasma it allows for quantitation of lowly concentrated cellular proteins which were not confidently observed in vW SWATH. Therefore, GPF SWATH is a useful approach to take advantage of large assay libraries for deeper proteome quantitation.

Keywords: Plasma, SWATH

MO02-04

All MS/MS ions monitoring acquired by data-dependent acquisition without dynamic exclusion: A new concept for in-depth protein quantification

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Introduction and Objectives

To alleviate the limitations of traditional DDA approach, strategies based on unbiased "data-independent acquisition" (DIA) have been received more and more attention, as exemplified by the most representative sequential window acquisition of all theoretical mass spectra (SWATH) method. This method permits the quantification with an accuracy and reproducibility comparable with selected reaction monitoring (SRM) across many samples. Though the DIA methods may be the trends of future development with the improvements in MS instrumentation, their scale of quantitative protein numbers and the flexibility of data processing are still not comparable with traditional DDA methods. In this report, we proposed a strategy that all MS/MS ions monitoring acquired by data-dependent acquisition without dynamic exclusion (AIM), to achieve the analogous purpose of DIA approach, but to use DDA mode.

Methods

The AIM method was developed using HeLa cell line as model organism and further applied to quantitative analysis of hepatocellular carcinoma cells (HCCs) with high and low metastatic potential (HCC-H/L). In brief, the proteins were extracted and digested with our previously developed i-FASP method. Afterwards, the digested peptides were fractionated by basic RPLC, and subjected to gas-phase fractionation with an m/z window respectively from 350 to 450 Th, 444 to 550 Th, 544 to 650 Th, 644 to 750 Th, 744 to 850 Th, 844 to 950 Th, 944 to 1050 Th and 1044 to 1250 Th combined with DDA acquisition mode with top 100 MS/MS fragments with 28 ms accumulation time without dynamic exclusion for mass spectrometry analysis.

Results and Discussion

With our method, 10,961 proteins, corresponding to 10,584 gene products were identified from HeLa cells, from which the common precursor and fragment ions were extracted for quantification. A total of 5,027 proteins were quantified at median ratios of 1.000, with 99. 6% of the ratios ranged from -1 to 1 (log2), and the corresponding standard deviations are 0.16. In addition, 64 differential proteins were confidently quantified from HCC-H/L cells.

Conclusion

In this work, a quantification strategy monitoring all MS/MS ions by data-dependent acquisition mode without dynamic exclusion was developed and was shown to be a powerful alternative for DIA quantitative proteomic studies that offers improved

MO02-04

identification/quantification accuracy and enhanced proteome coverage.

Keywords: Quantification analysis; All MS/MS ions monitoring; Data-dependent acquisition approach; Data-independent acquisition approach

MO02-05

Analyses of intact proteins by LC-FT-ICR mass spectrometry at 21 tesla

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Introduction and Objectives

Comprehensive knowledge of protein primary structure cannot be derived from the genome because translation of mRNA into protein does not dictate the composition of the final protein complement. Alternative splicing, single nucleotide polymorphisms, endogenous protein cleavages, and post-translational modifications (PTMs) expand the number of theoretically possible proteoforms to over one billion. Each proteoform has the potential to affect different biological outcomes, including phenotypes of health and disease. The ultimate mass spectrometry (MS)-based proteomics platform would be capable of unequivocally distinguishing closely related species and characterizing any PTMs, which requires intact protein analysis. To do this, fast spectral acquisition rates, high sensitivity, high resolving power, and efficient gas-phase fragmentation methods are needed to obtain optimal sequence information.

Methods

Fourier transform ion cyclotron resonance (FT-ICR) offers the highest achievable broadband mass resolving power and mass accuracy, which is especially important for characterization of intact proteins. The National High Magnetic Field Laboratory (NHMFL) recently installed a 21 tesla (T) FT-ICR mass spectrometer, the highest field ICR system to date. At 21 T, increased ion cyclotron frequency provides high mass resolving power at high scan rate. For example, the isotopic distribution of bovine serum albumin (66 kDa) is resolved by use of a 0.38 second detection interval and measured mass errors are routinely less than 1 ppm for proteins as large as 30 kDa. The instrument is equipped with an external quadrupole accumulator, which enables storage of multiple populations of fragmented (by CID or ETD) precursors prior to mass analysis of the product ions to facilitate acquisition of high signal-to-noise tandem mass spectra of intact proteins on a chromatographic time-scale.

Results and Discussion

Here, we report performance of the 21 T FT-ICR instrument for characterization of intact proteins and highlight results from ongoing proteomics projects. For example, observed mass errors are 0.2 and 3.1 ppm for light (24 kDa) and heavy (50 kDa) chains of an IgG1 monoclonal antibody, and 60% sequence coverage of the light chain is achieved in a single experiment. We demonstrate isotopic resolution of the G0F and G1F glycoforms of the heavy chain upon isolation of a single charge state. Combined CID and ETD fragment ion coverage exceeds 30% and glycosylation is site-localized. Finally, high-throughput proteome analysis of a human colorectal cancer cell line (DLD-1, KRas wt/G13D) results in identification of 684 unique protein sequences expressed as over 3,000 unique proteoforms at 1% false discovery rate.

MO02-06

Conclusion

21 T LC-FT-ICR MS/MS is a state-of-the-art platform for top-down proteomics. The instrument is part of the NHMFL ICR user facility and is available to all qualified users. Work supported by NSF DMR-115740 and the state of Florida.

Keywords: FT-ICR, 21 Tesla, Top-down proteomics

MP02-01

Analysis of Peptides and Proteins Using Ion Mobility Separation, Electron-Based Dissociation and Mass Spectrometry

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Introduction and Objectives

The combination of Ion Mobility with MS/MS can generate conformational, molecular weight and structural information. However, CID often results in only partial structure definition, whereas electron-based dissociation methods (ExD) generate peptide, protein and glycan sequence information while preserving labile modifications, and provide much more extensive glycan linkage information than CID.

Methods

We installed modifications to a 6560 IMS-QTOF MS (Agilent Technologies) and a 12-T SolariX™ Qh- FT-ICR MS (Bruker Daltonics), to enable IMS-ExD-MS and MS/MS experiments. In the IMS-QTOF MS, a radio-frequency-free electromagnetostatic cell (e-MSion) is placed between the quadrupole and collision cell. The FTMS is equipped with a prototype Trapped Ion Mobility Spectrometry (TIMS) device.

Results and Discussion

IMS separations enable the resolution of analytes on the basis of their collision cross-sections; even individual charge states of a single analyte often exhibit >=2 conformations. We are applying IMS-ExD-MS/MS to structural studies of native/modified peptides and proteins and to native/derivatized glycans and glycoconjugates. With these systems we are able to study conformation-specific ExD of linear oligosaccharides and branched milk sugars, glycopeptides, peptides and proteins. For charge states that exhibit multiple conformations, we have obtained distinctive ExD spectra for each state.

Conclusion

These approaches extend the capabilities of direct infusion and LC-MS/MS approaches to the separation and structural analysis of biopolymers, by adding an orthogonal separation that is based on molecular shape and taking advantage of electron based dissociation modes to provide increased sequence information while preserving labile modifications. Acknowledgements: NIH grant P41 GM104603 and a gift from Agilent Technologies University Relations Program. We are grateful to many colleagues at Agilent and Bruker for their assistance in these projects.

Keywords: electron capture dissociation, electron transfer dissociation, ion mobility separations, peptides, glycopeptides, protein structures

MP02-02

Extending the Depth of Coverage in SWATH® Acquisition with Deeper Ion Libraries

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Introduction and Objectives

SWATH® Acquisition has become a powerful tool in global protein discovery and quantitation and previous work showed that the extended dynamic range of the TripleTOF® 6600 system and its fast MS/MS acquisition speed in combination with variable window acquisition increased the depth of coverage.A less explored variable on depth of coverage is the impact of the size of the ion library. Here, the impact of using larger and deeper ion libraries on the numbers of peptides and proteins detected was explored and the quality of quantitation observed by processing the same data files from digested yeast as well as human cell lysates with increasingly deeper ion libraries was assessed.

Methods

Various ion libraries were generated by information dependent acquisition on a TripleTOF® 6600 system using 1D-nanoLC- as well as 2D-microLC-MS/MS strategies. In addition a pan yeast and pan human library were used for data extraction, generated from a large number of different cell lines and extensive protein identification experiments.SWATH® experiments of digested human HEK and yeast cell lysates were acquired on a TripleTOF® 6600 system using a NanoSpray® Source.IDA data were searched with ProteinPilot™ 5.0 Software. Library generation and SWATH® Acquisition data were processed using PeakView® Software 2.2.

Results and Discussion

The number of peptides and proteins increased from 12804 and 3371 (1D) to 197585 and 14425 (pan human) for human with increased library size and from 15627 (1D) and 2272 to 87535 and 4658 (pan yeast) for yeast. The reproducible detection of peptides from replicate injections of a yeast cell lysate can be greatly enhanced by 70% by using the pan yeast ion library vs the 1D library.For a HEK cell lysate gains in both peptides and proteins numbers were observed as the size of the ion library used for targeted data processing increased. The gain of 2D vs 1D library was 111% for peptides and 65% on a protein level. The numbers even increased using the pan human library for peptides and proteins by 332% and 118% respectively compared to the 1D library.Even as more data is extracted from the data files using the deeper libraries, the quality of quantitation is maintained even into the low abundant protein/peptide regime.Depending on the depth of the ion library needed and the available time for analysis, a researcher can balance the library generation effort required for a particular biological system. The comparison of detection rates of quantified peptides/proteins in each library to those present in total library showed that detection rate decreases as the ion library size increases. However the library generation time also increased. Microflow LC was used during creation of the 2D library, which can help speed up the process of library building.

Conclusion

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The use of extensive ion libraries highlights the deeper information that is contained in SWATH® acquisition over traditional DDA data.

Keywords: SWATH, DIA, ion library, proteomics, protein, peptide, quantitation, microflow

MP02-03

Chemical cross-linking of NMDA receptor signaling complex: promising strategy of complexes characterisation

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Introduction and Objectives

Chemical cross-linking followed by LC-MS/MS is one of the most promising technologies to analyse the protein-protein interactions in the complexes based on the MS data. The cross-linkers used in these methods are including the active molecules able to bind amino acids residues forming stable or MS cleavable bonds.

Methods

The NMDA receptor pathway is leading to the depression state development which makes its signaling complex components determination a task of high importance. Cross-linking with use of acidic and basic MS stable cross-linkers in comparison with MS cleavable DSSO in parallel is the promising strategy which is currently tested on the ribosome model to find the optimal settings for the method to work.

Results and Discussion

Conclusion

This technique can help to not only analyse the interactions between the proteins in the complex, but also to develop the active molecules able to disrupt it which can be used later as potential anti-depressant medicine.

Keywords: Cross-linking, mass spectrometry, NMDA receptor, ADH, BS3, DSSO

MP01-04

The ETD-like fragmentation for secondary metabolites

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Introduction and Objectives

Flavonoids are a class of secondary metabolites involved in many functions. An increasing number of studies are investigating their biological properties, focusing on the connection between antioxidant activity and chemical structures. Here we investigate their molecular structures using various fragmentation techniques (CID, HCD and UVPD) available on a modified Orbitrap Fusion Tribrid.

Methods

We performed direct infusion experiments of various flavones and conjugated flavonoids with a modified Orbitrap Fusion Lumos. We performed CID, HCD and UVPD fragmentation experiments on these molecules for structural investigation. The data acquired from the different fragmentation techniques were used to reveal the molecular structures of each flavonoid using mzCloud and Xcalibur Qual Browser.

Results and Discussion

Common fragment ions using all three techniques were observed (331.1000, 617.1477, 471.0903, 308.0291 and 185.0420) but the UVPD option also provided unique fragmentation channels in both low and high mass range: 121.0283, 149.0231 and 763. 204. These results present UVPD technique as a potential tool in the structural elucidation of conjugated flavonoid structures especially as CID and HCD fragmentation techniques are severely limited for flavonoid aglycones, O-glycosides, C-glycosides and acylated glycosides characterization. The diversity of chemical space in conjugated flavanoids is extremely large with the total number of combinations of conjugate sugars on different cores and their linking positions being unknown. UVPD surely offers the ability to gain unique fragmentation information which could lead to the position of conjugation that could be the key for identification.

Conclusion

UVPD is an unmatched fragmentation process required for complete and accurate molecular structural elucidation of flavonoids and conjugated flavonoids

Keywords: UVPD, Flavonoid, Orbitrap MS

MP02-05

Comparison of SWATH and iTRAQ in clinical study with multiple small protein samples

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Introduction and Objectives

The purpose of the study was to compare isotopic-labelling (iTRAQ) and label-free (SWATH) mass spectrometry (MS) quantification outcomes for reliability, robustness, sensitivity and possible other parameters for analysis of multiple small volume samples in a clinical study.

Methods

Tear samples (n=140) were collected by using Schirmer's strips at 5 different time points from the eyes of 28 glaucoma patients as part of a clinical trial testing glaucoma medication. The trial was approved by the Tampere University Hospital Ethics Committee and registered as EudraCT Number: 2010-021039-14. Tears were collected by using Schirmer strips. Tear sample was split between the two methods and used for further analyses. Samples were prepared for MS using iTRAQ, 25 or 50 µg and for SWATH, 6-50 µg, of total protein. For iTRAQ 4 µg and for SWATH 2.5 µg of sample was injected for MS analysis. Analyses were performed using the same 2 hour LC gradient on an Eksigent 425 NanoLC with ChipLC and by Sciex TripleTOF 5600+. Post processing was done by Sciex softwares and bioinformatics analysis by R software (v3.1.0.).

Results and Discussion

iTRAQ identified a total of 960 different proteins quantified in at least one sample (Unused ProtScore conf = 0.05 and FDR 1%). In individual samples using iTRAQ 106 to 444 proteins were identified, averaging 207 proteins. On average 110 proteins were seen in both technical replicates, but only 18 proteins were common in all individual samples. For SWATH analysis, a spectral library of 750 proteins (Unused ProtScore conf = 0.05 and FDR 1%) was generated out of which 697 proteins were identified and quantified from all individual samples in total. Repeatability was calculated by relative standard deviation (RSD) % of the technical replicate samples. Out of 207 proteins quantified by iTRAQ 65 had < 10 % RSD and 89 < 20 % RSD, corresponding to 31.5 % and 42 % of identified proteins, respectively. From 697 proteins quantified by SWATH, 215 showed < 10 % RSD and 330 < 20 % RSD, corresponding to 31 % and 47 % of all proteins. The two methods were further evaluated by comparing protein level fold changes of 43 proteins predominantly shared in 91 individual samples in iTRAQ and SWATH. Analysis revealed that the linear regression slope between the methods was 0.498.

Conclusion

Comparison of two MS-quantification methods, iTRAQ and SWATH, revealed label-free SWATH analysis as more suitable method for a clinical trial. The number of identified

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proteins was higher with iTRAQ and repeatability of the methods was equal. Both iTRAQ and SWATH showed similar relative quantification results for most of the shared proteins; however, the number of quantified proteins was much higher with SWATH. For proteomic analysis with limited amounts of protein SWATH provides more complete data without missing values and superior quantification performance when compared to iTRAQ.

Keywords: SWATH, iTRAQ, Clinical proteomics, Label free quantification

MP02-06

Quantification of Low-Abundance Serological Proteins as Potential Colorectal Cancer Biomarkers by Pseudo-MRM with Peptide-Affinity Enrichment

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Introduction and Objectives

Tissue inhibitor of metalloproteinase 1 (TIMP1) and protein tyrosine phosphatase kappa (PTPκ) are related to tumor development and have been suggested as a potential colorectal cancer (CRC) biomarkers. Therefore, the analysis of these proteins is demanded for the diagnosis of CRC. However, a bottleneck for analysis of these low-abundance proteins (a few dozen ng mL-1) is the wide dynamic range in the presence of high-abundance interfering proteins. For this reason, more sensitive method is required to analyze low-abundance proteins such as multiple reaction monitoring (MRM) using mass spectrometry (MS) and immunoaffinity enricement.

Methods

We have developed a method for quantitation of low-abundance proteins using a pseudo-MRM of hybrid Q-TOF MS. This approach was used for the quantification of TIMP1 and PTPκ, which were prepared with stable isotope standards and capture by anti-peptide antibodies (SISCAPA) in CRC serum. First, a pseudo-MRM analysis was conducted for synthetic peptides and spiked into tryptic digests of human serum. The pseudo-MRM was then used for the quantification of the tryptic peptides from two low-abundance proteins, TIMP1 and PTPκ.

Results and Discussion

By integrating multiple transition signals corresponding to fragment ions in the full scan MS/MS spectrum of a precursor ion, a pseudo-MRM showed an improved signal-to-noise (S/N) ratio and reproducibility. Finally, this method was used to detect femtomolar amounts of target tryptic peptides from TIMP1 and PTPκ, with good coefficients of variation (CVs <10%), using a few microliters of CRC serum (4.8 μL). These results demonstrate that the pseudo-MRM by hybrid Q-TOF MS provides a accurate and reliable quantitative assay of low-abundance proteins.

Conclusions

This study demonstrates that pseudo-MRM MS using hybrid Q-TOF MS combined with peptide-affinity enrichment (SISCAPA) has the potential to become a promising MS technique for the quantification of proteins in biomarker development, especially for the analysis of low-abundant proteins in complex blood fluids.

MP02-06

Keywords: Low-abundance serological protein, Peptide-affinity enrichment, Pseudo-multiple reaction monitoring

MP02-07

Shotgun proteomics using on-line Parallel Accumulation – Serial Fragmentation (PASEF)

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Introduction and Objectives

Ion mobility spectrometry (IMS) in combination with TOF instrumentation has shown promise to increase the analysis speed, selectivity and sensitivity of MS experiments. Trapped ion mobility spectrometry (TIMS) consists of a very compact ion tunnel (10 cm length) in which nitrogen gas is used to push ions against an electric field gradient until forces from gas flow and repulsing electric field come to an equilibrium. By stepwise lowering the electric field, ions are released from the device as a function of their ion mobility. We have recently demonstrated that synchronizing this sequential release of ions with the precursor isolation in the quadrupole can multiply the speed and sensitivity of MS/MS scans (Meier et al., J Proteome Res. 2015). Here we further extend the PASEF method to data-dependent acquisition.

Methods

All experiments were performed on a prototype TIMS-QTOF mass spectrometer (Bruker Daltonics). In PASEF mode multiple precursors per TIMS scan were selected by sub-millisecond switching of the quadrupole isolation window. To evaluate an on-line PASEF strategy, tryptic protein digests were HPLC separated on a 50 cm reverse-phase nano-column and coupled to MS via Captivespray ion source. Multidimensional PASEF-data were unfolded by integrating the corresponding mobility range for each individual precursor, resulting in a data set that can be processed with standard non-IMS processing software. A more sophisticated analysis was performed with MaxQuant, which was adapted for TIMS-QTOF data.

Results and Discussion

By separating ions according to their size-to-charge ratio, ion mobility adds a third dimension of separation to LC-MS experiments in addition to m/z and retention time. In an initial set of experiments we investigated the characteristics of the ion mobility dimension for complex biological samples. MaxQuant software was used to detect Isotope patterns (peptide features) that were present in all three dimensions of 90 min single shot HeLa digests. M/z and ion mobility resolved features cluster in several populations corresponding to their characteristics. About 250,000 features were detected after filtering for features with charge state > 1 and retention time between 15 min to 105 min. About 90% of the features detected showed collisional cross sections (CCS) between 339 and 867 Å2 and a median ion mobility peak width of around 0.7 ms. A novel algorithm allows real-time precursor ion determination in all three dimensions (LC retention time, TIMS elution time and m/z value). To reduce dead time precursor determination and data acquisition were parallelized which allows for selection of more than 10 precursors per

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100 ms TIMS scan.

Conclusion

Using ion mobility separation times of ~100 ms a 10-fold increase in sequencing speed could be obtained. This can be used to target more precursor ions and/or to acquire fragment spectra from weak precursors repeatedly to gain sensitivity. Additional method development should further improve these results.

Keywords: PASEF, TIMS, ion mobility

MP02-08

Development of an Online 2D RP-RP LC/MRM-MS Method for Targeted and Comprehensive Analyses of the Human Plasma Proteome

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Introduction and Objectives

LC/MRM-MS based quantitative proteomics is an increasingly attractive method for the detection and validation of plasma biomarkers relating to a wide range of disease states. However the multiplexing and sensitivity of MRM based approaches has been limited to relatively small panels (<300 proteins). Previous work has demonstrated the efficacy of an offline two dimensional reversed-phase - reversed-phase (2D RP-RP) separation. From this, we sought to develop an online 2D RP-RP method which would reduce processing time and increase sensitivity for targets of low abundance.

Methods

Plasma tryptic digests spiked with isotopically labeled standard (SIS) peptides were analyzed by RP-RP-LC/MRM-MS. Samples were separated using an Agilent 1290 UPLC system equipped with a quaternary pump and binary pump. The separation was performed using a stepwise high pH gradient (10 mM ammonium hydroxide) using a Waters Xbridge 4.6 x 150mm C18 column as the first dimension, and trapping the eluted peptides on an Agilent Poroshell 120 4.6 x 50mm C18 trap column. Separation in the second dimension was carried out at acidic pH on an Agilent Zorbax Eclipse Plus 150 x 2. 1mm C18. Eluted peptides were analyzed in MRM mode using an Agilent 6495 triple quadrupole mass spectrometer.

Results and Discussion

In order to test the efficacy of our online 2D RP-RP method, a panel of 210 peptides was selected with protein concentrations spanning several orders of magnitude (low ng/mL - mg/mL). Of these, we were able to reliably detect approximately 158 peptides. Compared to 1D LC/MRM-MS, our preliminary data provides major improvements in terms of coverage and sensitivity of the plasma proteome, notably for several targets of reportedly low abundance (ng/mL). Of these targets, approximately 20 have not been detected by traditional 1D LC/MRM-MS. By conducting the entire experimental workflow in an online manner, variations due to sample handling (fractionation, lyophilization, etc.) are minimized, and the experimental run time is reduced compared to the offline 2D RP-RP method, because there is no need to collect and manipulate the fractions from the first dimension. This reduces potential losses of low-abundance peptides onto the walls of the collection plate. Additionally, the use of analytical flow rates in this method allows for better sample to sample reproducibility than nanoflow based methods. From our preliminary results we found that the online 2D RP-RP method affords comparable sensitivities to our earlier offline method in terms of peptide detection while greatly shortening the experimental runtime.

Conclusion

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This represents the first use of an online 2D RP-RP LC/MRM-MS method at analytical flow rates for plasma proteomic measurements.

Keywords: Online 2D RP-RP MRM LC-MS Quantitiative Plasma Proteomics

MP02-09

Improved Qualitative and Quantitative Analysis of the Human Mitochondrial Proteome by Hybrid Acquisition

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Introduction and Objectives

Mitochondria are essential organelles for the regulation of cell life and death. Literature suggests the involvement of mitochondrial dysfunction in many human diseases, some strictly linked to mutations in the mitochondrial genome, and others connected to mitochondrial functionality by proteins sequence defects. At the same time, proteins possibly related to mitochondrial function await validation at the transcript and protein level. A novel hybrid acquisition mode, named Multi-Mode Acquisition, which is the product from combining of DDA and DIA in a single experiment, and associated analysis tools, are described for the analysis of the mitochondrial complement.

Methods

Data for tryptic digests of Human osteosarcoma (U-2 OS), neuroblastoma (SH-SY5Y) and cervix carcinoma (HeLa) samples which had been 1D gel separated were collected using 2 h reversed-phase gradients in combination with DDA, IM assisted DIA (HDMSE) and hybrid multi-mode acquisitions (MMA). The data were in all instances processed with dedicated peak-profile based detection algorithms. In the case of MMA acquisitions and experiments, the DDA and (IM) DIA data streams were merged and contrasted, and research informatics tools applied to create peak lists. Searches were conducted and compared with multiple probabilistic DDA search and de novo annotation algorithms.

Results and Discussion

Comparing DDA data and results processed using single scan peak profiling based peak detection vs. traditional algorithms increased the number of peptides and proteins identified, with a concurrent increase in amino acid sequence coverage. Moreover, the median of the score distribution and its connected FDR calculations based identification cut-off, increased proportionally. Next, the IM-DIA data of the same 1D gel bands were retention aligned with corresponding DDA data. Hybrid type spectra were generated by MMA processing data, which involves step-wise detection of DDA selected ions in the DIA data, assign drift times to DDA precursor ions, math DDA and DIA generated precursor and product ions, and algorithmically separating out chimeric DDA spectra. This process was applied to the HeLa data, providing an additional increase in identifications. The next level of data integration involved MMA processing across the 3 mitochondrial human cell lines. Overall, by combining the results of these 3 levels of data combination/comparing, a substantial mitochondrial proteome coverage increase. Finally, the enhanced association of precursor to product provides the means to search the resulting MMA spectra both traditionally by mass or de novo by sequence.

Conclusion

MP02-09

Single-scan processing for optimum rejection of co-fragmented peptides in individual and combined DDA/DIA data provides cleaner concurrent product ion spectra. A dramatic fragmentation spectra quality increase was observed, which in turns improves confidence and coverage of identifications compared to conventional acquisition and processing modes.

Keywords: DDADIAPeak detectionAlgorithmsIon Mobility

MP02-10

Development of A Novel Method for Detection of Glycoprotein Using Lectin Decorated Diamond Nanoparticles and Mass Spectrometry

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Introduction and Objectives

In recent years, researchers found that the process of modification of glycoprotein is very important for the mechanisms of molecular signaling networks. Among many analytical techniques, selective enrichment of important glycoprotein by nanomaterials have emerged as a new method with wide biomedical applications. Affinity purification with nanoparticles is an effective approach because these materials have high surface-to-volume ratio that improves the capacity for purification and the capability for high throughput screening.

Methods

We have developed an effective method for the selective enhancement of sensitivity of mass spectrometry (MS) for glycoprotein by mixing diamond nanoparticles (DNP) in the sample. In this method, the DNPs were functionalized with lectin, the commonly used receptor of many characteristic oligosaccharides due to lectin can site-specifically bind to sugar chains. The synthesis protocol is based on the preparation of functionalized DNP for the study of phospho- and sulfonated proteins reported previously in this laboratory. The quality for the modification of the lectin on the DNP is supported by transmission electron microscopy and infrared spectroscopy.

Results and Discussion

To characterize functionalized DNPs, Fourier transform infrared spectroscopy (FT-IR) was used to ensure proper fabrication of the surface modification. FT-IR spectroscopy provided clear evidence for the step-by-step surface modification including silannation and amine functionalization. After modification, the unique Si-O absorption bond was observed in the range from 1000 to 1100 cm^{-1}, indicating successful silanation on the DNP surface. The cross-linker conjugation on the DNP surface was confirmed by the present absorption peak at 1780 cm^{-1}, which corresponds to the C=O absorption band from the carboxylic acid group. The functionalzied DNPs were analyzed by transmission electron microscopy (TEM) to obtain information on size, shape and morphology. The observation of these images could assist for the quality control of synthetic progression. The captured target on nanoparticles was directly analyzed by MALDI-TOF MS. For example, at the minimum, only~1 pmol ribonuclease B (RNase B) is enough for detection.

Conclusion

In this method, the optimal DNP-based biomolecules separation system with high specificity and sensitivity were established. And the optimal MALDI instrument focuses on the detection of glycoprotein were constructed. All of the systems were demonstrated by glycoprotein standards (e.g. RNase B). We could efficiently purify the glycoprotein from

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mixtures by functionalized DNP and can be analyzed directly in MS. We are developing more types of functionalized DNP for improving detection limit of glycoprotein related biomarkers.

Keywords: Diamond nanoparticle (DNP), Lectin, glycoprotien, Mass sectrometry

MP02-11

A simplified approach to fast and accurate, high throughput targeted MS2 quantitation using internal standards

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Introduction and Objectives

Quantifying peptides is often performed using selected reaction or parallel reaction monitoring. These techniques are more sensitive and reproducible than standard data-dependent analysis; yet, the breadth of these approaches is limited and methods are hard to develop and maintain. We present a novel and simplified approach to fast and accurate targeted MS2 quantitation using internal standards.

Methods

All experiments were based around hybrid peptide samples, consisting of mixtures of unlabeled endogenous peptides combined with heavy internal standards. These samples were analyzed with an Orbitrap Fusion Lumos MS coupled to an EASY-nLC 1000 ultra-high pressure LC. The resulting LC-MS/MS data were searched using Proteome Discoverer 2.1 (1% FDR) and analyzed with Skyline.

Results and Discussion

Accurate quantification of high number of targets in a single analysis is obtained by reaching a balance between scan rate, sensitivity, spectral quality and scheduling. Generating an optimal method can be extremely time-consuming owing to intrinsic dependencies on matrix conditions, LC performance, and the need to balance the aforementioned properties. Herein we propose a very simple approach, which leverages the unique chemical properties of the heavy peptides and the high-resolution and accuracy of the Orbitrap analyzer to perform on-line¬ identification of the spiked-in standards without the need of a spectral library. We have compared the utility of this novel quantitative PRM-based method to traditional PRM analyses. We found that sensitivity and breadth of this novel PRM method was comparable to traditional approaches; yet, the method itself was much easier to setup and maintain.

Conclusion

Development of a simplified approach to fast and accurate, high-throughput targeted MS2 quantitation using internal standards.

Keywords: High throughput targeted Peptide quantitation,Simple approach,Internal Standard,Orbitrap MS

MP02-12

Comprehensive Relative Quantification of the Cytochromes P450 by Microflow LC and SWATH® Acquisition and Data Processing Using Cloud Computing

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Introduction and Objectives

Metabolism of drugs by the Cytochrome P450 superfamily is pivotal in determining their disposition, safety and efficacy. Since drugs may induce expression of several isoforms of cytochrome P450s, they may enhance their own turnover, increasing the risk of toxic metabolite formation or adverse interactions with co-ingested compounds. Thus P450 profiling is a fundamental aspect of drug safety evaluation. The cytochrome P450 proteins share extensive sequence homology, so that antibodies are incapable of discriminating every isoform, plus mRNA levels do not correlate well with protein. SWATH acquisition is a data-independent MS acquisition method for label-free quantification which enables closely-related proteins to be quantified retrospectively through post-acquisition extraction of specific peptide ions, and is thus perfectly suited to P450 profiling. The use of microflow LC technology allows for more robust and faster acquisition of samples, allowing for an increase in throughput and maximizing the use of instrument time. Together these two technologies improve the speed and robustness of proteomic data acquisition.

Methods

Mice were exposed to inducers of the cytochrome P450s, and pooled microsomal fractions were prepared from the livers. Following protein extraction and digestion, a database of microsomal proteins was generated by 2D microflow LC-MS/MS using information-dependent acquisition on a TripleTOF 6600® system (SCIEX). Individual samples were then processed and LC-MS data were acquired using the SWATH acquisition. SWATH data and its respective library was uploaded using the CloudConnect microapp and the data was processed using the OneOmics™ platform.

Results and Discussion

OneOmics platform separated induced and non-induced mice based on their overall protein expression pattern, and that of the P450s. Relative quantification of uniquely discriminatory P450 peptides enabled the induction profile of each compound to be ascertained in unprecedented detail allowing for the possibility to identify and quantify peptides unique to Cyp2C50 and Cyp2C54 despite the fact that the proteins share 92% sequence identity.

MO02-03

iST: Sample preparation for high throughput clinical proteomics- A novel, fast, sensitive and reproducible sample preparation for MS-based proteomics-

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Introduction and Objectives

Sample preparation workflows are a crucial part of routine mass spectrometry (MS) based proteomics measurements. Complex workflows, extensive sample fractionation and proteolytic digestion are highly time consuming and restrict the overall technical reproducibility limiting the overall applicability of MS-based proteomics for clinical applications. The accuracy and robustness of the MS platform is also strongly affected by sample quality reasoning for high quality proteomic samples. Here we present the straightforward and robust in-StageTip (iST) method for streamlined sample processing of complete proteomes.

Methods

The iST method is a 3-step procedure performed in a single, enclosed volume which thereby circumvents the likelihood of contamination and sample loss. Due to the straightforward nature, the method can readily be performed in a 96-well format on liquid handling robotic system. The method is highly compatible with established and novel StageTip based pre-fractionation methods and thereby allows in-depth analysis of complex proteomic samples.

Results and Discussion

Applying the procedure to the well-studied cancer cell line HeLa allowed us to estimate protein copy-numbers of 9,667 proteins. The results demonstrated excellent reproducibility (R2 = 0.97) in quadruplicates measurements reflecting the overall strength of the method. In addition, we applied this sample-preparation workflow to process urine samples. We collected urine samples from 6 healthy male donors on 3 consecutive days. We identified a total of 3284 proteins in urine and calculated the label-free quantification values of 2200 proteins in average per sample, with MS-signals spanning 6 orders of magnitude.

Conclusion

The in-StageTip method opens up opportunities for high-throughput clinical applications enabling exceptional sample quality at low cost and effort.

Keywords: Sample PreparationClinicalHigh Throughput

MO09-01

Structural characterization of protein phosphorylation and antibody complexes by top/middle-down mass spectrometry

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Introduction and Objectives

Accurate structural characterization of a specific protein PTM isoform is challenging due to the difficulty of separating it from other isoforms as well as the limitations of the traditional structural methods. In addition, structural characterization of large proteins such as antibody complexes at high resolution remains challenging. Hydrogen/deuterium exchange coupled with mass spectrometry (HDX-MS) has become an indispensable technique for higher-order structural characterization of proteins. The traditional peptide-based bottom-up approach suffers from significant back exchange and only peptide-level resolution. Also, the connection between multiple PTMs is lost in the bottom-up approach. By using top-down MS and the further improved middle-down approach we developed recently [Pan et al, Chem. Sci. 2016, 7: 1480], we are able to selectively characterize the structure of specific phosphorylation isoforms and also the antibody-antigen complexes at single-residue level.

Methods

Subzero temperature HPLC was carried out at -20º C. LC-MS data were acquired on an Orbitrap Fusion mass spectrometer with ETD capability (Thermo Scientific). The AGC target was set at 2e5, and the maximum injection time was 100 ms. Online ETD experiments were done by selecting one charge state for each peptide/fragment ion in a single HPLC run. Intact protein fragments were detected using LC-MS using a scan range of 300-2000 m/z.

Results and Discussion

By using selective top-down ETD, we were able to identify six specific phospho-forms of Calmodulin (CaM) out of 16 theoretical possibilities. Phosphorylation of CaM at four sites by CK2 was found to follow a sequential order. By combining top-down HDX/ETD with subzero temperature HPLC separation, the impact of phosphorylation on CaM's structure was elucidated in a isoform-specific manner.The antibody-based drug Bevacizumab (BEV) is currently used for treatment of a number of cancers. The BEV-VEGF system was used as our model antibody-antigen complex. Intact protein measurements showed that the light chain and heavy chains of BEV acquired fewer deuterium atoms in the complex than in BEV alone. Maximum differences were observed at an HDX time of 4 h, with +9 Da on the heavy chain and +3 Da on the light chain. Online ETD was used to locate the protected sites, and HDX differences were only observed on ions from the N-terminus, not the C-terminus. Amino-acid-level deuteration data on the heavy chain showed that the differences were on residues 58-67 and 82-84, plus little on residues <39. Data analysis for the light chain is also in progress. A targeted middle-down technique is also developed to determine the binding site on the antigen VEGF at single resiue level.

MO09-01

Conclusion

Selective structural characterization of specific PTM isoforms is achieved by using top-down HDX/ETD with subzero temperature HPLC separation. A novel middle-down technique is developed for amino acid level structural analysis of antibody complexes.

Keywords: Phosphorylation, Electron transfer dissociation, top-down, middle-down, hydrogen deuterium exchange, antibody

MO09-02

Trapped Ion Mobility Spectrometry: An Additional Dimension of Separation for Proteomics Applications

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Introduction and Objectives

Ion mobility spectrometry (IMS) is an analytical method that separates ions based on their transport through an inert gas. In this sense, IMS is a gas phase ion chromatography technique analogous to liquid chromatography (LC). Importantly, for purposes of coupling, the timescale of an IMS separation (<1 s) is between that of LC and time-of-flight mass spectrometry (TOFMS) – opening the possibility of an additional dimension of separation, thereby improving peak capacity, and dynamic range over LC-TOFMS alone. In recent years, IMS has been coupled with mass spectrometry (IMMS) and commercialized. As interest in IMMS has grown, the development of new technology is bringing IMS to a level of maturity worthy of consideration in many analytical fields.

Methods

Trapped ion mobility spectrometry (TIMS) is one such new technology. In a TIMS analyzer a retarding electric field holds ion in place against a moving gas. Ions, initially trapped in the TIMS analyzer, elute according to mobility – lowest mobility first – as the strength of the retarding electric field is reduced. In the present work, a TIMS analyzer was incorporated as a section of an ion funnel of a prototype TIMS-QTOF mass spectrometer (Bruker Daltonics). Ubiquitin (Sigma-Aldrich, St. Louis, MO) was diluted to a concentration of 1 µM in 70:30 H2O:acetonitrile. Samples of isobaric peptides were prepared at concentrations of 1 to 5 µM in 50:50 H2O:acetonitrile. Trypsin digest of Bovine Serum Albumin (Michrom Bioresources Inc. PTD 00001/15) was diluted to a final concentration of 0.33 pmol/µl in 50:50 H2O: acetonitrile.

Results and Discussion

TIMS promises a simplicity, flexibility, and sensitivity unprecedented in the world of IMMS. Specifically, TIMS analyzers are small (~10 cm in length) and operate at low voltages (~200 V) as compared to conventional IMS analyzers, but can achieve a relatively high resolving power (~300) and duty cycle (up to 100%). Because the user has direct control of the retarding analytical field, the TIMS experiment can be optimized to suit the application. This presentation discusses the basics of TIMS and its performance characteristics in a prototype TIMS QTOF mass spectrometer especially as it relates to the field of proteomics. Results from the analysis of ubiquitin demonstrate that TIMS can be operated in a “soft” manner – i.e. preserving conformations representative of native states. The high resolution analysis of peptides,

in some cases reveals previously unresolved conformations. The analysis of PTM peptides (specifically PFGK modified with propionyl v propanal carbonyl acyl groups) shows that such isobaric regioisomers can be nearly baseline resolved at mobility resolving powers of about 160. In a TIMS-MS analysis of BSA digest the TIMS separation was shown have a peak capacity of about 45.

Conclusions
A TIMS-QTOF mass spectrometer has been shown to have both high performance and flexibility in the analysis of proteomic samples.

Keywords: Trapped ion mobility mass spectrometry, isobaric PTM, digest

MO09-03

Qualitative and Quantitative Characterization of a Novel Scanning Quadrupole DIA Method for Omics Analysis

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Introduction and Objectives

Targeted LC-MS based assays are increasingly applied in the post-discovery omics area with emphasis on validation, the first of many phases in translational analyses, or in studies that are aimed at gaining the understanding of biological systems, drug development and treatment. Context is driving current omics experiments, thereby driving the development of LC-MS acquisition methods that can provide both qualitative and quantitative information in a single experiment. An alternative DIA mode operation, whereby a resolving quadrupole is scanned during high-resolution precursor and product ion acquisitions, was applied for targeted lipidomics and proteomics quantitation experiments using transition extraction list and compound library based approaches.

Methods

Human plasma samples were treated with isopropanol and centrifuged to precipitate proteins. The lipid-containing layer was collected and a second aliquot of the plasma samples was tryptic digested and analysed separately. DIA data were acquired on a tandem quadrupole/oa-time-of-flight (ToF) mass spectrometer. The m/z isolation range of the quadrupole was continuously and repetitively scanned with MS data acquired using a high-resolution acquisition system capable of delivering up to 2000 spectra/s. Alternate MS scan data comprise precursor and CID product ions. The quadrupole mass range and resolution were investigated to determine the optimal balance between sensitivity and specificity. The resulting 2D data, m/z (ToF) vs. m/z (quadrupole), were processed and quantified using Progeneis QI and Skyline open source informatics. Control, diabetic and obese plasma samples of varying phenotype were analysed and differently spiked with standards, acting as pseudo QCs, and quantitative lipid and protein changes determined.

Results and Discussion

Quantitative proof-of-concept data were acquired by diluting, lipid standards in protein precipitated plasma and a four protein digest mixture into an E.coli digest. For both experiments, pseudo transition extraction lists were utilised to obtain quantitative information, indicating quantitative response of the method for the two complementary analyte types. Discovery analysis of the 2DMS DIA data sets identified differentially expressed lipid classes (FA, PC, TG, and PS) and apolipoprotein peptides, respectively, across the three conditions of interest quantified.

Conclusion

Analysis of the data indicates that scanning quadrupole DIA enables is over an order of magnitude more specificity than a static quadrupole operated with the same resolution, providing reduced FQR values and higher dynamic range. It was found that quadrupole

MO09-03

transmission windows of 5 - 10 and 20 - 30 Da provided optimum lipid and protein identifications. The obtained lipidomic and proteomic application results were in good agreement with previous discovery studies and the expected changes in relation to both disease and phenotype.

Keywords: Targeted quantitation, discovery, proteomics, lipidomics, precursor quadrupole scanning 2DMS DIA

MO09-04

ProteusQC™: the Versatile Standard for Bottom-up Proteomics

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Introduction and Objectives

The majority of current research within the field of MS proteomics has focused on the relative/absolute quantitation of candidate protein biomarkers using bottom-up LC-MS in conjunction with stable isotope-labeled standards (SIS). While these methodologies are being designed within a fit-for-purpose approach, development is often slowed by the absence of standards for response normalization and quality control (QC) assessment. Further, the methods that incorporate SIS often do so at the peptide level, which is not ideal since these cannot adequately account for the variability that may arise during proteolysis. Toward minimizing these aforementioned limitations, we developed the ProteusQC™ standard for bottom-up proteomics.

Methods

ProteusQC™ consists of a balanced mixture of 15N-labeled human ApoA1 and six of its 13C/15N-labeled tryptic peptides (New England Peptide Inc.). An aliquot of this mix was diluted then spiked into a biosample (e.g., human plasma or cerebrospinal fluid, CSF) for bottom-up proteomic analysis. Sample preparation involved denaturation, reduction (with dithiothreitol), alkylation (with iodoacetamide), and enzymatic digestion (at a 10:1 protein: trypsin ratio). Processing of the peptide mixture was achieved by reversed phase LC-MS/MS, with the Quantiva QqQ (Thermo Scientific) operated in the dynamic MRM mode. Data analysis for peak assessment, quality control, and quantitation was accomplished in Skyline.

Results and Discussion

Toward improved development time and data quality for bottom-up proteomic experiments, a SIS mixture of ApoA1 and its peptides was prepared. Development of ProteusQC™ involved a number of optimizations (e.g., LC gradient, MRM transitions) and evaluations (e.g., proteolytic digestion kinetics, SIS formulation). The final method yielded peptides that are dispersed over the entire chromatographic space and are well suited to serve as standards for retention time indexing, method/platform QC, and absolute protein quantitation. These merits were demonstrated in the analysis of 62 high-to-moderate abundance proteins (inferred from >300 peptides) in human plasma, with application to human CSF in progress. Also ongoing is the stability testing of the mixture under various storage conditions. In our short-term evaluations, for example, the targets of ProteusQC™ in a plasma digest were found to deliver stable peptide ratios over 2 weeks of storage at 4°C. To be presented is the development, implementation, and application of ProteusQC™ to control biosamples.

Conclusion

Through rigorous assessment, we have developed a versatile standard for retention time

MO09-04

indexing, QC assessment, and absolute quantitation in bottom-up proteomic studies. These afforded merits are expected to help advance the development and application of proteomics in basic and clinical research studies.

Keywords: indexing, plasma, proteomics, MS/MS, quality control, quantitation

MO09-05

Sampling of tissues for proteomics by soft laser ablation

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Introduction and Objectives

Sampling of tissue can be cumbersome, especially if the tissue is tight. During mechanical homogenization often insoluble material remains, which contains many proteins and in addition is absorbing further proteins. Usually after homogenization of tissue this insoluble material is removed by centrifugation and thus the proteins within this material are lost for identification and quantification. Furthermore this problem adds an additional error reducing reproducibility because the efficacy of mechanical homogenisation is difficult to control. A further point introducing a change of the protein composition during homogenisation of tissue is enzymatic activities. Homogenization of tissue by irradiation with a picosecond infrared laser (PIRL) vaporizes the tissue very softly (cold vaporization). In this study the efficacy of the photonic homogenization and conventional homogenisation of diverse tissues was compared with respect to the yield of identifiable individual proteins and the degree of proteolytic degradation.

Methods

Tissue from tonsils and pancreatic tissues each were divided into two comparable parts and homogenized either mechanically by bed mill (Tissue Lyzer) or by PIRL. The homogenates were subjected to SDS-PAGE, the whole lanes of each separation were cut into pieces following the bands and pieces digested with trypsin. After desalting the tryptic peptides were analyzed with LC-MS/MS using an orbitrap mass spectrometer. The mass spec data were processed with Proteome-Discoverer and MaxQuant yielding qualitative and quantitative data about the protein compositions of the homogenates.

Results and Discussion

In PIRL homogenates significant larger numbers of proteins were identified. This observation can be explained by the absence of insoluble material in the PIRL homogenates in contrast to the mechanical homogenates. In addition it was shown that also the number of protein species was significantly higher. An explanation for that phenomenon is that enzymes under PIRL homogenization conditions have less time for converting proteins because the tissue vaporization is ultrafast and the tissue aerosol was frozen immediately after having left the tissue. This explanation also fits to the results about proteolysis. SDS-PAGE migration profiles, calculated for each protein from relative quantification of proteins by mass spectrometry, uncovered the rate of proteolysis. In the PIRL homogenates significantly less proteolysis was detected in comparison to mechanical homogenisation.

Conclusion

Homogenization of tissues by PIRL is advantageous in comparison to mechanical homogenization because the yield or proteins is significantly higher and the degree of

enzymatic degradation of proteins is smaller. Thus homogenization of PIRL should give a view on the composition of proteomes which is more close to the composition in the intact tissue prior to homogenization.

Keywords: Tissue homogenization, Laser ablation, Label free quantification, Proteolysis

MP03-01

A robust and sensitive capillary flow LC – high-resolution accurate-mass MS platform for discovery and targeted proteomics

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Introduction and Objectives

Capillary flow LC-MS (capLC-MS) with 100-500 µm inner diameter (ID) columns and flow rates from 1 to 10 µL/min is a useful alternative to analytical flow LC-MS (>100 µL/min) due to increased MS sensitivity, lower solvent consumption, reduced contamination of MS and consequently, higher LC-MS uptimes. Additionally, capLC-MS can provide higher throughput in comparison with nano flow LC, while maintaining the same sensitivity by loading higher sample amount. We describe a reliable and easy-to-setup capLC-MS platform for sensitive targeted and shotgun analysis of peptides and proteins. The obtained results were compared with nano and analytical flow LC-MS analysis and recommendations for optimal LC and ESI interface configuration for different proteomics applications were formulated.

Methods

The UltiMate 3000 RSLCnano system with capillary flow meter configured in pre-concentration mode for trap and elute experiments was used for peptides separation on 0.3 x 150 mm or 50 mm, 2 µm Acclaim PepMap C18 columns. The integrated micro flow pump of the RSLCnano system was used for fast sample loading at 100 µL/min onto a 0.3 x 5 mm Acclaim PepMap trap cartridge. The capLC system was coupled to a Q Exactive HF or TSQ Quantiva with an EASY-Spray ESI source and a micro EASY-Spray transfer line with a 20 µm ID silica emitter. Data were acquired in Full MS, data dependent acquisition or parallel reaction monitoring modes with Chromeleon 7.2 and processed with Proteome Discoverer, BioFinder and Skyline.

Results and Discussion

We systematically investigated the effect of flow rate and column ID on MS sensitivity using samples with different complexity, such as Cytochrome C protein digest, peptide retention time calibration mixture, HeLa cell lysate digest and monoclonal antibodies (mAb). The sensitivity of capLC-MS was around 20 times higher at 5 µL/min in comparison with analytical flow LC-MS analysis at 450 µL/min. The optimized capLC-MS conditions resulted in high-throughput separations with ~ 10 min total analysis time for targeted quantification of peptides at amole level. A long term precision study showed RSD values smaller than 15% with and without internal standards for complex biological samplers. Peak capacities above 500 were achieved for capLC that is comparable with nano flow LC separations. For identical sample loading amounts this resulted in identification rates in the shotgun proteomics experiments of at least 65% of protein compared to nanoLC-MS experiments. However, the loading capacity of capillary columns is around 10 times higher that allows to minimize differences.

Conclusion

Capillary flow LC together with plug-and-spray EASY-Spray emitters for electrospray

MP03-01

ionization unites the advantages of high-throughput analytical flow LC separations with high sensitivity of nano flow LC-MS analysis. It is a valuable tool for targeted peptide quantification as well as bottom-up proteomics profiling of large sample cohorts for translational medicine studies.

Keywords: Capillary flow LC MS, shotgun, targeted

MP03-02

Application of baculovirus expression vector system in producing human high molecular weight kininogen, a highly glycosylated secretion protein.

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Introduction and Objectives

Human kininogen I (KNG1) is one of the key components in the kinin-kallikrein system (KKS), which is an endogenous multi-protein cascade and activation of which leads to trigger molecular mechanisms, including blood coagulation, fibrinolysis, complement, and renin-angiotensin system. In recent years, KNG1 was also reported as potential biomarker in kidney injury disease, multidrug-resistant Tuberculosis and oral squamous cell carcinoma. Because of its highly glycosylation and secretion pathway through signal peptide, it is hard to be generated by E. coli expression system for further utilities.

Methods

To obtain large amount of soluble and functional high molecular weight kininogen (HMWK), an insect cells, Sf21, based baculovirus expression vector system (BEVS) was employed in this study. To facilitate the purification of HMWK in culture medium, a green fluorescent protein was co-expressed to monitor the intact of insect cells by a bi-cistronic baculovirus expression vector.

Results and Discussion

The C-terminal Hisx6 tagged HMWK could be purified from serum-free medium by one step of Immobilized metal affinity chromatography (IMAC) and showed expected high molecular weight and glycosylated modification as in mammalian cells. Protein identification was confirmed by both Western blotting and LC-MS/MS analysis. Glycosylation sites was further identified by O18-labeling combined LC-MS/MS protocol.

Conclusion

The yield was about 5 mg from per liter medium with >95% purity reveals that BEVS is of great benefit for production of human secretion protein with highly glycosylation, like KNG1.

Keywords: KNG1, high molecular weight kininogen (HMWK), glycosylation, secretion.

MP03-03

Primary structure analyses for protein therapeutics using mass spectrometry-based methods

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Introduction and Objectives

Protein therapeutics have been one of the major forces that drive biotech industry forward, and their sales grew at an amazing rate in the past years, especially when compared with other pharmaceuticals. Meanwhile, the past decade has seen a rapid advancement in mass spectrometry (MS), in terms of resolution power in mass analyses, consistency of ion fragmentation and accessibility of high-performance instruments, which thus predicts extensive application of mass spectrometry in quality control and quality assessment of protein therapeutics. Primary structure analyses always provide the preliminary parameters that allow us to evaluate the functional competence of these therapeutics.

Methods

In the past, bottom-up approaches have been the mainstay of MS-based analyses, but it is very difficult to distinguish the actual changes from the artefacts introduced by long and complicated procedures used for sample preparation. The enormous improvement in high-resolution instruments, on the other hand, renders effective top-down analyses much more plausible. Intact protein therapeutics below certain mass limits can be directly discerned as ion clusters, which are made of MS signals that are closely juxtaposed but clearly separated in an extremely short mass range. To decipher such MS codes, there is a growing need in development of new informatics tools. Here, we proposed a new approach to target the characterization of proteins bearing small-MW modifications such as disulfide formation and amidation/deamidation.

Results and Discussion

We used this approach to develop an informatics-based system which is able to identify the delicate unforeseeable changes of expressed proteins. For example, accurate determination of the mass losses enables us to uncover two truncated rEGF species, whose new ends have been successfully verified using tandem mass spectrometry. Moreover, we have developed a new approach to document small-MW modifications, e.g. disulfides, in intact proteins. For example, our analyses reveal the presence of three disulfide bonds in rEGF and its two truncated derivatives.

Conclusion

With these state-of-the-art instruments and informatics methods available, we will have an excellent opportunity to effectively reveal protein variations caused by minuscule structural modifications.

MP03-03

Keywords: primary structure; intact protein; small-MW modification; informatics; high-resolution MS

MP03-04

Thermostable plasma proteome and its potential applications in biomarker discovery

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Introduction and Objectives

Common approaches to reduce complexity of plasma proteome usually needed costly consumable reagents and/or materials. However, economical prefractionated method is rarely documented. This study adopted thermal treatment to reduce plasma complexity and characterized thermostable proteome in search of potential benefits in biomarker discovery.

Methods

Thermal treatment of human plasma was performed by a temperature-controlled water bath. Thermolabile proteins were removed by centrifugation. Thermostable fraction was characterized by SDS-PAGE, Western blotting, 2-DE, and mass spectrometry. Reproducibility of proteome profile was evaluated by 2-D image analysis. Literature search was performed to highlight disease-related thermostable plasma proteins.

Results and Discussion

There were dramatic changes in protein band pattern and proteome profile of thermostable plasma fraction as compared to the control untreated plasma. Western blotting revealed albumin, IgG heavy chain and alpha-1 microglobulin were markedly depleted, whereas haptoglobin subunits were enriched after thermal treatment. Reproducibility of thermostable proteome profile was acceptable as demonstrated by visual recognition and spot volume analysis. Literature search revealed that a number of thermostable proteins might be of particularly usefulness in discovery of cancer biomarkers.

Conclusion

Thermal treatment provided the consistent result and can be used to reduce plasma protein complexity in the light of economical benefit and accessibility for plasma proteomic analysis. Potential benefits of thermostable plasma proteome in discovery and validation of cancer biomarkers should be validated in the future.

Keywords: Plasma; Prefractionation; Proteomics; Sample preparation; Thermostable protein

MP03-05

A new method for label free quantification in the Proteome Discoverer framework

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Introduction and Objectives

Proteome Discoverer is a node-based workflow engine and platform for analysis of mass spectrometry-based proteomics data. Here we present a new workflow for label-free quantification that provides the full suite of quantitative capabilities only available previously to isotopically-labeled quantification. The workflow will be demonstrated on several datasets, including proteins and peptides spiked into complex matrices at known ratios.

Methods

The peptide retention time standard calibration mixture (Thermo Fisher Scientific) was spiked into a standard HeLa tryptic digest sample at three different concentrations and the data were acquired on a Q Exactive instrument (Thermo Fisher Scientific, Bremen, Germany). Data for UPS1 proteins spiked into a complex yeast digest were downloaded from PRIDE (PXD002099). A preliminary version of Proteome Discoverer 2.2 was used to interpret the data. Sequest HT was used as the primary search algorithm. Datasets were normalized on total peptide abundance and scaled abundances were subsequently used to compare the protein and peptides across the replicates and datasets.

Results and Discussion

The quantification values for proteins are calculated by simply summing the abundances of the peptide groups. The peptide groups abundances are also calculated by summing the abundances peptide features associated with the identified PSMs. For datasets with missing PSMs, the label-free workflow looks for those features in the remaining datasets using a “feature mapping” step.The “HeLa spike-in” dataset produced over 5000 protein identifications and over 34000 unique peptides, the majority of which were quantified. 80% of the proteins in the HeLa proteins were within 0.8 to 1.2 in the comparisons of the three different samples. The quantitative ratios for the various spiked-in peptides range from 1.6 to 2.2 for the 20 fmol/10 fmol ratio while the ratios ranged from 10 to 17 for the 1000 fmol/10 fmol ratio with the majority of measurements above 14, indicating that spike-in on the 100 fmol sample may have been a little bit more than expected.

Conclusion

A new untargeted label-free quantification workflow based on the Minora algorithm has been demonstrated on a dataset with proteins at known concentration and is shown to be more accurate and sensitive than the previously available label-free quantification approaches from previous versions of Proteome Discoverer. The combination of the label-free quantification workflow integrated into the scaling, normalization, and study management features of Proteome Discoverer provide a powerful means for analyzing highly complex proteomics data.

Keywords: Label free quantificationProteome Discoverer framework

MP03-06

Studying protein phosphorylation with ultra-high temporal resolution using a microfluidic device

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Introduction and Objectives

Cells can rapidly transmit signals through PTMs such as phosphorylation. Although signaling can occur within millisecond to seconds our knowledge on temporal profiles of early signaling events is limited. This may be attributed to the lack of tools allowing reproducible and fast mixing for stimulation, quenching/lysis with high temporal resolution. We developed a µmixer µfluidic device that allows conducting fully controlled temporal stimulation and quenching/lysis of cells to study early signaling (e.g. 0.10 ± 0.08 s after stimulation). We combined our device with quantitative phosphoproteomics to study early EGFR signaling events.

Methods

Our µmixer device comprises 2 mixing segments for (i) cell stimulation and (ii) quenching/lysis. Dynamics was first modelled by the finite element method (FEM) and verified using fluorescent dyes and confocal fluorescence microscopy. We stimulated EGFR-overexpressing HEK293 cells with EGF for 0 s, 0.5 s, 1.0, and 5.0 s, and without EGF as controls. On-chip generated lysates were digested with trypsin and phosphopeptides enriched using TiO2. Samples were analyzed by nano-LC-MS/MS using label free DDA on an Orbitrap Fusion Lumos for discovery, and parallel reaction monitoring (PRM) on a Q-Exactive HF for validation, including the use of stable isotope labeled (SIL) reference (phospho)peptides. Data were analyzed using MaxQuant and Perseus for DDA and Skyline for PRM.

Results and Discussion

Label free quantification allowed following temporal profiles of 6,540 confidently localized phosphopeptides on 2,240 proteins (FDR <1%). As expected, most phosphorylation sites did not significantly alter over time. For more than 500 sites we observed changes within the first 5 s, and 26 sites in 15 proteins showed more than 5-fold upregulation.We used PRM and SIL peptides to precisely quantify EGFR auto-phosphorylation and changes in known EGFR downstream targets, such as SHC1 and MAPK1. Our preliminary data demonstrate that EGFR auto-phosphorylation occurs already after 500 ms while most downstream targets show initial changes after 1-5 s. Reproducibility of sample processing and data analysis were confirmed throughout experiments.

Conclusion

We developed a µfluidic device capable of fully mixing cells with stimulant or quenching/lysis reagents in 80 ms. It allows studying early signaling events upon receptor/ligand interaction with a high temporal resolution and reproducibility. We combined our device with quantitative phosphoproteomics to study early EGFR signaling. We currently implement devices for 100 ms, 3 s, and 10 s of stimulation to better follow

the signaling pathway over time. We will expand our method to study aberrant signaling of EGFR mutants and upon treatment with novel EGFR inhibitors. Novo et al. Analyst 2016; 141(6):1888-905.Beck et al. Blood 2014; 123(5):e1-e10 123.Engel et al. Angewandte Chemie (in press)

Keywords: µfluidics,signaling,ultrahigh temporal resolution

MP03-07

Improving proteome coverage via efficient, complementary and automated digestion using high content magnetically immobilized Trypsin and Chymotrypsin

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Introduction and Objectives

Protein digestion is a critical part of bottom-up mass spectrometry workflows with proteins are cleaved into their surrogate peptides prior to analysis. The most widely used enzyme for this application is Trypsin, but other proteases are gaining in popularity primarily due to different cleavage specificities. This allows for generation of complementary peptide sets resulting in improved sequence coverage and more confident protein identifications. This information is particularly valuable when studying closely related species such as isoforms, monitoring of point-mutations (requisite during manufacture of protein-based biologics or biosimilars) as well as post-translational modification studies. Of particular interest is Chymotrypsin, due to its preference for hydrophobic residues, digesting orthogonally to Trypsin. This makes Chymotrypsin especially effective for digestion of membrane proteins, which constitute approximately one third of the total proteome. Here we show that the use of immobilised Trypsin and Chymotrypsin results in higher sequence coverage and depth of proteome cover. We further evaluate the stability of the immobilized enzymes using digestions under denaturing conditions. Protocols are also automated using a magnetic handling station.

Methods

Digestion of a standard a four protein mix containing was performed using immobilized Trypsin and Chymotrypsin under native and denaturing conditions (2-5M Urea and 0.1-0.5 % SDS) in both manual and automated manner. Peptide analysis was performed on an AB Sciex 6600 TripleTOF coupled to a Dionex RSLC (micro mode). Data processing was performed using PEAKS Studio 6 software (Bioinformatics Solutions Inc) or Protein Pilot v5 (AB Sciex). Work is currently on the way to assess the performance of the immobilized enzymes in complex cell lysates.

Results and Discussion

Use of immobilized Trypsin and Chymotrypsin resulted in improved sequence coverage when combining datasets from Trypsin and Chymotrypsin digestions. Stabilization through multi-point attachment allowed for protein digestion under denaturing conditions. In the case of immobilized Trypsin activity was retained in the presence of 5 M Urea and up to 0.5 % SDS. This resulted in significant improvements in terms of protein sequence coverage. The high content immobilization of Trypsin and Chymotrypsin on magnetic microparticles allowed for the development of rapid automated digestion workflows.

Conclusion

Efficient and rapid protein digestion was achieved using immobilized Trypsin and Chymotrypsin. Combining the LC-MS/MS datasets from the digestions proved complimentary and provided improved sequence coverage. Immobilised Trypsin retained

MP03-07

activity in presence of Urea and SDS. LC-MS/MS peptide sequencing showed that digestion in the presence of Urea provided improved sequence coverage without compromising specificity. The digestion protocols were compatible with robotic handling stations for workflow automation.

Keywords: Sample prep, digestion, proteolysis, MagReSyn®, Trypsin, Chymotrypsin

MP03-08

2D-SWATH® mass spectrometry was applied in quantitation of membrane proteome of rat brain hippocampus

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Introduction and Objectives

SWATH®-MS is a data independent acquisition (DIA) method widely used in quantitative proteomics study. However, one dimentional chromatography was used in almost all of reported SWATH studies, and quantitation coverage is limited in 1D-SWATH. There are no reports applying two dimensional (2D) chromatography to analyze complex sample using SWATH®-MS. Obviously, if 2D-LC is used in SWATH experiment, number of quantified proteins will be increased dramatically. In this study,2D-LC system coupled with SWATH®-MS (2D-SWATH®) was first time used to large-scale quantitative measurements of rat brain hippocampus membrane proteome.

Methods

The rat brains were removed from the skull and the hippocampus extracted quickly and easily from rats#.The proteins were extracted and the crude membrane fractions were isolated with differential centrifugation. Tryptic digestion was performed with the filter assisted sample preparation (FSAP) method followed by high pH reversed-phase separation and fractionation. Mass spectrometric data were acquired in IDA and SWATH® mode on a SCIEX TripleTOF® 6600 system with Eksigent Ekspert™ nanoLC system to generate the proteome libraries and quantitative information,respectively,and processed with SWATH®2.0 plugin in Peakview®2.2 software.

Results and Discussion

Rat brain hippocampus membrane proteins digest was fractionated with high pH RP-LC system into 6 fractions and then the mass spectrometric data were acquired on a TripleTOF 6600 system both in IDA and SWATH modes for each fraction. Equal amount of Bovine serum albumin (BSA) and Beta Galactosidase digest (BGAL) were added before protein digestion and mass spectrometry acquisition, respectively. After processing the MS data with SWATH2.0 software, all the quantitative area information were extracted and combined for signal normalization with BSA and BGAL setting as references. Statistical testing shows that the quantitative results possessed a normal distribution with logarithmic value of zero as mean. Totally 6,064 proteins (1%FDR) with 33,481 distinct peptides were identified as for the ion library. Of these, 4,520 proteins with 20,317 distinct peptides were quantified with the characteristics that more than 90 percent of the proteins' quantitative results meets the 20% Coefficient of variation (CV%) specification. Finally 812 2-fold up-regulated and 300 2-fold down-regulated proteins with p-value less than 0. 05 were found in the rat brain hippocampus prolonged simulated microgravity model.

Conclusion

2D-SWATH® was first time used to large-scale quantitative measurements of rat brain hippocampus membrane proteome.Totally 6,064 proteins (1%FDR) with 33,481 distinct peptides were identified as for the ion library, and 4,520 proteins with 20,317 distinct

MP03-08

peptides were quantified with the characteristics that more than 90 percent of the proteins' quantitative results meets the 20% Coefficient of variation (CV%) specification.

Keywords: 2D-SWATH, Proteome quantitation, membrane proteome

MP03-09

Systematic exploration of subcellular redox status by methionine containingpeptide enrichment

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Introduction and Objectives

Aims: In Photofrin-mediated photodynamic therapy (PDT), cell fate can be modulated by the subcellular location of Photofrin. PDT triggers oxidative damage to target cells, including the methionine (Met) oxidation of proteins.

Methods

Here, we developed a new Met-containing peptide enrichment protocol combined with SILAC-based quantitative proteomics, and used this approach to explore the global Met oxidation changes of proteins in PDT-treated epidermoid carcinoma A431 cells preloaded with Photofrin at the plasma membrane, ER/Golgi, or ubiquitously (designated as conditions I, II and III, respectively). We also explored potential Photofrin-binding proteins in A431 cells using affinity purification coupled with LC-MS/MS.

Results and Discussion

Results: Across the three PDT conditions, we identified a total of 449 Met-peptides corresponding to 313 proteins that underwent severe oxidation upon PDT. We observed overrepresentation of proteins related to the cell surface, plasma membrane, ER, Golgi, and endosome under all three conditions, suggesting that these locations are the most susceptible to Photofrin-PDT. The most frequently oxidized Met-peptide sequence was “XXXMGGMGXG/MDGA.” We also identified 398 proteins that are highly likely to act as Photofrin-binding proteins, confirmed the bindings of EGFR and cathepsin D with Photofrin, and showed that the enzyme activities of both proteins were significantly reduced by Photofrin-PDT in vitro.

Conclusion

Innovation and Conclusion: Our results shed light on the global and site-specific changes in Met-peptide oxidation among cells undergoing Photofrin-PDT-mediated oxidative stress originating from distinct subcellular sites, and suggest numerous potential Photofrin-binding proteins. These findings provide new insight into the molecular targets through which Photofrin-PDT has diverse effects on target cells.

Keywords: Photodynamic therapymethionine oxidationSILACoxidative stressreactive oxygen speciessubcellular

MP03-10

Identification of metal species by ESI-MS/MS through release of free metals from the corresponding metal-ligand complexes

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Introduction and Objectives

All organisms require essential micronutrients for their normal life. Plant secreted organic acids or phytosiderphores to chelated and absorbed essential metals from the soils. Therefore, different plant species owned their specific metal-phytosiderphores complex, and due to low abundance concentration in plants, it's difficult to identify metal complexes with analysis tools for structure elucidation, like NMR.

Methods

In this wok, we develop a convenient and fast method to identify metal complex with using ESI-MS/MS in the case of analysis of metal-deoxymugineic acid (-DMA) and metalnicotianamine (-NA) complexes. Using ESI-MS/MS, metal-DMA/NA complexes were identified through releasing the free metals from the corresponding metal complexes. MS/MS data further allows to obtain the possible fragmentation pathways for different metal-DMA/NA complexes and to select the highest abundant fragments that may be useful for quantitative analysis using multiple reaction monitoring.

Results and Discussion

Different metal–DMA/NA complexes were simultaneously identified under different physiological pH conditions with this method. We further demonstrated the application of the technique for different plant samples and with different MS instruments.Simultaneous identifications of different metal-DMA/NA complexes were also conducted under different physiological pH conditions using the method. We here presented the ESI-MS/MS based identification of metal species.

Conclusion

This approach can be applied in the identification of different metal-ligand complexes, especially for metal species whose MS spectra peaks are clustered close together. Potential applicability of the approach in biological samples such as different plant samples are also studied.

Keywords: metal-phytosiderphores complex, metal-ligand complexes, ESI-MS, NA, DMA

MP03-11

Sensitive and accurate quantitation of phosphopeptides using TMT isobaric labeling technique

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Introduction and Objectives

New advances in mass spectrometry (MS) enable comprehensive characterization and accurate quantitation of complete proteomes. The most accurate isobaric mass tagging (eg, Tandem Mass Tag™ (TMT™)) quantitation on high dynamic range complex mixtures can be accomplished by employing the Synchronous Precursor Selection (SPS) MS3 method available on the Thermo Scientific™ Tribrid™ mass spectrometer family. This method has also been applied to phosphopeptide quantitation; however, due to differences in phosphopeptide fragmentation patterns during tandem MS, the number of phosphopeptide identifications drops significantly. In this study, we developed and optimized instrument methods to address this limitation to provide high phosphopeptide identifications as well as accurate quantitation.

Methods

The first set of samples HeLa cells were lysed, digested, labeled with TMT10plex reagents, and mixed at ratios of 16:8:4:2:1:1:2:4:8:16. Yeast digest (Promega) was labeled and spiked into above HeLa digest with the last 5 channels in equal amount (0:0:0:0:1:1:1:1:1). The second set of samples is the A549 cells grown at control, insulin-treated, and IGF-1 treated conditions, followed by lysis, digestion and labeling using TMT10plex reagents in triplicated channels for different conditions. Labeled yeast digest was spiked into the sample with three channels 127C, 129N and 130C present. The mixed samples were further enriched for phosphopeptides and subjected for LC-MS/MS analysis on Thermo Scientific™ Orbitrap™ Fusion™ MS and Orbitrap Fusion Lumos™ instruments.

Results and Discussion

Ratio distortion was observed for multiplexed quantitative phosphopeptide analysis on HeLa and yeast two proteome mixture, when using traditional MS2 based multiplexed workflows. This was due to the interfering ions co-isolated with the precursor ions. The application of SPS MS3 method significantly improved the quantitation accuracy to recover the high dynamic ratios in the samples. While the moderate compromise of identification numbers was expected due to the slower cycle time in MS3 method, a nearly 50% of loss in the number of phosphopeptides was observed. This was probably due to the strong presence of the neutral loss peak specific to phosphopeptide, which limited the identifications from CID MS2 spectra. We developed and optimized MS3 instrument method to reduce the loss of phosphopeptide identifications to less than 30%, whiling maintaining quantitation accuracy benefits given by SPS MS3. The new MS3 method was applied to differential expression analysis of proteins in A549 cells lines, with the presence of yeast proteome as interference. Upon stimulation with insulin or IGF-1, many phosphoproteins were shown to be regulated. Results demonstrated increase up to 30% in identification and quantitation rates.

MP03-11

Conclusion

Two novel instrument methods were developed for achieving sensitive and accurate quantitation of phosphopeptides.

Keywords: TMT, sensitive, accurate, quantitation, phosphopeptides

MP03-12

Quantitative analysis of methylation on histone H3 during cell cycle

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Introduction and Objectives

Histone is a fundamental building block of nucleosomes that is important for packing of genomic DNA. These can be heavily decorated with covalent modifications on their N-terminal tails, including various post translational modifications (PTMs). Accumulative evidence indicates that PTMs of histones can control the activity of transcription of surrounding DNA. Recently, H3.1 and H3.2 were shown to be exclusively incorporated during S phase, whereas the deposition of H3.3 occurred outside S phase.

Methods

A MRM-based quantitative approach for the measurement of PTMs of histone H3 has been developed in the study of epigenetic regulation in cell cycle. MRM method is used to PTMs of human core histones H3.1, H3.2 and H3.3 of Karpas224 cells arrested in G1, S, G2/M phase and then treated with EI1. We intended to find out the changes of modifications on lysines 27 and 36 on histone H3 during cell cycle and clarify the behavior of H3.1 and H3.3 for the checkpoint of certain cell cycle phase comprehensively. In our strategy, histones were separated by HPLC and then derivatized with newly developed method. Finally digestion was carried out by trypsin to produce suitable length of fragments for analysis by MS and MS/MS. We applied MRM technique for absolute quantifying changes of modifications on histone H3 between G1, S, G2/M phases and EI1 (HMT inhibitor targeted on Ezh2) treated ones. H3.3 is shown of much lower abundance compared to H3.1 in Karpas cells. As H3.3 variants is involved in the establishment of “active” chromatin, more modifications related to activation occur on H3.3.

Results and Discussion

We initially intended to focus on peptide K27-R40 from histone H3, and find out all the modifications patterns on this peptide change during cell cycle. This would help us clarify the mechanism of histone modification for the checkpoint of certain cell cycle phase. We found significant increase in unmodified H3K9 and dramatically decrease in H3K9me2 from G1 to S phase And H3K9me1 increased a little at the same time. During G2 phase, we detected a small increase in H3K9me0, along with decrease in H3K9me2 after S phase. Among them, H3K9 di-methylation harbored a 30% increase in G1 phase as compared to control, but this modification decreased a lot as cells moved on to S. As expected, a great range of decrease of peptides with K27me3 was detected in EI1 treated cells in H3.1 and H3.3, and correspondingly H3.1K27me0, H3.1K27me1 increased dramatically.

Conclusion

A rapid, specific, and sensitive MRM assays with an optimized mixture of 45SIS peptides for histone H3 has been developed. The abundance of H3.1 and H3.3 based on the peptide K27-R40, and the modifications corresponding to this peptide on H3.1 and H3.3 can be quantitatively analyzed and detected in an exclusive way by using this method.

MP03-12

This analytical platform can be used to investigate modification changes on histone H3 during cell cycle. And we discovered H3.1 harbors more abundance than H3.3.

Keywords:

MP03-13

A Novel Tandem Quadrupole Mass Spectrometer for the Quantitative Analysis of Peptides using a Multi-point Internal Standard Calibration Method

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Introduction and Objectives

Translational and biomarker verification studies are challenged in that they not only require the analysis of large sample cohorts with high-throughput, but also demand high sensitivity, high resolution and selectivity over a large dynamic range. Targeted LC-MS/MS based assays afford analyte quantification with the reproducibility and throughput required in order to rapidly assess biomarker performance. Multiple Reaction Monitoring (MRM), using tandem quadrupole mass spectrometry, is an enabling technology that provides speed and selectivity, whilst miniaturized LC systems offer additional improved sensitivity. Here, the application of micro-fluidics coupled to a novel tandem quadrupole MS/MS system, using a multi-point internal calibration method for the quantitation of peptides and proteins is presented and considered for speed, sensitivity, accuracy/ bias and selectivity.

Methods

Results and Discussion

Initial benchmarking of the MS system, fitted with a novel ion guide that enables improved and selective ion transmission, alongside with detector advances, was conducted by contrasting the sensitivity and precision performance for six stable isotope labelled peptides spike into biological matrix (tryptic digested, undepleted human plasma) against four other MS systems, including two tandem quadrupole and two high-resolution time-of-flight instruments, all operated in MRM mode of acquisition. On average, S/N was found to be two-fold better compared to the next most sensitive instrument in MRM mode of acquisition. %CV performance values were found to be similar to the two other tandem quadrupole instruments and approximately two fold better than the time-of-flight instruments. A multi-point internal calibration method was used to achieve within sample calibration. This technique was assessed with regard to quantitative precision. In addition, since external standard response calibration runs are not required, throughput gains were determined.

Conclusion

Performance metrics of the method are demonstrated and the results contrasted with those obtained with conventional MRM quantitation approaches for the direct analysis, without applying any type of fractionation or enrichment, of three human plasma peptides, representing putative blood-based markers of cardiovascular disease that span >5 orders of dynamic range.

Keywords: MRMTargeted QuantitationMicroflowCardiovascularSerum

MP03-14

Streamlined hands-free sample preparation using Pressure Cycling and PCT µPestle sample containers for tissue biopsy profiling by SWATH-MS.

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Introduction and Objectives

Modern proteomics techniques, such as SWATH-MS, require high quality sample prep methods that preserve sample integrity and minimize sample loss to maximize proteome coverage. Fueled by the demands of precision medicine, high–throughput proteomic analysis of tissue biopsies presents particular challenges with respect to complete protein extraction, solubilization and digestion. Traditional methods of tissue sample preparation typically involve manual sample handling and multi-step procedures that inevitably lead to loss of analytes due to sample transfer steps. The utility and efficiency of the Pressure Cycling Technology (PCT) sample preparation approach, that employs PCT MicroPestle-based tissue homogenization and subsequent proteolysis in the same sample container, have been reported previously (Shao, et al., J Proteome Res. 2016 Jun 3;15(6):1821-9). The goal of the current study was to further optimize this protocol in order to minimize manual sample handling and sample preparation time, while maximizing quantitative recovery of proteotypic peptides.

Methods

We optimized several critical steps of the previously reported tissue preparation protocol using 2-3 mg rat liver tissue samples as a model system. Several permutations of the PCT-based homogenization and digestion protocol were tested to examine the influence of various sample preparation steps on the number of identified proteins and quantitative recovery of proteotypic peptides, while minimizing sample handling time. These permutations included combining tissue homogenization with reduction/alkylation steps or with Lys-C digestion, as well as the optimization of pressure and chemical environment during digestion with Lys-C and/or trypsin. The resulting peptides were analyzed using IDA and SWATH-MS on SCIEX Triple-TOF® 6600 mass spectrometers.

Results and Discussion

Our data strongly suggest that the use of pressure cycling technology (PCT), and an optimized sample preparation protocol, increases the quantitative recovery of observed peptides and significantly improves sequence coverage. Sample preparation time, as well as the operator hands-on time were also much reduced. The most effective protocol in our study took less than 3 hours to process 16 samples from start to finish, with approximately 30 minutes of hands-on time, and resulted in the identification of an equivalent number of proteins as an earlier-published 4-hour protocol that required over an hour of operator involvement.

MP03-14

Conclusion

We emphasize reduced time, improved quantitation and sequence coverage in a new, optimized, PCT-based tissue sample preparation protocol that combines hands-free tissue homogenization and digestion followed by IDA and SWATH-MS data acquisition.

Keywords: Precision medicine; Tissue proteomics; Sample preparation, Homogenization, Proteolytic digestion, Automation, Pressure Cycling Technology (PCT); SWATH data acquisition.

MP03-15

A zebrafish SWATH-MS platform for protease substrate identification

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Introduction and Objectives

The zebrafish (Danio rerio) is an attractive animal model for modelling disease pathogenesis. However, its full usefulness is hampered by the lack of antibodies for tracking signalling events at the protein level. Therefore, a SWATH-MS (data-independent acquisition technology coupling high throughput protein identification with reproducible and accurate quantitation) platform would be an invaluable resource for applications in zebrafish biology. To this end, we generated a comprehensive zebrafish reference ion library, and implemented it in identifying substrates of the protease BMP1 (bone morphogenetic protein).

Methods

To generate the ion library, a range of zebrafish material (from early embryos to adult tissues) was used with extensive fractionation followed by data-dependent acquisition analysis. For the BMP1 substrate identification experiment, fin clips from 3 months old wild-type and BMP1 mutant zebrafish were analysed using SWATH-MS data acquisition.

Results and Discussion

Currently, our zebrafish reference ion library exceeds 10,000 proteins derived from 200, 000 peptide spectra and 3.5 million transitions. Using this library, we performed a SWATH-MS-based analysis of a zebrafish BMP1 mutant harbouring a dysfunctional BMP1 protease. We observed the expected elevation of either N- or C-terminal regions of known BMP1 substrates such as COL1A1, COL1A2 and COL5A1, indicating the feasibility of using SWATH-MS for identifying BMP1 substrates. Moreover, we identified 563 differentially expressed proteins in the BMP1 mutant fin, of which a majority of them are involved in collagen assembly and extracellular matrix organisation.

Conclusion

We have constructed the most extensive and high-quality zebrafish reference ion library to date. We demonstrated the utility of this resource by applying it with SWATH-MS to analyse a zebrafish BMP1 mutant, and successfully recapitulated the cleavage pattern in known BMP1 substrates. Importantly, our zebrafish SWATH-MS platform can be potentially extended to identify novel substrates of BMP1, and further the understanding of its role and mechanism.

Keywords: SWATH-MS, zebrafish, reference ion library, protease substrate identification

MP03-16

Multiplex Pseudo-Isobaric Dimethyl Labeling for Proteome Quantification

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Introduction and Objectives

The accurate and precise proteome quantification with parallel processing (i.e. multiplexing) is of great significance to satisfy the increasing demand of modern biology and medicine. In our work, a multiplex pseudo-isobaric dimethyl labeling method was proposed for simultaneous quantification of six proteomic samples. Fragment ion pairs in tandem MS scans were used to provide quantitative information. The method showed excellent accuracy, precision, coverage and plexing capacity for real sample analysis.

Methods

The multiplex pIDL was based on the different dimethyl labeling selectivity of amino groups in the N- termini and C- termini of peptides under different pH conditions. Also, peptides could be differentially labeled based on the mass defects of $^{12}C/^{13}C$ and $^{1}H/^{2}H$ with 5.84 mDa mass shifts between the fragment ion pairs which can be detected by high-resolution mass spectrometry. With different isotope labeling reagents, our labels are isobaric in MS between six-plex but different in MS/MS, and we extracted the intensities of fragment isotopologues for quantification.

Results and Discussion

By this method, six samples could be quantified simultaneously, and more than 99% of all identified proteins could be quantified. The six differently labeled HeLa cell digests were mixed at ratio of 1:1:1:1:1:1. The quantification results are almost free from precursor interference with relative error<5% and CV<10%. When it came to the dynamic range of 20-fold, the relative error was still less than 3% with CV<15%, demonstrating the wide dynamic range in complex sample analysis. Besides, expression dynamics of proteins in EMT process was investigated by this method.

Conclusion

Six different protein samples could be analyzed simultaneously by multiplex pIDL method with highly accurate and precise quantification results and 20-fold dynamic range. All these results demonstrate the multiplex pIDL method may have great potential in fields such as life science, precision medicine and food safety.

Keywords: proteome quantification, pseudo-isobaric dimethyl labeling, high throughput

MP03-17

Reductive Amination Combining Dimethylation for Quantification of Early Stage Protein Glycation

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Introduction and Objectives

Glycated proteins are formed by a non-enzymatic reaction between reducing sugar with amino groups located in the N-terminal position or in lysine and arginine residues. It plays critial role in aging disease, including diabetes, alzheimer's and parkinson's disease, while the quantification of endogenous protein glycation remains a problem.

Methods

Here, we developed a method combining reductive amination with dimethylation (RAD) for early stage protein glycation quantification. Using NaBH3CN and NaBD3CN to reduce early stage protein glycation, it can not only stabilize early stage protein glycation, but also introduce 1 Da mass shift between the two samples. After that, isotopic dimethylation (CH2O, CD2O) was used to enlarge the mass shift, and ionization was improved at the same time.

Results and Discussion

RAD showed good linear (R2> 0.99) and stability (CVs<1.6%) across tow magnitutes in the analysis of glycated myoglobin. The method was applied to profile the proteome difference of endogenous glycated proteins in the serum of diabetes and diabetic patients with complicated retinal detachment. The glycation level of human serum albumin was found to be upregulated in diabetic complication sample.

Conclusion

RAD approach can be a powerful tool for endogenous glycated proteins quantification.

Keywords: Protein glycation, Quantification, Reductive amination

MP03-18

Applications of solid phase peptide synthesis using the MultiPep Continuous Flow synthesizer with real-time UV-monitoring and automated feedback

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Introduction and Objectives

The automated assembly of peptides via solid phase synthesis following the Fmoc-strategy is a well established and commonly used method. Difficulties during peptide synthesis are sequence inherent, mostly due to length of the peptide, occurrence of hydrophobic stretches and sterically hindered amino acids. To gain insight into the most critical coupling and deprotection steps during peptide synthesis implies valuable information in terms of yield and purity of the resulting peptides. Accordingly, the crude product should be obtained in the highest possible purity. In the end this saves time and costs during the following purification steps. Here, we present examples of automated peptide synthesis of so called “difficult sequences” and pharmacological active peptide on a continuous flow synthesizer with real-time UV monitoring on a standard and low loaded resin.

Methods

Three different test peptides were chosen from literature. For all three peptides it is reported that they are difficult to synthesize with standard protocols and building blocks following the Fmoc-strategy. • Jung & Redemann-10mer: H-WFTTLISTIM-OH• β-Amyloid (1-42): H-DAEFRHDSGYEVHHQKLVFFA EDVGSNKGAIIGLMVGGVVIA-OH• Bivalirudin, a direct Thrombin inhibitor: H-fPRPGGGGNGDFEEIPEEYL-NH2Peptides were synthesized in a scale of 100 µmol on the MultiPep CF system using 5-fold excess of standard Fmoc-building blocks with HCTU as coupling reagent followed by a capping step. Fmoc deprotection was performed with 20% piperidine in DMF, minimum repetitions for deprotection were set to 3, maximum repetitions to 7 respectively. If deprotection was detected to be difficult, the following coupling cycle was automatically adapted as double coupling with elongated coupling time. As solid support, standard (0,5 mmol/g) and low loaded (0,2 mmol/g) resin, manufactured by Intavis, were used. Additionally, in case of β-Amyloid (1-42) a shortened coupling cycle was applied to prevent on-resin aggregation. Cleavage was performed by treating the resin with Reagent K for 2 hours. After precipitation in cold diethyl ether and washing steps, the crude peptides were obtained and further analyzed by mass spectrometry and RP-HPLC.

Results and Discussion

Obtained data are summarized in Table 1. Collection of corresponding

MP03-18

HPLC-chromatograms for synthesis of β-Amyloid (1-42) and MS-spectra are collected in Figure 3.

Conclusions

• Continuous Flow System with automated feedback was successfully applied for solid phase peptide synthesis •Difficult peptides were successfully synthesized in high quality. •Resin with lower base loadings gave throughout better results, in case of Bivalirudin only slightly better results. •Purity was improved for β-Amyloid (1-42) peptide when short coupling times were applied.

Keywords: peptide synthesis, MultiPep CF

MP03-19

Development of a novel nano separation device for robust and accurate gradient delivery with intelligent diagnostics.

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Introduction and Objectives

Matching modern HR/AM nano-ESI MS analysis robustness and reliability demands from the front-end LC separation the absolute highest quality and reproducibility of sample separation.To ensure such reproducible system performance, we developed a device with deeper system integration and novel approaches to quality control as well as intelligent system diagnostics.

Methods

A predigested human cell line (Promega), dissolved in 0.1% TFA was used as a QC. 200ng were injected to avoid blank injections and to reduce carry-over. The tryptic peptides were separated on a novel nano-UHPLC system using a 50 min reverse phase gradient, on standardized 15 cm 75 µm bore columns with C18 resin. Data was acquired on an impact II MS (Bruker Daltonik) equipped with a nanoESI source.

Results and Discussion

The novel LC system configuration minimizes the risk of introducing leaks in the system and additionally automates trouble-shooting by pin-pointing the exact leak position. The design incorporates a valve configuration allowing software controlled switching between “trap and separation column mode” and “separation column mode”. The elimination of manual intervention removes the risk of damaging a well-functioning system by mounting or un-mounting a trap column. 3 different LCs instruments were installed in 3 different labs; all applying the same described setup with their own instrumentation. High reproducibility of identification results was achieved, showing only ±6.5% variation [KD1] of protein identifications and ±16% variation of peptide identification between the labs when comparing average value of 2 to 4 analyses per lab.

Conclusion

We have developed a novel nano-UHPLC system with outstanding robustness, deep system integration and ease-of-use without compromising top performance.

Keywords: RobustnessReproducibility

MP03-20

A method for mass spectrometry-based absolute quantification reveals rhythmic variation of mouse circadian clock proteins

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Introduction and Objectives

Absolute values of protein expression levels in cells are crucial information for understanding cellular biological systems. Precise quantification of proteins can be achieved by liquid chromatography (LC)-mass spectrometry (MS) analysis of enzymatic digests of proteins in the presence of isotope-labeled internal standards. Thus, development of a simple and easy way for the preparation of internal standards is advantageous for the analyses of multiple target proteins, which will allow systems-level studies.

Methods

We developed a method for absolute quantification in which the reconstituted cell-free protein synthesis system (PURE system) was used for the preparation of internal standards. The PURE product is synthesized by mixing a liner DNA and 20 amino acids containing Lys(6) and Arg(10) with the PURE system's reagents. The product has 3 components of FLAG-tag, quantification-tag and target peptide sequence of interest, which are seperated by lysine residues at which the product can be cleaved by tryosin. After the purification of the FLAG-tag, the concentration of the product was determined by MS-quantification with the quantification-tag, in parallel, the product was added to biological samples as internal standard and followed by absolutely quantifying the target peptide in the biological sample in a manner of targeted proteomics. The developed method was termed MS-based Quantification By isotope-labeled Cell-free products (MS-QBiC).

Results and Discussion

This MS-QBiC was applied to a systems-level dynamical analysis of mammalian circadian clock proteins, which consist of transcription factors and protein kinases that govern central and peripheral circadian with low copy numbers. Sixteen proteins from 20 selected circadian clock proteins were successfully quantified from mice livers over a 24-hour time series, and 14 proteins had circadian variations. Additionally, the quantified values were applied to detect internal body time using a previously developed molecular timetable method, showing that single time point data from mice can predict the endogenous state of the circadian clock. These results suggest that the usefulness of multiplexed and sensitive quantification of absolute amount of target proteins and the quantitativity of MS-QBiC.

Conclusion

A method for absolute quantification of proteins for targeted proteomics was developed. It

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introduces a simple and high-throughput synthesis of internal standards for peptide quantification and thereby facilitates both multiplexed and sensitive absolute quantification of proteins. Application of this method to the systems-level dynamical analysis of core circadian clock proteins and detection of internal body time using quantified values of circadian clock proteins were shown. The results demonstrate the validity of the developed method.

Keywords: absolute quantification; mass spectrometry; cell-free protein synthesis system; mammalian circadian clock protein

MP03-21

The future of mass spectrometry-based protein clinical tests

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Introduction and Objectives

Mass spectrometry has played an important role in protein biomarkers discovery. Yet, very few of those candidate biomarkers have been validated, and mass spectrometry-based protein tests have not found their way into clinical laboratories. To date, there are about a dozen MS-based protein clinical tests, all of them in a form of lab developed tests (LDTs). Offered in this presentation is a unique perspective on the future of mass spectrometry protein tests, in view of the following determinants: the true demand for such clinical tests, end-users requirements, platforms and systems design, sample preparation bottlenecks, analytical and clinical validation, and regulatory approval. Fresh thoughts and attitudes toward these tests are required in order to move them toward clinical utilization and diagnostic use en masse, with critical emphasis on content, simplicity, and cost. Some possible killer-apps and solutions will be discussed.

Methods

Results and Discussion

Conclusion

Keywords: mass spectrometry, protein, laboratory test, clinical, diagnostic, plasma, serum

MP03-22

A novel method to separate high-molecular-mass proteins by an N, N' -methylenebisacrylamide gradient gel electrophoresis (BIS-gradient APAGE)

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Introduction and Objectives

Many high-molecular-mass (HMM) proteins (MW>100 kDa) are known to be involved in many human diseases, including Duchene and Becker muscular dystrophies (dystrophin: Mr~400 kDa), hypothyroidism (thyroglobulin: Mr~330 kDa), and cardiomyopathies (cardiac myosin heavy chain: Mr~200 kDa). Though a variety of proteomic techniques have been described, at the moment purification of a HMM protein remains a difficult task. A two-dimensional gel electrophoresis method employing agarose gels in the first dimension (agarose 2-DE) , which is sufficiently good at separating high-molecular-mass (HMM) proteins as large as 500 kDa and as much as 1.5 mg in total, has been used for separation of proteins from various tissues and cells with diseases. Agarose gel, when used for IEF, can analyze much larger proteins than the polyacrylamide gel can. However, the second dimension of the agarose 2-DE system was a conventional SDS-PAGE. Therefore, HMM proteins were often trapped at the top of the stacking gel. The use of fixed-concentration acrylamide and agarose hybrid gels that have a gradient of increasing N,N'-methylenebisacrylamide concentration (BIS-gradient APAGE) can separate and analyze HMM proteins effectively.

Methods

The first dimensional IEF of the agarose 2-DE method was according to the method of Oh-Ishi et al (2000). Preparation method of the second dimensional bisacrylamide-gradient gel (200 mm x 120 mm x 1.5 mm) is described briefly. The solution A includes 0.5% agarose and 4.5% acrylamide. The solution B includes 0.5% agarose, 10% glycerol, 4.5% acrylamide, and 0.24% bisacrylamide. Each solution was boiled in a microwave oven until the solution became clear. Urea powder was put into each solution (final concentration of urea was 4M). The bisacrylamide gradient was formed with a gradient-maker connected with a perista pump. Each solution was set on each chamber of the gradient-maker, then TEMED and ammonium persulfate were added to each solution. After polymerizing acrylamide at room temperature, the slab gel was kept at 4 C over night until the agarose formed a gel. The second-dimensional gel electrophoresis was started with a constant current at 40 mA for 1 h and continued at 70 mA until the end of the run.

Results and Discussion

To get a good resolution for proteins more than 200kDa up to 400kDa, we used a 0-0. 24% BIS gradient gel with with 0.5% agarose and 4.5% acrylamide. When the BIS-gradient APAGE system was used as Agarose 2-DE, chicken atrial and ventricular myosin heavy chain isoforms were successfully separated from each other. Moreover, the BIS-gradient APAGE gel is easier to handle than the conventional 5% SDS-PAGE gel.

Conclusion

The BIS-gradient APAGE system has a good resolution, low cost, and high reproducibility. Therefore, the use of the BIS-gradient APAGE would be one of the good choice for proteomic analysis of HMM proteins.

Keywords: SDS-PAGE, high-molecular-mass proteins, bisacrylamide-gradient

MP03-23

Precision and Accuracy in Proteomics

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Introduction and Objectives

The terms precision and accuracy are used to refer to a number of properties of an instrument, a method or a measurement. Unfortunately, the terms are often used ambiguously, even within the field of mass spectrometry-based proteomics. Here, it is important to know the mass measurement uncertainty for any information used to identify peptides or proteins. For well-calibrated spectra, the mass measurement accuracy is limited by resolving power and mass measurement precision. This uncertainty is typically plugged into the search engine used to identify the peptides and proteins in the first place.

Methods

We also talk about quantitative precision, or how well the abundance of a particular species can be determined. Again, for well-calibrated measurements in combination with proper internal standards, reference materials and standard curves, the quantitative uncertainly should be limited by the inherent measurement precision. All quantitative proteomics measurements to date, as far as we are aware, are indirect using proxies. In targeted measurements, these proxies are individual peptides, selected to be representative of the protein of interest. In label-free experiments, whether based on ion intensity or using spectral counting, we achieve higher protein sequence coverage, but then need a method to weigh the information from multiple peptides to one number for the protein. As proteomics is largely gene-centric, the word “protein”, unless otherwise specified, refers to all products from a particular gene present, irrespective of allelic variants, splicing, or post-translational events. To be more specific, the term proteoform has recently been coined to refer to a well-defined molecular species, with the same amino acid sequence and pattern of post-translational modification. This is a third type of precision – namely how precisely the measurands define to what the measurements refer.

Results and Discussion

Similar phenomena occur in other proteomics methods, such as the antibody-based proximity extension assays and the DNA-aptamer based assays now being able to quantify a hundred or more proteins simultaneously in many samples. Only top-down proteomics can precisely identify and quantify individual proteoforms in a single experiment. Although a great tool for discovery, top-down proteomics brings particular challenges, for example with regard to generating isotopically labelled standards, and for now remains outside the scope of clinical laboratories.

Conclusion

We have here defined the different meanings of precision and discussed what precision means – practically – in different approaches to proteomics. We will use examples from literature, but also from our own work and that of our collaborators. The examples will include large, many-peptide and label-free studies in human populations, few-peptide, multiplexed MRM-based measurements using isotopically labelled standards, several

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affinity-based methods and classical biochemical assays. Each precise in their own way.

Keywords: Precision; Proteomics

MP03-24

Capillary Electrophoresis - Mass Spectrometry for Top Down Proteomics

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Introduction and Objectives

The majority of the proteomics workflows use a bottom-up approach, where a protein or protein mixtures are subjected to enzymatic digestion, followed by LC separation of the resulting peptides and MS-MS identification of those peptides. While the bottom-up workflows are developing into a routine tool in the life sciences, a disadvantage is the loss of information on posttranslational modifications (PTM). Top down proteomics with whole proteins overcomes this disadvantage and allows a complete molecular characterization of the protein in question, but does require effective protein separation methods compatible with MS detection. In this paper, we explore the use of capillary electrophoresis mass spectrometry for top down proteomics under capillary zone electrophoretic conditions on an Orbitrap MS.

Methods

We employed for the CEMS interface a sheath flow design from CMP Scientific coupled to a Thermo Fisher Scientific Orbitrap mass spectrometer. Using 50 um ID capillaries, both with a cationic and neutral coating, we optimized the separation conditions for model protein mixtures ranging in molecular mass from 12 kD to 70 kD. To be compatible with a mass spectrometer, the background electrolyte was acidic acid of different concentration, ranging from 10 mM to up to 70% and covering a pH range for 2 to 4. The sheath flow composition ranged from 10 to 30 % methanol or isopropanol with 1% formic acid. On the mass spec side, we recorded the accurate mass and in MS/MS experiments a number of different fragmentation methods such as HCD, ETC and UVPD was used.

Results and Discussion

In optimizing the CE separation conditions, we found that only a relatively narrow pH range of 2.5 to 3.0 resulted in symmetrical protein peak shape of 30 to 60 seconds at the base. This allowed us enough time for the fragmentation methods employed since we wanted to get a minimum of 12 points across the protein peak. Typical separation times using a total capillary length of 80 to 100 cm and electrical fields of 30kV were 20 to 30 minutes. For the sheath flow, changing the organic modifier did not result in any different electrospray behavior. The optimized CE conditions were then used to separate small heat resistant fish allergens, paravalbumins with a molecular weight of 11 kD. The different MS/MS fragmentation modes allowed a coverage of up to 85%. These conditions were then also applied to other protein samples, such as hemoglobin and the NIST standard for an antibody.

Conclusion

Capillary electrophoresis mass spectrometry coupling is an interesting alternative to LCMS for protein separations. CEMS conditions were optimized to obtain accurate protein mass and MS/MS data for protein characterization.

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Keywords: CE-MS couplingtop down proteomicsoptimization of CE conditions for protein separationsMS/MS of whole proteins

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Advanced peak detection, deconvolution, and centroiding algorithms improve MS/MS spectral quality and peptide identification

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Introduction and Objectives

Correct peptide identification depends upon several factors, including accurate masses obtained from MS/MS experiments and the ability to distinguish signal from noise. This is especially important in shotgun proteomics experiments, where the complexity of the sample mixture might contain peptides with nearly-isobaric fragments that can overlap into unidentifiable peaks, especially if scanned by low resolution mass analyzers. In this study, we compare the results and quality of peptide identification lists after processing raw files with either Standard Centroiding or PeakInvestigator™ (an advanced peak detection, deconvolution and centroiding software service).

Methods

Baker's yeast was purchased from a grocery store. Cells were lysed with 8 M urea in 50 mM ammonium bicarbonate. Proteins were processed using 5 mM DTT for reduction (1 hour at room temperature) and 20 mM iodoacetamide for alkylation (30 min in dark). For digestion, urea was diluted to 1 M and trypsin was used at a 1:50 enzyme:sample ratio (w/w) overnight at room temperature. The peptide mixture was analyzed using nano liquid chromatography (Easy-nLC, Thermo Scientific) equipped with a C18 analytical column. nLC was coupled online with an Orbitrap Velos Pro (Thermo Scientific), and data were acquired using data-dependent acquisition. MS was performed in the orbitrap at 60,000 resolution. MS/MS was performed either in the orbitrap at 7,500 resolution, or in the ion trap (for comparison) using normal scan mode. Raw profile data were centroided using the Direct Access Web Portal for PeakInvestigator (v1.2, Veritomyx). Database searching was performed using Proteome Discoverer (v1.4, Thermo Scientific) with the yeast database (UniProt), no variable modifications and precursor mass tolerance set to 10 ppm. False discovery rate threshold was set to <1%.

Results and Discussion

We performed database searching using several mass tolerances after processing raw files with either Standard Centroiding or PeakInvestigator™. By using highly stringent MS/MS mass tolerances (<0.1 Da for ion trap), PeakInvestigator provided longer lists of confidently identified peptides, using a standard false discovery rate (<1%). By using a more tolerant search, both PeakInvestigator and Standard Centroiding led to a similar number of confidently identified peptides; however, the peptides identified by PeakInvestigator showed a drastic improvement in the identification score.

Conclusion

PeakInvestigator enhances confidence, and thus sensitivity, in proteomics data identification when using lower resolution instruments.

Keywords: proteomics MS/MS database peptide identification sequencing yeast

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Higher multiplexing with NeuCode SILAC metabolic labeling

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Introduction and Objectives

NeuCode is a quantitative proteomics technology that enables higher SILAC multiplexing through the use of near isobaric amino acid isotopologs. NeuCode amino acids have the same nominal mass and structure but are labeled with different combinations of 2H, 13C and 15N stable isotopes that can be resolved by modern high resolution MS scan. Here, we describe new isotopologues of lysine (e.g. +4, +8, and +12 Da), that permit metabolic labeling with 7 unique NeuCode channels.

Methods

A549 and HepG2 cells (ATCC) were cultured with SILAC media containing 10% dialyzed FBS and one of 8 different lysine isotopologs K000, K202, K040, K602, K341, K080, K642, and K390 (lysine with designated number of 13C, 2H, and 15N, respectively). Cell growth and viability was measured using MTT and AlamarBlue assays. After 5 doublings, cell lysates were reduced, alkylated and digested with LysC. Protein and peptide concentrations were determined using the Pierce™ BCA Protein Assay Kit and the Pierce™ Quantitative Colorimetric Peptide Assay, respectively. Resulting peptides were combined for multiplex analysis, and analyzed by Thermo Scientific™ Orbitrap™ Fusion or Orbitrap Elite mass spectrometers operating at an MS1 resolving power of 500K @ m/z 200 and 480K @ m/z 400, respectively. MaxQuant software modified for NeuCode was used for peptide identification and quantification.

Results and Discussion

Cells cultured with the different lysine isotopologs exhibited similar viability and growth for two human cell lines. NeuCode amino acid incorporation was also assessed over time by LC-MS of labeled protein digests. Incorporation rates for the different amino acids were also similar with ~95% labeling observed after 5 cell doublings. Equal amounts of labeled peptides from each condition were mixed and analyzed high resolution Orbitrap instruments with alternating MS1 scans of low (e.g. 60K) and high resolving power (e.g. 500K). The low-resolution MS1 scans was used to identify peptides in three isotopic clusters at +4, +8, and +12 Da with the high-resolution MS1 scan used to resolve near isobaric lysines for relative quantitation. A modified version of MaxQuant software was used to automatically extract the quantitative signatures present in these data. Analysis of our equal-mixed samples resulted high correlation to expected ratios and demonstrate feasibility for higher multiplex SILAC analysis.

Conclusion

New isotopologs of heavy lysine enabled higher multiplex quantitation using NeuCode SILAC metabolic labeling.

Keywords: NeuCode, SILAC, quantiative proteomics, multiplexing, MaxQuant

MP03-27

Urine protein preparation workflow for urine proteomics

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Introduction and Objectives

High purity and yield of proteins in sample preparation is necessary for proteomics of urine samples especially to discover urine biomarkers for diseases. We compared protein preparation methods and propose a consistent best protocol for urine protein preparation from frozen stored urine samples. Protein conditions of frozen and fresh urine are quite different. The method for preparing high purity sample from frozen urine is necessary for high accuracy urine proteomics.

Methods

Frozen urine samples were used for protein preparation by precipitation methods using ethanol, isopropanol, acetonitrile, acetone or methanol/chloroform. Recovery of protein was examined by measuring protein amounts using a protein measurement kit before and after precipitation. The prepared proteins were treated with trypsin in solution for peptide generation. After trypsin digestion, peptides were purified by C18 column and the amounts were measured by Nanodrop. Then, the peptides (500ng) were used for mass spectrometry (MS) (QExective, Thermo fisher scientific). After MS analysis, proteins were identified by Proteome discoverer 2.1.

Results and Discussion

Ethanol, isopropnol and acetone precipitation showed the highest recovery rate, nearly 80% at average. Acetonitrile and methanol/chloroform precipitation showed lower recovery rate of approximately 60% at maximum. All of precipitated samples contained more or less of colored substances as contaminants such as urobilinogen, especially in ethanol, isopropanol and acetone precipitation. In these samples, colored substances were not removed by washing of precipitates. While, acetonitrile and methanol/chloroform samples became clean by washing. In trypsin digestion step, proteins in EDTA containing solution was well digested. Finally, the number of protein identification was increased in these samples. In addition, the number of miss-cleavage sites for identified proteins was less in the peptide samples generated in the presence of EDTA. The number of identified proteins was not related to the recovery rate and probably to sample purity and trypsin digestion efficiency.

Conclusion

Judging from results of each precipitation methods, M/C precipitation was a best way for protein preparation from frozen urine. The sample prepared by M/C precipitation was more clean than samples using other method. Sample which was treated by EDTA containing solution indicated good result in MS analysis. It has possibilities that EDTA contributed for well dissolving of precipitated protein. This effect made homogenize condition of solution, and benefit for well digestion of protein. Peptide purification by C18 column was enough for keeping good condition of peptide sample. In addition to general

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purification method, methanol wash contributed for remove the almost stained substances. Prepared peptide sample according to this total procedure kept good condition for using MS analysis.

Keywords: Urine protein preparation, protein precipitation, peptide purification

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Next Generation Protein Sequencing (NGPS) enables full-length de-novo protein and antibody sequence determination

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Introduction and Objectives

Determination of a protein sequence without prior knowledge is an important and rate limiting step in the analysis of poorly characterized protein samples, such as samples derived from un-sequenced organisms, environmental samples, and microbiome. This is also the case for antibodies where the genomic sequence of the variable region is unknown. For biopharmaceutical applications, sequence assessment of monoclonal antibodies and other biosimilars is necessary, especially considering that they are produced by different cell clones in different manufacturing processes, and any unpredicted sequence variation might impact quality, safety and potency of the therapeutic protein. Despite the need, current proteomic techniques do not provide de-novo full length protein sequencing capabilities. To address this need, we developed Next Generation Protein Sequencing (NGPS), a method for full-length de-novo protein sequencing in high throughput.

Methods

NGPS is based on cleavage of a protein at semi-random sites, enrichment of nLC-MS/MS amenable peptides, nLC-MS/MS analysis, de-novo peptide tag sequencing and assembly of peptide tags into consensus contigs. To benchmark NGPS and test its performance, the method was applied to samples of bovine serum albumin (584 amino acids), equine myoglobin (153 amino acids), bovine fetuin (342 amino acids), and two monoclonal IgG antibodies, the sequence of one of which was unknown.

Results and Discussion

Excluding leucine/isoleucine and glutamic acid/deamidated glutamine ambiguities, end to end, full-length sequencing was achieved with 99-100% accuracy for all benchmarking proteins, including the antibody whose sequence was known. Application of the method to the unknown antibody resulted in 3 assembled contigs: Contig1 corresponding to the full length light chain, contig2 covering the N-terminal of the heavy chain, including the entire variable region and part of the constant region, and contig3 covering the rest of the heavy chain constant region. The sequence was validated, and post-translation modifications were mapped on the sequence as well.

Conclusion

NGPS represents a breakthrough method for database independent, high throughput protein sequencing. Currently NGPS can achieve de novo full length sequencing of monoclonal antibodies and other pure proteins. We anticipate that with instrument development, which will facilitate better peptide separation and faster scanning, NGPS will become instrumental in sequencing proteins of unknown sequences such as mixtures of antibodies, T-cell receptors and even metaproteomes. Furthermore, due to its ability to determine both protein termini with precision, NGPS represents a new strategy to address

challenging questions, such as determination of signal peptides or substrate cleavage sites by proteases.

Keywords: NGPS, de-novo , antibodies, variable region, full length

Protons From Gas Phase Molecules Enhances the S/N of peptides in ESI

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Introduction and Objectives

ESI-based mass spectrometry has enjoyed great success in its application to proteomics. However, in order to increase the number of quantifiable proteins, digested peptides have to be separated by liquid chromatography (LC). Organic acid, such as formic or acetic acid, has been commonly used as a modifier in the mobile phase in LC. TFA, the most effective modifier for intact protein separation, causes severe ion suppression in ESI. Recent studies have shown that such suppression can be eliminated by using organic acid as assisting vapor to enhance S/N of analyte ions. However, the mechanisms for such enhancement are not known.

Methods

We have manufactured enclosed ESI ion sources utilizing Coanda effect (CEESI ion source). The nano spray tip is positioned at a stagnation point where gas flow is minimized due to Coanda effect. This significantly increases the lifetime of the tip. Since the ion source is not open to atmosphere, background ions from the environment are eliminated, resulting in much lower noise level. In the CEESI ion source, different assisting vapor can be introduced into the source chamber and mixed, their vapor pressure controlled individually. In optimized conditions, S/N of peptide ions can be significantly enhanced. To elucidate the source of protonation in ESI, caffeine was chosen as the test compound since it does not contain any exchangeable hydrogen.

Results and Discussion

When caffeine was dissolved in pure water and D2O vapor was introduced into the closed ionization chamber. $[M+D]^+$ was observed as the predominant molecular ion. When deuterated acetic acid (CH3COOD) was introduced as vapor, the same result was observed. However, when caffeine was dissolved in D2O and pure water vapor was introduced, the predominant molecular ion observed was $[M+H]^+$. Furthermore, when ultrahigh purity (UHP) nitrogen was introduced into the ionization chamber to replace vapor, ion signal was quenched. These observations strongly suggest that the source of protonation comes from the gas phase molecules, not from the ESI solvent. Conceivably, the H^+ concentration around the Taylor cone can be enhanced by changing the vapor pressure in the ionization chamber, thereby increasing the S/N of analyte ions. Comparing signal intensity of peptides with and without vapor assistance using the CEESI source, a 2-fold enhancement could be obtained in general. A 5-fold enhancement was observed for singly phosphorylated peptides, while a doubly phosphorylated peptide could be enhanced by 27-fold. For a tryptic digest of HepG2 cell lysate, an increase of 15% in protein identification could be obtained using vapor assistance.

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Detection and Quantification of Proteins From DIA Data without Spectral Libraries

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Introduction and Objectives

SWATH™ and other Data independent analysis (DIA) data contain both extensive chromatographic information as well as spectral Information. Traditional SWATH/DIA workflows place a heavy burden on the end user to develop high-quality spectral libraries that can detract from the real biological analysis. Here we present a novel targeted extraction method for SWATH data which works with or without Spectral Libraries. Removing of the dependence on spectral libraries, FASTA files of protein sequences can be used directly to extract data from SWATH files allowing for a novel data processing. Such a method opens the potential for the extraction of modified peptides or peptides hypothesized from DNA sequencing while iterating data processing allows for hypothesis development.

Methods

A novel method for the detection of peptide signals in SWATH data was applied which uses a novel scoring algorithm. Publicly available data from Selevsek et al (Mol Cell Proteomics, 2015) was used in this study. The library which was used in this study was compared to the library generated in- silico for the quantified proteins. The extraction parameters for the data were set to remove the retention time by using an extraction time width corresponding to the total analysis time. A different number of randomly chosen peptides for the proteins were selected for quantitation in the insilico method. The quantitative outcomes of these analysis were compared to the results from the use of the specific library with the publish data.

Results and Discussion

The peptide sequences were extracted using the classic SWATH algorithm, with the experimentally derived library as published, and our novel method with both the experimental library and insilico library. The results indicate that peptides which were extracted from the published library could also be extracted when an equivalent insilico library is generated. Insilico generated libraries for all of the targeted proteins indicate that the same population of peptides/proteins can be extracted as in the published library but the addition of new peptides into the library provide higher sequence coverage of the extracted proteins. When random peptides are chosen for each of the targeted proteins through an insilico library we show that the number of proteins extracted is equivalent with the published library methods at a given FDR rate. Assessment of the quantitation values for the insilico library show that the extracted data supports the same hypothesis of the publication but removed the need for the development of sample specific libraries.

Conclusion

The use of FASTA / raw sequence data for the extraction of Quantitative information from SWATH data looks appealing. But the major power of this technique comes in the

analysis of proteins and modifications for which there are no library entries for i.e. splice variants etc. This opens a new way to utilize Genomics data with proteomics SWATH data

Keywords: Library free targeted DIA analysis. denovo data processing of SWATH data.

A "Solution" for High Sensitivity Phosphoproteomic Analysis with TMT Labeling

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Introduction and Objectives

Isobaric labeling strategies allow quantitating the multiple samples simultaneously, and have been used as powerful tools to evaluate the dynamics of cellular signaling network. Undesirable charge-enhancement on phosphopeptides with TMT would affect purification efficiency on both TiO2 and IMAC systems. Here, we aimed to develop a desirable workflow for increasing the sensitivity of TMT labeled phosphopeptides.

Methods

HeLa cell lysates were digested by phase transfer surfactant-aided trypsin digestion protocol. For TiO2, the phosphopeptides were firstly loaded onto the surface of TiO2 and labeled with TMT in HAMMOC tip. For IMAC, tryptic peptides were firstly labeled with TMT and purified by optimized IMAC condition. After elution, the purified TMT labeling phosphopeptides were analyzed by LC-MS/MS.

Results and Discussion

After TMT labeling, the content of triply charged phosphopeptides were increased from 28% to 44% and the number of identified phosphopeptides were decreased from 4472 to 1296, where 90% of the decreased fraction was singly phosphorylated peptides. Especially, the content of Lys-containing singly phosphorylated peptides was dramatically decreased from 52% to 19% by TMT labeling. Similar phenomenon was also observed in Fe3+-IMAC. Up to 55% of phosphopeptides has been lost during phosphopeptides purification. These results indicated the positive charge of TMT and the phosphate group might form intramolecular salt bridge which neutralizes the negative charge of phosphate group and decrease the binding affinity between phosphopeptides and TiO2 or Fe3+ ion. Here, we has established a new analytical strategy for TMT labeling phosphopeptides in both TiO2 and IMAC system. Briefly, the phosphopeptides are firstly loaded onto the surface of TiO2. Once the bounded phosphopeptides are released from TiO2 by phosphate buffer followed by mixing with TMT reagents, the phosphopeptides are immediately labeled with TMT tag. Compared with the original TMT labeling strategy, the number of identified TMT labeled phosphopeptides was increased from 1296 to 4247 with over 90 % labeling efficiency from only 50 µg tryptic peptides. On the other hand, the recovery and specificity of IMAC has been controlled by the pH and concentration of organic acid. The loss of TMT labeled phosphopeptides can be recovered by decreasing the

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Performance considerations for ultraviolet photo-dissociation using the Nd:YAG 5th harmonic (213nm)

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Introduction and Objectives

Ultraviolet photo-dissociation (UVPD) of peptides and proteins has been largely performed using excimer lasers. Few UV light sources, outside of excimer lasers, produce light of the appropriate wavelength to dissociate uniformly along the amide backbone of polypeptides. Recent advancement in solid state (SS) laser technology has enabled robust production of the Nd:YAG 5th harmonic (213nm). We demonstrate that 213 nm light is sufficient in photon energy and near enough to the absorbance band of the amide backbone to produce high quality UVPD spectra from polypeptides. A detailed comparison between excimer and SS laser generated spectra has been established via sequence coverage, fragment ion identifications, and fragment ion partitioning. Finally we explore strategies for over-fragmentation protection and fragmentation energy normalization.

Methods

An ArF excimer (193nm) and a SS (213 nm) laser were each separately coupled directly to an Orbitrap Fusion Tribrid mass spectrometer. All irradiation of ions took place within the dual cell linear RF ion trap assembly. No additional light focusing optics were utilized, however, to maintain a photon beam with a diameter similar to that of the ion cloud an iris was used with the excimer laser to transmit only a ~1.0mm diameter light beam. Normalization of the energy delivered per pulse was performed to facilitate direct comparison between laser wavelengths. Over-fragmentation protection was achieved through application of broadband RF waveforms during irradiation. A fragmentation energy calibration was developed to account for MW vs. number of laser shots.

Results and Discussion

Myoglobin has been utilized in a preliminary comparison between the excimer and SS lasers. In this comparison, fragmentation spectra were generated using the 15, 19, and 24 plus charge states of apomyoglobin. In this comparison, we show that sequence coverage between the two wavelengths is approximately the same (nominally >60%). The types of fragment ions observed are also very similar; predominantly a/x ions with some b/y and c/z ions with similar numbers of identified fragment ions of each type. For example, fragmentation from the [M+15H]15+ion produced 141 of 200 (70.5%) a/x type fragment ions using 213 nm , compared to 146 of 219 (66.7%) using 193nm. These percentages hold across the various charge states we studied. Moreover, the SS laser outperformed the excimer in terms of raw fragment ions identified (229@213nm vs. 212@193) at optimized fragmentation energy. The SS laser is capable of producing equivalent quality fragmentation spectra without the apparent downsides of an excimer laser, namely large footprint, requirement of rare noble gas mixture, and over-classed per pulse energy.

Conclusion

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UVPD fragmentation using a 213nm light source on the Orbitrap Lumos platform. We explored approaches for over-fragmentation protection and energy normalization to achieve optimum conversion of precursor ion to product ions.

Keywords: photo-dissociation, UVPD, FTMS, Top-down, parking

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The SOMAscan® assay and SOMAmer® reagents: Translatable tools from high-throughput biomarker discovery to targeted.

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Introduction and Objectives

SOMAmer® reagents are novel affinity binders made from single-stranded DNA engineered with amino-acid like side chains. These reagents combine the best properties of antibodies and aptamers – high affinity to thousands of proteins, reproducibly made synthetically. The hydrophobic nature of these binding interactions results in exquisite shape complementarity between the reagents and their protein targets. SOMAmer reagents have been proven effective in biomarker discovery, diagnostic products, and research tools.

Methods

SomaLogic has developed a proteomic assay called the SOMAscan® assay for biomarker discovery that transforms protein concentrations in a biological sample into a corresponding DNA signature that can be measured using any DNA quantification technology. The commercially available SOMAscan assay measures over 1,300 human proteins simultaneously in biological samples. Recent SOMAscan data have shown that certain SOMAmer reagents are able to distinguish between proteins resulting from single-nucleotide polymorphisms (SNPs) and wild-type proteins in human plasma. Applications of the SOMAscan assay range from broad proteomic profiling of thousands of proteins to exquisite specificity measurements for certain analytes. In addition, hundreds of the same reagents are available as individual life science tools for direct translation of biomarker discovery results to targeted assays.

Results and Discussion

Recent SOMAscan data have shown that certain SOMAmer reagents are able to distinguish between proteins resulting from single-nucleotide polymorphisms (SNPs) and wild-type proteins in human plasma. Applications of the SOMAscan assay range from broad proteomic profiling of thousands of proteins to exquisite specificity measurements for certain analytes. In addition, hundreds of the same reagents are available as individual life science tools for direct translation of biomarker discovery results to targeted assays.

Conclusion

SOMAmer reagents can be used in a variety of applications from biomarker discovery to targeted assays.

Keywords: Biomarker discovery, SOMAmer, protein binding, affinity

MP03-34

New methods for qualitative and quantitative proteome analysis

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Introduction and Objectives

Besides the complexity of samples, the dynamic change with time and space brings great challenges for proteome analysis. Therefore, in our recent work, great effort has been made on the development of new methods for qualitative and quantitative proteome analysis.

Methods

To achieve the deep-coverage proteome profiling, we proposed an ionic liquid based FASP protocol (i-FASP), by which with 1-dodecyl-3-methylimidazolium chloride (C12Im-Cl), both soluble and insoluble proteins could be efficiently extracted from cells, and denatured simultaneously at 95°C with DTT added, followed by on-filter alkylation, digestion and desalting. Besides, to improve the throughput for proteome sample preparation, we developed a hollow fiber membrane-aided fully automated sample treatment (FAST) method, by which samples could be denatured, reduced, desalted and digested via “one-stop” service.

Results and Discussion

Different from the typical FASP protocol, most C12Im-Cl could be easily removed by ammonium bicarbonate. Contributed by the stronger extraction and solubilization efficiency of C12Im-Cl than SDS, and the higher tryptic digestion capacity for proteins dissolved in C12Im-Cl buffer, the identified protein and membrane protein number in HeLa cells was obviously improved within reduced time. Furthermore, such a protocol was successfully applied in the label-free quantification of tissues and cells. By FAST, the whole procedure of sample preparation could be finished within 6 min. Through the on-line combination of FAST with nano-LC-ESI-MS/MS, we further established a fully integrated platform for high-throughput proteome quantification, and demonstrated its capacity to analyze sub-nanogram starting materials.

Conclusion

In summary, both i-FASP and FAST were successfully applied to achieve the proteome quantification, and showed advantages of high accuracy, precision, coverage and throughput.

Keywords: Proteome quantification, sample preparation, ionic liquid, integrated platforms

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Ultralong and Ultranarrow-Bore Capillary Columns for Proteome Analysis

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Introduction and Objectives

Proteome research is challenged by the complexity and the low abundance of proteins with significant biofunctions. Therefore, it is urgent to develop new columns with high sepration efficiency and ensure high sensitivity when coupled with MS. In our recent work, we prepared several novel ultralong and ultranarrow-bore capillary columns with hybrid silica monolith or open-tubular coating as the stationary phase, which showed great promising in proteome analysis.

Methods

The 140 cm-length ethylene-bridged hybrid monolith was in-situ prepared in the capillary by the one-step sol-gel co-condensation of bis(triethoxysilyl)ethylene and vinyltrimethoxysilane, followed by modification of C18 groups via thiol-ene “click” chemistry. The 20-μm-i.d. C18-functionalized amine-bridged hybrid monolith was prepared by the sol-gel reaction of bis[3-(trimethoxysilyl)propyl]amine and allyltrimethoxysilane followed by modification of C18 groups via thiol-ene click chemistry. The 10-μm-i.d. open-tubular capillary columns coated with C18 polymer brushes was prepared by surface initiated atom transfer radical polymerization of octadecyl methacrylate on the inner wall of the capillary. The integrated gold coated emitter with stable nanoelectrospray was prepared at the end of such narrow-bore separation columns for coupling to MS. By the hyphenation with MS/MS, the analysis of proteome samples was achieved.

Results and Discussion

The 140 cm-length and 100 μm-i.d. ethylene-bridged hybrid monolith was successfully used for the separation of peptides with column efficiency up to 120,000 N/m. For the nanoRPLC-ESI-MS/MS based analysis of 1 μg HeLa cell lysate digests, 22523 tryptic peptides corresponding to 4423 proteins were identified, much better than the reported results obtained with 2 mm packing materials. For trace sample analysis, the 20 μm-i.d. and 25 cm-length C18-functionalized amine-bridged hybrid monolith showed the advantages of high resolution and low non-specific adsorption, by which 721 protein groups were identified from 10 ng digests of HeLa cells in a single run. Furthermore, with 10 μm-i.d. and 300 cm-length open-tubular capillary columns, ~1000 proteins could be identified from 10 ng digests of Hela cells in a 50 min run. Totally, 3035 peptides corresponding to 1327 proteins were identified in three runs.

MP03-35

Conclusions

In summary, the seultralong or ultranarrow-bore capillary separation columns with novel separation stationary phases have shown advantages of high efficiency and high sensitivity in nanoRPLC-MS/MS based proteome analysis, which might be of great promising in the large scale label-free proteome analysis.

Keywords:

Proteome analysis, ultralong column, ultranarrow-bore column, bridged hybrid monolith, nanoRPLC-MS

MP03-36

Quantitative dot blot analysis (QDB), a low cost, high-throughput immunoblot method to complement Mass Spec studies in a regular lab.

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Introduction and Objectives

With the rapid progress of MS-based proteomic research, novel protein candidates come out constantly to greatly advance our understanding, diagnosis, prevention and treatment of human diseases. On the other hand, the broad distribution of the protein content of single protein among individual animal and human raises a big challenge to all the scientists in the field: How to evaluate the statistical significance of these candidate proteins in MS studies.

Methods

In this study, a convenient high-throughput immunoblot analysis derived from traditional dot blot analysis, quantitative dot blot analysis (QDB), is proposed to meet this demand in a common laboratory. Using newly developed Zestern plate, QDB was established over multiple proteins through combined Western blot analysis and dose studies. The consistency and accuracy of the analysis can be further improved through combined QDB analysis of target protein and tubulin, a commonly used housekeeping protein for loading control.

Results and Discussion

QDB is able to quantitatively measure the result of immunoblot analysis of a large number of samples simultaneously, and the convenience, effectiveness and efficiency of this method were examined at both cellular level and in animal studies. The characteristics of low cost, and easy to set up, reliability,flexibility, and the minimum technical requirement of this method make it possible to be performed in any research laboratories.

Conclusion

Our results demonstrate that QDB is a reliable method for high throughput immunoblot analysis in a regular research lab. to complement MS studies for the evaluation of candidate proteins. More importantly, it provides an efficient tool for any research lab. to perform high throughput immunoblot analysis to meet their specific needs.

Keywords: Quantitative dot blot analysisQDBHigh throughputZestern plate

MP03-37

Simultaneous MRM quantitation of 200 proteins from a dried blood spot

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Introduction and Objectives

Quantitation of protein biomarkers in blood offers diagnostic opportunities for many diseases, including cancer. The simplicity and less-invasive nature of dried blood spot (DBS) sampling make this an attractive methodology for blood-based biomarker testing, not only for the detection of diseases, but also for monitoring disease progression in individual patients. This requires accurate quantitation of multiple proteins simultaneously and is extremely challenging due to the overwhelming complexity and diversity of the components in blood. Recently we reported the quantitation of 97 proteins in DBS using multiple reaction monitoring [PMID:23221968]. Here, we report the development of a new LC/MRM-MS assay for quantitating 200 proteins in DBS samples from whole blood. This is the most highly multiplexed blood-based MRM assay to date.

Methods

Blood samples for method development were made from pooled human blood. Samples for biological application are from 50 patients. Protein extraction, reduction, alkylation, and digestion were carried out using a protocol we recently developed. The stable isotope-labeled standard (SIS) peptides were synthesized in-house. The peptide digests were separated with a RP-UPLC column on an Agilent 1290 Infinity LC system interfaced to a 6495 triple-quadrupole mass spectrometer. The MRM data was visualized and processed with MassHunter Qualitative and Quantitative Analysis software.

Results and Discussion

Our initial target panel was composed of 1182 unique tryptic peptides, corresponding to 526 human proteins. This panel of target proteins was selected from our in-house library of SIS peptides which are surrogates of many FDA-approved and potential biomarkers for lung cancer, cardiovascular disease, and other diseases in various biofluids. After the first round of LC/MRM-MS screening in DBS using five transitions per peptide, a single peptide mixture was then prepared and subjected to another round of interference screening, with 3 transitions per peptide. Interference free peptides were then used for quantitation of the corresponding 200 target proteins. The overall dynamic range of the assay spanned more than 6 orders of magnitude. Biological applications will be carried out using 50 individual patient samples. Results from these measurements will help to establish the normal concentration range of these 200 target proteins, and evaluate their diagnostic potential for various diseases. Although this method used 15 microliters of blood for method development, this volume can be lowered to less than 10 microliters for real sample measurements. This is advantageous especially for monitoring disease progression where multiple samples need to be taken from a patient over time.

Conclusion

A highly multiplexed LC/MRM-MS assay was developed for quantitating 200 candidate

protein biomarkers from whole blood and dried blood spots.

Keywords: Multiple reaction monitoring, dried blood spot, biomarker, protein quantitation, LC-MS

MP03-38

Ionic liquid-based filter-aided sample preparation for in-depth proteome analysis

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Introduction and Objectives

Generating the map of the human proteome would facilitate revealing the disease mechanism and discovering new drug targets. However, the extremely hydrophobic proteins are easily lost during the sample preparation process. To achieve an in-depth proteome analysis, it is imperative to establish an efficient and robust sample preparation method for discovering these hydrophobic proteins.

Methods

In our work, combined with 1-dodecyl-3-methylimidazolium chloride (C12Im-Cl) and FASP method, we developed a novel sample preparation method, ionic liquid-based FASP (i-FA SP), to simultaneously achieve the high efficiency extraction, solubilization and reduction of both hydrophilic and hydrophobic proteins, the in situ alkylation and digestion of the proteins, and the facile removal of C12Im-Cl using NH4HCO3 buffer.

Results and Discussion

This efficient method improved protein and membrane protein identification in HeLa cell lysates by 15.9% and 29.2%, respectively, over the conventional FASP method, and the analysis time was reduced by 1.3-fold. In contrast to the substantial sample loss observed with the FASP method, average of 3,300 proteins were identified with a 5 h sample preparation and a 1 h separation by our strategy from 1,000-cell samples. This method was further applied to the label-free quantitative proteome analysis of human liver cancer tissue, a total of 4,353 proteins were confidently quantified, among which 125 proteins were considered as differential proteins.

Conclusion

In this work, based on the strong dissolving ability, high trypsin digestion compatibility, ease of removal and compatibility with subsequent LC-MS/MS of C12Im-Cl, combined with an ultrafiltration device for its high efficiency for detergent removal, we developed a novel sample preparation method named i-FASP, which was successfully applied in an in-depth qualitative and quantitative proteome analysis. With the advantages of high throughput, high digestion efficiency and high recovery, our strategy has a high potential to be an updated version of FASP and to play an important role in proteome analysis.

Keywords: Ionic liquid; Filter; High recovery; Label-free quantitative

MP03-39

High-throughput de novo proteome identification aided by translatome sequencing

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Introduction and Objectives

De novo peptide sequencing do not rely on reference databases, thus suitable for identifying peptide variations, modifications and non-tryptic peptides. However, it was not practical for high-throughput proteomic analysis due to the lack of appropriate FDR control method. In order to employ de novo peptide sequencing for proteome analysis of complex samples, we present a new strategy to use translating mRNA (RNC-mRNA) as such a control.

Methods

We used RNC-mRNA data as a reliable quality control for de novo peptide sequencing. Peptides were identified by using appropriate peptides sequence tags.

Results and Discussion

We have applied this strategy on the HCD mass spectra of three hepaocellular carcinoma cell lines (Hep3B, MHCC97H and MHCCLM3) and identified 8124, 8569 and 7924 proteins, respectively. Among them, 1033, 1282 and 1206 proteins could not be identified by conventional database search, respectively. Notably, 297 missing proteins (PE2~4) and 8 PE5 were identified, most of which were identified with at least 2 unique peptides. For these extra proteins, we verified 74.4% of specific peptides which from 82 candidate peptides by using MRM method, including unique peptides from 17 missing proteins. Additionally, we identified fusion peptides, amino acids alterations and modifications directly from the MS spectra with confidence provided by RNC-mRNA evidence.

Conclusion

We successfully performed large-scale de novo proteome analysis with the aid of translating mRNA. This strategy provides unique power on resolving proteome at least in steady-state cells beyond the traditional target-decoy search strategy.

Keywords: De novo peptide sequencing / High-throughput proteomic analysis / Translating mRNA / HCD mass spectra / Peptides sequence tags

MP03-40

Comparison of different sample preparation protocols for protein and phosphopeptide identification

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Introduction and Objectives

Mass Spectrometry (MS) proteomics for the identification of proteins and characterization of complex biological systems rely on consistent protein lysate preparation and digestion using an endoprotenase. The optimal lysis buffer used depends upon the types or source of samples, and the sub-class of proteins required (e.g. membrane proteins). Using mouse lung tissue as a model, we investigated several experimental parameters, including protein solubilization with common denaturation reagents (urea, sodium deoxycholate (SDC), sodium dodecyl sulfate (SDS)), digestion conditions (in-solution vs filter-aided digestion). By applying label-free quantitative MS approaches, the aim of this study was to assess the impact of different comparative sample preparation conditions on the lung tissue sample at both proteome and phosphoproteome levels.

Methods

Two hundred and fifty grams of mouse lung tissue were homogenized with freshly prepared lysis buffer, urea, 2% SDC, 4% SDC and 4% SDS. The suspension was then transferred to centrifuge tubes and clarified by centrifugation, and the protein concentration was measured using a 2-D Quant assay (GE Healthcare, Life Sciences). Thirty to fifty micrograms of proteins were digested with in-solution and filer-aided spin column format. Five hundred micrograms of proteins were used for the phosphopeptide enrichment with titanium dioxide (TiO2). A 2-hour gradient on a QExactive mass spectrometer was used to run all the samples with technical replicate. The data analysis was performed using MaxQuant and Perseus software. Pathway analysis is done with Database for Annotation, Visualization and Integrated Discovery (David) and Ingenuity Pathway Analysis (IPA).

Results and Discussion

The preliminary results of the in-solution digestion of lung tissue lysate prepared with urea, 2%SDC and 4%SDC buffer, showed that the 4% SDC lysis buffer produced the highest number of proteins, 3,686, followed by 2%SDC (3,425), and then the urea buffer (3,248) with a 2-hour gradient using a QExactive instrument. A Database for Annotation, Visualization and Integrated Discovery (David) pathway analysis revealed that, with in-solution digestion strategy, cytoskeleton proteins were enriched the most from the urea buffer prepared sample, while proteins structurally constituent of ribosome and organelle inner membrane proteins are enriched in the 2% SDC and 4% SDC lysis buffer, respectively.

Conclusion

The pilot results clearly demonstrate the choice of lysis buffer needs to be considered as it may have a profound impact upon the numbers and types of proteins identified.

Keywords: Lung, protein extraction, phosphopeptides, sodium deoxycholate, sodium dodecyl sulfate, urea

WO07-05

Comprehensive draft of the HeLa proteome to a depth of more than 13, 000 proteins.

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Introduction and Objectives

Shotgun proteomics has seen an enormous growth in sequencing capabilities, ranging from identification of only a few peptides two decades ago till the presentation of the human proteome drafts two years ago. This innovation has largely been driven by improved sample preparation and separation methods coupled with analytical improvements of high-resolution mass spectrometry instrumentation, as well as computational analysis tools. The HeLa cervix carcinoma cell line is the most commonly used model for studying human cell biology. Here we present a high-definition LC/LC/MS/MS method for comprehensive analysis of the HeLa proteome in just a few days of analysis time and further show that it can be extended to other human cell lines and tissue samples.

Methods

Rapid and reproducible sample preparation protocol, where lysis, denaturation, reduction, and alkylation were performed in a single step achieved high protein and peptide recovery. The resulting peptide mixtures were fractionated by high-resolution off-line reversed-phase chromatography and each fraction was analyzed by nanoflow LC-MS/MS on a Q Exactive HF mass spectrometer with short LC gradients and very fast scanning HCD methods. For evaluation of the established method multiple enzymes, various cell lines and human tissues were compared to each other. We additionally compared the obtained data to state-of-the-art RNA-seq, and previously published proteomics results. All raw data analyses and bioinformatics were performed using the MaxQuant software suite.

Results and Discussion

Our strategy of high-resolution off-line separation of peptides into many fractions is ideal for massive peptide sequencing by short LC gradients coupled to very fast MS/MS scanning. Using this strategy we find that the HeLa proteome contains at least 13,000 proteins. Such a comprehensive proteome dataset can reproducibly be achieved in 25.3 hours of instrument time generating approximately 1 million fragment scans with a >40% identification rate. Our proteome analysis is comparable to in depth RNA-seq datasets and is to our knowledge the deepest single cell type proteome obtained so far. Our approach is extendable for analysis of other cell types and tissues with similar proteome depth and very few false positives. For proteome studies, the protease trypsin seems sufficient to cover the expressed proteome. However, comparable analyses of HeLa digests derived by chymotrypsin, Lys-C and Glu-C digestion, respectively, significantly improves identification of protein isoforms and increases the average sequence coverage to approximately 50%, which in turn allows identification of thousands of phosphorylation sites without specific enrichment.

WO07-05

Conclusion

The streamlined technology presented here achieves an unprecedented proteome depth, which is further underscored by the fact that our combined dataset contains more unique peptide sequences than any large peptide databases.

Keywords: Deep proteomes, thorough enzyme comparisons, modifications without enrichment.

MO03-01

Quantitative activity-based profiling of kinase inhibitor binding and selectivity on protein microarrays containing >300 human protein kinases

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Introduction and Objectives

Protein kinases are critical components of cellular signal transduction cascades within a cell and play a vital role in many diseases such as cancers so have become an important class of drug target. The presence of a common, highly conserved ATP-binding pocket has enabled the development and exploitation of numerous synthetic chemical inhibitors as drug candidates, yet simultaneously creates problems of inhibitor selectivity within the protein kinase family. Kinase drug discovery processes therefore typically initially profile large numbers of candidate inhibitors against a single kinase of interest, followed by profiling promising hits or lead against larger panels of representative kinases. Here we describe the development and application of a miniaturised protein microarray-based kinase inhibitor selectivity assay that enables high throughput, quantitative and reproducible measurement of the binding of inhibitors to specific targets, as well as quantitation of off-target interactions.

Methods

The catalytic domains of individual human kinases were cloned and expressed in SF9 insect cells; a C-terminal BCCP tag enabled in vivo biotinylation. Protein microarrays were fabricated by printing nL volumes of crude insect cell lysates onto custom streptavidin-coated, PEG-derivatised microarray surfaces, resulting in single-step, in situ purification and oriented immobilisation of each kinase in a spatially-defined array. A photo-labile, ATP analog was evaluated as a novel, broad specificity, kinase Activity-Based Probe (ABP), using a representative microarray of 15 human kinases. Quantitative, competitive displacement inhibitor binding assays were then developed based on this ABP and applied to protein microarrays containing up to 336 folded, functional recombinant human kinases. Using this ABP-based protein microarrray platform, ligand binding curves were generated for the kinase inhibitors staurosporine, PKC-412, Erbstatin and Iressa for each arrayed kinase.

Results and Discussion

Using our ABP-based, miniaturised protein microarray-based platform, we have generated quantitative data on the binding affinity and selectivity of a four kinase inhibitors against a panel of fifteen human protein kinases, as well as against a small panel of clinically-relevant epidermal growth factor receptor (EGFR) mutants, resulting amongst others in information on the differential selectivity of binding of Iressa and Erbstatin to specific EGFR mutants. Furthermore, we have used our ABP-based protein microarray platform to screen >300 human kinases for binding to Iressa, through which we have identified 47 kinases, including EGFR itself, that may represent biologically relevant

MO03-01

targets of Iressa.

Conclusion

Our novel platform is ready to enable differential kinase inhibitor binding and selectivity phenomena to be studied now across the human kinome for hits or leads as part of drug discovery programs.

Keywords: Activity-Based ProbeCompetitive binding assayHuman protein kinaseKinase inhibitor selectivityProtein microarray

MO03-02

Novel Hybrid Platform for Rapid, highly sensitive and specific quantification of proteins and their post-translational modifications

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Introduction and Objectives

Detection of low-abundance proteins and their post-translational modifications (PTMs) remains a great challenge. Enzyme-linked immunosorbent assay (ELISA) is one of the gold standards for rapid and accurate protein detection. However, it is not sensitive enough to detect low-abundance PTMs and suffers from nonspecific detection.

Methods

Recently, we have devolped a rapid, highly sensitive and specific platform integrating ELISA with proximity ligation assay (PLA), named ELISA-PLA. ELISA-PLA achieves a high specificity by multiple recognition using multiple kinds of antibodies and obtains its good sensitivity by DNA amplification.

Results and Discussion

For GFP, the limit of detection (LOD) was decreased by two orders of magnitude compared to that of ELISA. ELISA-PLA was also used to quantify the O-GlcNAcylation of AKT, c-Fos, CREB and STAT3, which is faster and more sensitive than IP-WB method. To quantify target glycoprotein without site-specific glyco-antibody, lectin was further introduced in ELISA-PLA to form a novel method named LEP. In LEP, non-priming DNA sequence was linked with glycan of capture antibody to block the recognition of lectin. The introduction of DNA can not only block the inherent glycan of the antibody to reduce the background signals, and can be used for DNA amplification to increase the sensitivity of detection.

Conclusion

In conclusion, ELISA-PLA and LEP could be a promising platform for rapid, sensitive and specific for proteins and PTMs detection.

Keywords: ELISA; proximity ligation assay; Glycosylation

MO03-03

A highly sensitive probe for fucosylated glycans for biomarker discovery

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Introduction and Objectives

Glycan functions are based on their well-regulated expression which is often dysregulated in various diseases including cancer. Particularly, we and other groups have demonstrated that fucosylated glycans are profoundly involved in several diseases such as chronic obstructive pulmonary disease (COPD) and various cancer types with dysregulated expression of fucosylated glycans. To develop novel glycan-related biomarkers and drug targets, it is needed to detect target glycans with good sensitivity and specificity, but the current detection methods are not sufficient. We aimed to develop a novel probe for the detection of fucosylated glycans using metabolic glycan labeling method in combination with click chemistry approach.

Methods

We have synthesized various novel fucose analogs having an azide- or alkynyl group which can be utilized for the detection by click chemistry. To select a fucose analog with the highest incorporation into glycans, we carried out in vitro fucosyltransferase assays. To characterize its sensitivity and cytotoxicity, cellular glycans having these analogs were tagged by click chemistry and detected by western-blotting and immunostaining. We also pursued its cellular metabolites by structural analysis of sugar nucleotides and N-glycans by LC-MS techniques.

Results and Discussion

We found that one of our new fucose analogs, 7-alkynyl-fucose, was the best substrate for various fucosyltransferases compared with other fucose analogs. Consistent with this, by using 7-alkynyl-fucose we detected cellular fucosylated glycans with higher sensitivity and lower toxicity than existing probes. Moreover, our MS analyses revealed that after incorporation into cells this analog was correctly converted to the GDP-form and incorporated into cellular glycans mainly at the N-glycan core (Kizuka et al., Cell Chem Biol., in press). In combination with click chemistry and subsequent biochemical approaches, this glycan labeling method can also be applied to highly efficient identification of target fucosylated glycoproteins from biological samples.

Conclusion

Our new fucose probe can detect fucosylated glycans with high sensitivity. A chemical biology approach using this probe will enable us to easily detect and analyze fucosylated glycans, proteins and lipids. Our new fucose probe is a highly sensitive and powerful tool for both basic and medical research in the future particularly for discovery of

MO03-03

fucose-related biomarkers.

Keywords: Click chemistry, Fucose, Glycosylation, Glycan imaging

MO03-04

A novel set of isobaric peptide labeling reagent enabled proteomic quantification over 10 different samples

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Introduction and Objectives

Isobaric labeling of protein digests enables sensitive and reproducible quantitation of proteins among different samples. However, current isobaric peptide labeling reagents have several limits, such as the high cost for labeling and disposable consuming of the whole tube of reagents. Herein, we developed 10-plex isobaric tags (IBT) with high stability and relatively low cost, which were an improved version of 6-plex DiART reagents developed in our lab.

Methods

Tryptic peptides were labeled with IBT-10plex in an organic buffer for 2h at room temperature and then subjected to Q-Exactive LC-MS/MS for identification and quantificaiton. For phosphopeptide quantification, the Hela peptides were labeled by IBT-10plex, enriched by TiO2 beads and separated on a high pH reverse phase spin column. For data analysis, commercial Proteome Discoverer 1.4 and our own iPEAK-iQUANT were used.

Results and Discussion

Firstly, the MS identification parameters were optimized to identify the labeled peptides. The resolution of 35,000 for MS2 was found good enough to resolve the tiny difference among the report ions with 6.3mDa and get maximum amounts of the peptide identification; while 30% NEC was proper to keep the best balance of identification and report ion quantification for the same peptide. Secondly, the labeling processing was optimized for optimal labeling ratio between samples and label reagents, labeling time and labeling solution, which was evaluated by labeling efficiency. We found that the reaction with a ratio of 20:1 for IBT tag /peptide for 2h reached the plateau of label efficiency curve as well as the maximum identification. Interestingly, we found the solution of 200mM TEAB for peptide resuspension gave 99.5% labeling efficiency, but 1M TEAB only did 82% label efficiency for N-terminal and K residue labeling. Thirdly, we evaluated the performance of IBT, including the quantification bias of each individual tag, the dynamic range of quantification, and interference from matrix. The labeling for complex samples proved that there was higher quantification correlation for IBT compared to iTRAQ, little quantification bias among different tags, the accurate quantification within the dynamic range of 50 folds and matrix effect played little effects on IBT quantification. Fourthly, IBT 10plex reagents were applied for quantifying phosphorylation changes caused by EGF treatment in 10 time points. Totally 7962 unique phosphopeptides were identified and > 85% phosphopeptides showed <20% variabilities. The identification and quantification results were supported by other reports; however, the identification number

MO03-04

of proteins was only about 80% of that from iTRAQ labeling due to higher MS2 resolution and less total spectra.

Conclusion

The IBT 10-plex labeling reagent was demonstrated a good performance in protein quantification. This reagent therefore offers another choice for labeling quantification of proteomics due to its low cost and easy use.

Keywords: Isobaric labeling, quantification, phosphorylation

MO03-05

TMTcalibrator™ Enhances Biomarker Discovery in Peripheral Fluids

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Introduction and Objectives

TMTcalibrator™ is an innovative mass spectrometry workflow designed to overcome the sensitivity challenges in early biomarker discovery. Although an ideal source of biomarkers, the high dynamic range of proteins in plasma and cerebrospinal fluid (CSF) and the predominance of hyper-abundant proteins limit the detection of low abundant biomarkers in peripheral body fluids.

Methods

The TMTcalibrator™ workflow uses isobaric Tandem Mass Tags® (TMT®) to label diseased tissue or cell line digests in parallel with an appropriate set of labelled body fluids. The disease tissue is mixed into fluid samples at a range of concentrations providing a 4-point calibration curve and in sufficient amounts to ensure the majority of data dependent MS/MS acquisitions are made on tissue derived peptides. This increases the chance of identifying low abundant, tissue-derived biomarkers and overcomes the aforementioned issues associated with plasma and CSF biomarker discovery. Fragmentation of the unique TMT® tag allows the relative abundance of all peptides present in both the calibrator material and in the samples, even those with a low abundance, to be determined relative to the calibration curve created by the reference tissue calibrator. The method is compatible with Synchronous Precursor Selection, a form of MS3 analysis, eliminating quantitative interference from co-isolated ion species and allowing direct analysis of 6 individual non-depleted, unfractionated fluid samples. Downstream bioinformatic processing and data analysis determines relative levels of peptides and proteins based on TMT® reporter ion intensity, identifying proteins regulated between the disease and control samples.

Results and Discussion

To verify the approach, we designed a TMTcalibrator™ experiment to identify biomarkers of Alzheimer's disease (AD) in the CSF that correlate with disease severity. Post mortem brain tissue from AD patients classified according to the level of tau deposition, was used as the reference calibrator material, generating a 4-point reference curve with 4 of the ten TMT® 10plex tags. The remaining tags were used to label 3 CSF samples from AD and 3 non-AD controls. Peptides common to the AD brain and the CSF samples were identifiable, even those present at low abundance in the CSF, as MS/MS was triggered by the peptides present at higher levels in the brain material. Comparing the levels of each TMT® reporter ion generated upon fragmentation revealed which peptide sequences were differentially expressed in AD CSF compared to non-AD individuals. The diseased tissue used in this study was classified based on the presence of phosphorylated tau. As a validation of the TMTcalibrator™ workflow, the levels of tau and phosphorylated tau were investigated.

Conclusion

MO03-05

We found 87 peptides common to the CSF and AD tissue, 11 of which were significantly upregulated in the CSF of AD patients compared to the non-AD controls. This and further data from the TMTcalibrator™ workflow will be presented at the meeting.

Keywords: TMTcalibrator

MP04-01

The mechanism of Nav1.7 regulating the metastasis of prostate cancer cells as revealed by toxin probes and proteomic analysis

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Introduction and Objectives

Voltage-gate sodium channel subtype Nav1.7 has been found to be highly expressed in the prostate cancer cell line Mat-LyLu and to modulate the metastasis of this cell line, but the mechanism underlying this process is not clarified so far. JZTX-Ⅰ and HNTX-Ⅲ, isolated from Chinese spider Chilobrachys jingzhao and Selenocosmia hainana, could inhibit inactivation and activation of Nav1.7 respectively.

Methods

In this study, JZTX- I and HNTX- III were used as molecular probes to study the differential expression of plasma membrane proteins during the process of matastasis of Mat-LyLu cells. 2-DE was used to separate the purified plasma membrane proteins. The differentially expressed proteins were then identified by MALDI-TOF-TOF.

Results and Discussion

Among the Identified proteins, 29 was found in the JZTX-Ⅰ treated group and 79 was found in the HNTX-Ⅲ treated group. these proteins were involved in cell adhesion (Muskelin), tumor metastasis (Moesin, Fascin) and Energy metabolism (Isocitrate dehydrogenase, Phosphoglycerate kinase, Glutamate dehydrogenase 1). The Rho GTPase signaling pathway plays a central regulatory role during this process. Inhibiting the activity of Rac or Rho can result in decreasing the Mat-LyLu cells metastatic activity.

Conclusions

Toxin molecular probes could be a useful tools to discover the underlying mechanism of cancer metastasis.

Keywords: Toxin probes, Nav1.7, Tumor metastasis, Proteomics, Rho GTPase

MP04-02

A deep proteome analysis of the hemolymph, brain, and antennae extends the mechanistic understanding of honeybee resistance to Varroa destructor

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Introduction and Objectives

Varroa destructor is the major cause of global colony losses of the Western honeybee (Apis mellifera) and hygienic behavior (HB) is a collective response by adult bees to defend against parasites and diseases which is involved in resistance towards Varroosis. Despite efforts to elucidate the molecular mechanism underlying HB, it is far from being completely understood.

Methods

We studied the proteomic correlated to HB using a honeybee line selected for Varroa-specific HB for over a decade. Here, we individually identified worker bees from this line that showed HB and compared the proteomes of their mushroom bodies (MBs) and antennae to those in workers that came from the same set of colonies, but did not show the behavior. Also we compared the pupal hemolymph of worker bees from the selected and a control line using mass-spectrometry-based proteomics.

Results and Discussion

A total of 8609 proteins were identified from the 3 tissues covered an unprecedented depth >55% of the honeybee proteome. The uniquely found functional classes and pathways in each tissue suggest that hygienic bees have shaped distinct proteome settings to underpin the HB. The up-regulated proteins in pupal hemolymph suggest that the HB-line has adapted a unique strategy to drive pupal organogenesis and boost individual and social immunity via energy metabolism and protein biosynthesis. The up-regulated proteins implicated in synaptic vesicles and calcium channel activities in the MBs of hygienic bees indicate that their neuronal sensitivity is enhanced to promote the execution of HB. Moreover, the up-regulated proteins associated with olfactory senses and signal transmissions manifest the fact that the neural sensitivity is also enhanced to input a strong signal to the MBs and thus initiating the HB.

Conclusion

These findings significantly extend our understandings on the molecular underpinnings of HB. The enhanced olfactory, neuronal sensitivity, individual and social immunities are involved in combating Varroa infestation. Also the identified wide array of markers may be useful for accelerating marker associated selection of HB to save the honeybees.

Keywords: Apis mellifera, Varroa destructor, hygienic behavior, hemolymph, antennae, mushroom body

MP04-03

Functional and Proteomic Investigations Reveal Roles of Major Royal Jelly Protein 1 in Anti-hypertension in Mouse Vascular Smooth Muscle Cell

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Introduction and Objectives

Vascular smooth muscle cells (VSMCs) are a major cell type of the arterial wall and their functionality is associated with blood pressure regulation. Although royal jelly (RJ) has reported effects on anti-hypertension, the mechanism of blood pressure regulation by major royal jelly protein 1 (MRJP1), the most abundant RJ protein, is still unknown.

Methods

The mrjp1 gene was inserted into lentiviral vector followed by transduction the mouse VSMCs to investigate how MRJP1 influences VSMC functionality by functional and proteomic analysis.

Results and Discussion

After the lentiviral transduction, mouse VSMCs are able to express proteins of MRJP1. And the expression of MRJP1 in VSMCs significantly reduced cell contraction, migration, and proliferation, suggesting a potential role in decreasing hypertension via action on VSMCs. These anti-hypertension activities were further observed in the changes of the proteome setting of mouse VSMCs. Among 675 different proteins after MRJP1 expression, 646 were down-regulated and significantly enriched in pathways implicated in VSMC contraction and migration, including muscle filament sliding, actin filament polymerization and assembly, and ATPase activity, which suggest that MRJP1 lowers VSMC contraction and migration by inhibiting muscle filament movement. The down-regulated proteins also enriched pathways in proliferation such as carbohydrate/nucleoside metabolic processes, RNA splicing, and transport, indicating that MRJP1 hinders VSMC proliferation by reducing the supply of energy and genetic material.

Conclusion

This provides sound clues and potential novel approaches for the treatment of hypertension and relevant cardiovascular diseases using genetically-engineered, natural products in gene therapy.

Keywords: Major royal jelly protein 1; Vascular smooth muscle cell; Hypertension

MO08-02

Proteome Dynamics Reveal Temporal Regulation of O-GlcNAcylation/phosphorylation in Determining Apoptosis of Activated B Cells

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Introduction and Objectives

Evidence in recent years shows that selected phosphorylation sites on proteins may be further modulated upon O-GlcNAcylation, thus complicating the intricate networks that regulate important cellular functions. In the immune system, the molecular switch that integrates the apoptotic pathway with B cell receptor (BCR) activation signaling has remained unclear. Here, we attempted to decipher the molecular mechanisms bridging B cell activation and apoptosis mediated by post-translational modification (PTM).

Methods

Quantitative phosphoproteomic analyses were used to investigate temporal shifts in the site-specific phosphorylation. Mouse splenic B cells were treated with either anti-IgM or OGA inhibitor prior to the anti-IgM treatment; untreated B cells were used as the control. Extracted proteins were enzymatically digested and phosphopeptides were enriched by using automatic pH/acid-controlled immobilized metal affinity chromatography (IMAC) procedure, followed by triplicate LC-MS/MS analysis. IDEAL-Q algorithm was used for label-free quantification. To definitively map O-GlcNAc sites on Lsp1, vectors expressing Flag-EGFP-tagged Lsp1 and OGT were co-transfected into human embryonic kidney (HEK) 293T cells. Lsp1 that was purified by anti-Flag was subjected to both collision-induced dissociation (CID) and electron-transfer dissociation (ETD) MS/MS to identify O-GlcNAcylation sites.

Results and Discussion

We found that O-GlcNAcase inhibition enhances B cell activation and apoptosis induced by B cell receptor (BCR) cross-linking. The proteome-scale analysis of the interplay between protein O-GlcNAcylation and phosphorylation in stimulated mouse primary B cells revealed 313 O-GlcNAcylation-dependent phosphosites on 224 phosphoproteins. Further functional studies have confirmed the proteomic results and demonstrated that temporal regulation of the O-GlcNAcylation and phosphorylation of lymphocyte-specific protein-1 (Lsp1) is a key switch that triggers apoptosis in activated B cells. O-GlcNAcylation at S209 of Lsp1 was identified as the major O-GlcNAc site on Lsp1 by both electron-transfer dissociation (ETD) and

collision-induced dissociation (CID) tandem mass spectrometry. This O-GlcNAc modified site was shown as a prerequisite for the recruitment of its kinase, PKC-β1, to induce subsequent S243 phosphorylation, leading to ERK activation and down-regulation of BCL-2 and BCL-xL, which resulted in the increase of activated B cell apoptosis.

Conclusions

We demonstrate the critical PTM interplay of O-GlcNAcylation and phosphoetlation on Lsp1 that transmits signals for initiating apoptosis after BCR ligation.

Keywords: O-GlcNAcylation/ phosphorylation/ proteomics/ B cells/ apoptosis

MO08-03

Subtilisin for large scale (phospho)proteomics – the beginning of a wonderful love story?

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Introduction and Objectives

Bottom-up proteomics using trypsin is still the method-of-choice for large scale (phospho-)proteomics. Several alternative enzymes have been introduced in order to increase the accessibility of the proteome. Due to the limitations of commonly used enzymes, we evaluated whether the power of high sensitivity and high resolution MS instrumentation can be exploited to make use of a broad specificity protease, namely subtilisin, as a real alternative for large scale (phospho-)proteomics.We demonstrate that subtilisin (1) enables a fast and reproducible digestion, (2) provides access to 'hidden' areas of the proteome (3) and can be used for quantitative proteomics with chemical labels/label-free.

Methods

A431 cell pellets were lysed, carbamidomethylated and 8 aliquots were digested either with trypsin or subtilisin, each. Both enzymes were compared using a Q-Exactive HF based on 4 criteria. (1) In-depth proteome analysis by analyzing high pH RP fractions. (2) Reproducibility: 8 individual digests per enzyme were iTRAQ 8plex labeled and pooled in ascending/descending amounts (e.g. Subtilisin 113-121=1:2:3:4:5:6:7:8; trypsin accordingly 8:7:6:5:4:3:2:1) to determine whether iTRAQ reporters reflected expected ratios and digests were reproducible. (3) Large-scale quantification and susceptibility to reporter ion interference: Aforementioned subtilisin and trypsin pools were mixed to evaluate whether 1:2:3:4:5:6:7:8 (subtilisin) and 8:7:6:5:4:3:2:1 (trypsin) ratios could still be determined from the supercomplex mixtures. (4) Phosphoproteomics: (1) and (3) after TiO2 enrichment and HILIC fractionation.

Results and Discussion

(1) Subtilisin improves the in-depth coverage of the proteome. More than 400 proteins were exclusively found in the subtilisin data set, and ~3000 proteins had higher sequence coverage.(2) Subtilisin can be used for reproducible digestion. For both enzymes, iTRAQ reporter ratio box plots reflected the pre-mixed sample amounts well, confirming reproducible digestions even for the broad-specificity protease.(3) Subtilisin can be used for large-scale iTRAQ quantification. After mixing subtilisin and trypsin digests, respective peptide IDs still reflected the original and opposing ratio distributions.(4) Subtilisin can be used for large-scale iTRAQ-based phosphoproteome quantification, once phosphopeptide quantification was possible. Interestingly, phosphopeptide enrichment with both TiO2 and ERLIC showed many novel regions of the phosphoproteome were accessible via subtilisin, among those a specific enrichment of Pro-rich regions. Finally, we evaluated if subtilisin can be used for super-fast protein digestion: Indeed, it produces efficient proteolytic digests even after 1 min at 56 °C.

MO08-03

Conclusion

We demonstrate that subtilisin is a veritable alternative to trypsin, although it offers slightly lower overall proteome coverage, it grants access to hidden areas of the proteome and may be used for super-fast proteome analyses.

Keywords: missing proteins,alternative enzymes,new phosphorylation sites

MO08-04

Profiling Kinome Activities Using Kinase-Specific Substrate Peptides

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Introduction and Objectives

Protein phosphorylation mediated by protein kinases is important for cellular signal transduction networks. Large-scale phosphoproteomics enables us to overview protein phosphorylation events in the cell signaling. However, it is still challenge to clarify a role and regulation mechanism of individual kinase from the phosphoproteome analysis since crosstalk between signaling pathways is complicated and kinase-substrate relationships have not been completely cleared yet. In this study, we designed kinase-specific substrate peptides and developed a method for direct monitoring of kinase activities at the kinome-wide scale.

Methods

To design the kinase-specific substrate peptides for each kinase, in vitro reactions of recombinant human kinases in HeLa cell extracts were individually performed. The phosphorylated substrates were identified with quantitative phosphoproteomics approach based on hydroxy acid-modified metal oxide chromatography and nanoLC-MS/MS. Based on the in vitro substrates, the most suitable substrate peptides which show high specificity and sensitivity for monitoring of kinase activity were determined in combination with position weight matrix (PWM), phosphorylation motifs and their scores. Activities of protein kinases were measured against a crude extract of HeLa cell. The designed substrate peptides were reacted with the cell extract in the presence of ATP and magnesium chloride. The phosphorylated peptides were determined with nanoLC-MS using Q Exactive or LTQ (Thermo fisher).

Results and Discussion

As a result, we profiled 395 kinds of wild-type human kinases by using in vitro kinase assay, and then more than 180,000 kinase-substrate relationships were identified. Based on the kinase-substrate relationships, about 1,500 phosphorylation motifs were extracted and PWMs were calculated for each kinase. The fold-enrichment of the obtained phosphorylation motifs showed a high correlation with phosphorylation stoichiometry of the substrates. Using the kinase-substrate relationships and phosphorylation stoichiometry, about 100 kinase-specific substrate peptides targeting for 30 protein kinases were designed. In vitro kinase assay using synthetic peptides and a recombinant kinase showed most of the designed substrate peptides were exclusively or highly-selectively phosphorylated with a specific kinase. Using the kinase-specific substrates, we quantified kinase activities in the crude cell extract with high linearity and reproducibility. Spiking recovery test revealed some substrate peptides showed low recovery due to a degradation by endogenous proteases. Modification at N and C terminus of the substrate peptides improved the sensitivity of measurement of kinase activity.

MO08-04

Conclusion

We designed kinase specific substrate peptides targeting for 30 human protein kinases based on the in vitro kinase-substrate relationships, and successfully quantified activities of the protein kinases in a crude cell lysate in vitro.

Keywords: phosphoproteomicssignal transduction kinome protein kinase

MO08-05

Tyrosine phosphorylation changes due to calcium signaling cascade post-sampling prevented by enzyme heat inactivation

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Introduction and Objectives

Post sampling events trigger a signaling cascade in the cells of the sample, primarily mediated by calcium. This causes rapid, enzymatic driven, changes to protein phosphorylations, primarily loss of phosphorylations, and other molecules. The post-sampling changes distort the in vivo levels making interpretations of results difficult or erroneous. In the presented work, we have done standard bottom-up phospho-proteomics with affinity enrichment comparing either ordinary snap frozen samples with samples where the enzymes were heat inactivated just after removal to investigate the effect of post-sampling events on tyrosine protein phosphorylations

Methods

Mouse brains, N=3x2, was rapidly removed after decapitation and either directly snap frozen in isopentane, dry ice cooled, or heat inactivated prior to freezing. Samples were homogenized and proteins were extracted using an 8M urea based buffer and digested to peptides prior to affinity enrichment of tyrosine containing peptides using the pT1000 antibody. Enriched peptides from each sample were analyzed twice using state-of-the-art MS and differences in detected intensity analyzed between samples.

Results and Discussion

Over 1100 tyrosine phosphorylations were detected across all samples. Roughly one third showed at least 50% higher levels in samples where the enzymes had been heat inactivated. 62 phosphorylations were only found in the heat inactivated samples whereas only 1 peptide was unique to the snap frozen samples. GO and KEGG pathway analysis shows large changes in the phosphorylation of proteins for many pathways and biological functions. This is particularly for enzymes involved in calcium signaling, e.g. the NMDA receptor which is massively dephosphorylated in the snap frozen samples. This is consistent with calcium mediated signaling occurring rapidly post-mortem as part of the membrane depolarization.

Conclusion

The result of the present study indicate that protein phosphorylations change rapidly post-sampling and/or during extraction and significantly affect detected levels for a large portion of phosphorylations. Detected changes are consistent with a calcium driven signaling cascade that is well established in the literature to occur post-mortem. General enzyme inactivation using heat denaturation is a promising approach to address enzyme driven change post-sampling.

Keywords: Post-sampling, heat inactivation, tyrosine phosphorylation, calcium signaling

MP05-01

Phosphotyrosine proteomics reveals modulation of kinase activity in colorectal cancer cell lines with the resistance to Cetuximab

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Introduction and Objectives

Phosphorylation modification on serine, threonine and tyrosine residues, is one of the most important posttranslational modifications that control biological functions. Particularly, abnormality in phosphotyrosine (pY) status is closely related to malignant transformation of cells. Thus, tyrosine kinases have become important targets in cancer therapy. Although many tyrosine kinase inhibitor (TKI) has been approved or is currently in the clinical trials, the emergence of treatment-resistant cancer by TKI administration has been also reported. Therefore, understanding of acquired resistance mechanism by TKI has a large social significance that leads to establish “precision medicine”.

Methods

In this study, we carried out global profiling of pY signaling networks for the purpose of overcoming resistance to Cetuximab in colorectal cancer cell lines. Comparison of pY status between Cetuximab-sensitive cell lines (LIM1215 and DLD1) or Cetuximab-resistant cell lines (HCT116 and HT29) were performed with pY proteomics which was optimized in our lab. We found activated kinases in resistant cell lines with several informatics methods such as kinase-substrate enrichment analysis. Then, we checked effect of TKI specific to the activated kinases on cell proliferation.

Results and Discussion

Our pY proteomic method identified 1323 pY peptides in these four colorectal cancer cell lines at 0, 24 hours post Cetuximab treatment. By using information on physiological function or kinase-substrate relationship in PhosphositePlus database, 13 activated kinases in resistant cell group have been extracted. Treatment of two specific TKIs showed significant inhibition of cell proliferation in resistant cell lines.

Conclusion

The results of this research indicate that global profiling of pY signaling networks could be applied for screening target kinases in anti-cancer therapy. In addition, our optimized pY proteomics might contribute to comprehensive understanding of pY signaling networks in a kinome-wide fashion.

Keywords: phosphotyrosine, kinome, treatment-resistant cancer, Cetuximab

MP05-02

Nuclear phosphoproteomic view unravel clade-specific signaling pathways and transcriptional dynamics

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Introduction and Objectives

Nucleus, the regulatory hub is a dynamic system that serves as modulator of signaling events dictating cell fate decisions. Nuclear accessibility of regulators deciphers flexibility in unpredictable environment by restraining transcription factor, chromosome territory remodeling and changing DNA conformation by PTMs. Reversible protein phosphorylation, a sub-stoichiometric PTM can act as specific determinant of eukaryotic cell. Repercussions of phosphorylated nuclear proteins in context with cellular function are largely unknown.

Methods

The proteome and phosphoproteome was developed with nuclear enriched fraction using combination of 1-DE, 2-DE and subsequent Pro-Q Diamond staining, SCX, IMAC and MOAC. Proteins were identified using ESI-MS/MS and Triple-TOF/MS. 2-DE immunoblot was performed for hydroxy amino acid phosphorylation. Further, a protein-interactome model was developed.

Results and Discussion

To better understand the role of protein phosphorylation in cross-kingdom integration and co-ordination of nuclear function, we have developed the nuclear phosphoproteome and proteome in crop plants. Mass spectrometric analysis led to the identification of more than 2000 phosphoproteins and 3000 proteins, presumably involved in variety of biological functions viz. signaling and gene regulation, cell division, chromosome territory remodeling and innate defense. Comparative data analyses revealed new nuclear phosphoproteins of unknown functions along-with the presence of unexpected phosphoproteins which have never been associated with nucleus. In silico prediction and mass spectrometric identification of site-specific phosphorylation of amino acids indicated their possible effect on nuclear signaling network. Cluster and network analyses identified significant nuclear phosphoprotein and protein hubs. Further, cross kingdom clade species comparison deciphered organism-specific nuclear proteome dynamics.

Conclusion

Our study provides novel imminent in nuclear dynamics and elucidated the phosphoprotein network that branches to several hormonal and signaling pathways. To our knowledge, this is the first report on the comprehensive understanding of the complex phosphoprotein network in cellular signaling operating in plant nucleus.

Keywords: Phosphoproteome, Nucleus, Immunity, Signaling, Transcriptional dynamics, Interactomics, Network hub, Plant

MP05-03

Integrated analysis of phosphoproteome and global proteome reveals key protein phosphorylation by MCM2 in lung cancer cells

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Introduction and Objectives

Minichromosome maintenance protein 2 (MCM2) with licensing factor activity, a crucial regulator of DNA replication initiation, has been widely used as a proliferation biomarker in many types of cancer. However, the molecular regulation mechanisms underlying MCM2 expression in lung cancer cells are poorly understood. In this study, we investigated the regulation role of MCM2 in lung adenocarcinoma.

Methods

A comparative analysis of two lung cancer cell lines using integrated phosphoproteome and global proteome was performed to uncover MCM2 downstream networks. We conducted phosphoproteome profiles by selective enrichment of phosphorylated peptides using titanium dioxide for MCM2-silenced H1299 and MCM2-overexpressed A549 cells and proteome profiles of MCM2-silenced H1299 cells using iTRAQ coupled with LC-MS/MS.

Results and Discussion

A total of 2361 phosphosites, 753 phosphoproteins, and 2639 proteins was identified, where 148 phosphoproteins and 46 proteins were significantly changed in response to MCM2-overexpressed A549 and silenced H1299 cells. Common downstream phosphosites were found with opposite regulation without changes in protein abundance. Further analysis revealed that MCM2 is highly expressed in clinical lung adenocarcinoma samples and its regulation of cell proliferation and migration by inducing cell cycle arrest at G1/S phase in lung cancer cells. Moreover, phosphorylation of Ser99 on HMGA1 was specially found to regulate lung cancer cell proliferation.

Conclusion

Our results provide a comprehensive insight into the regulation role of MCM2 in lung cancer and also reveal that MCM2 promotes cell proliferation through regulation of HMGA1 phosphorylation. This study enhances our capability to therapeutically target cancer-specific phosphoproteins.

Keywords: MCM2, HMGA1, lung cancer, DNA replication initiation, protein phosphorylation, phosphoproteome, proteome

MP05-04

Particulate Matter Dephosphorylates Ezrin/Radixin/Moesin (ERM) Protein on BEAS-2B Cell

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Introduction and Objectives

ERM (Ezrin, Radixin, Moesin) proteins play role of corss-links between the plasma membrane and the F-actin and regulate the function of cell migration, morphology and adhesion and cell signal processes. The ERM proteins are consisted of three domains ; N-terminal FERM domain; a central α-helical domain; and an actin-bind C-terminal domain. The ERM proteins are phosphorylated on their C-terminal threonine or serine residue are active in their cross-linking activity. Inhalation of the airborne particulate matters (PM) like titanium dioxide and silica dioxide can induce or aggravate the acute and chronic airway diseases. But, the underlying mechanisms of this response remain poorly understood. Therefore, to explain this mechanisms, we investigated the connection between the effect of PM and the dephosphorylation of ERM using a proteomics approach.

Methods

In vitro study BEAS-2B cells were treated with TiO2 particles which were diluted with endotoxin free water. BEAS-2B cells were treated with the TiO2 particles of the concentrations of each 0, 0.4, 4, 4㎍/㎖. protein extracts in RIPA buffer were validated using by wester blot using Ezrin (3C12), Radixin, Moesin (38/87), Ezrin (phospho T567), Radixin (phospho T564) , Moesin (phospho T558) antibodies. mRNAs were validated by RT-PCR using Ezrin, Radixin, Moesin primers. In vivo study Animal model established that female Balb/c mice (6weeks old). received 40㎍/㎖ of TiO2 particles through the trachea. The state of changed phosphoprotein by TiO2 particles in lung was estimated by western blot, immunohistochemistry. We established the epithelial cell and the animal model that was treated with PM particle. In according to treat PM particle, the state of changed phosphoprotein was estimated by western blot, immunohistochemistry staining.

Results and Discussion

We showed that phosphorylated ERM proteins indicating dose-dependent decrease on treatment of epithelial cells with PM particles. These data were validated by western blot and the degree of ERM phosphorylation was decreased in PM treated epithelial cells compared with that of the non-treated. Dephosphorylation of ERM proteins was also observed in animal model. The expression of phosphorylated ERM proteins was definitely lower in lungs of PM treated mice than those in control mice.

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Conclusions

We have provided evidence that dephosphorylation of the ERM proteins is concomitant with the PM treatment. This process may be associated with working of various phosphatases and kinases. Therefore, further studies should be need to research about the phosphatase and kinases inhibitor related signal pathway. In that case, inhibition sites of dephosphorylation in ERM proteins may act as diagnosis maker for the pulmonary disease patients stimulated with PM.

Keywords: ERM (Ezrin, Radixin, Moesin), Particulate matter(PM)

MP05-05

Deep Phosphoproteome Analysis Reveals Neurobiological Underpinnings for Nurse and Forager Honeybee Workers (Apis mellifera ligustica)

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Introduction and Objectives

The life transition from nurse to foraging honeybee worker is key to accomplish age-dependent tasks. The honeybee brain is comprised of a nervous system that sufficiently regulates this life transition. Knowledge about how protein phosphorylation functions in regards to the neurobiological activities in the honeybee brain to drive the age-specific labor division is still lacking. The cerebral phosphoproteome of nurse and forager honeybee workers was investigated using Ti4+-IMAC phosphopeptide enrichment, mass-spectrometry-based proteomics. In all, 3,077 and 3,056 phosphosites, residing on 3,234 and 3,110 phosphopeptides from 1004 and 958 phosphoproteins were identified in the brains of nurse and forager bees, respectively. The age-specific phosphoproteins, phosphosites, kinase classes and functional pathways found in the nurse and forager bees suggest that specialized phosphorylation networks are needed to drive distinct cerebral activity. Phosphorylation were not correlated with protein abundance and expression level, either for the entire dataset or for individual proteins, indicating protein phosphorylation in the bee brain is independent of protein abundance and expression levels. Also proteins responsible for key biological functions and pathways were phosphorylated with age development: glycerophospholipid metabolic process, transport, vesicle-mediated transport, phosphorylation, intracellular signal transduction, phosphate-containing compound metabolic process and metal ion transport. These observations make manifest the fact that dynamic phosphorylation tunes protein activity to optimize and regulate cerebral behavior of the brain according to age. Moreover proteins and phosphoprotein expression play complementary roles to support the neuronal activity of honeybees and maintains the unique biochemistry in each age group. These data represents the only in vivo dynamic phosphoproteomic study of the honeybee brain to date and elucidate the regulatory mechanism of phosphorylation events that underline the neural activity in honeybees.

Methods

Results and Discussion

Conclusion

Keywords: Brain, foragers, honeybee, nurses, phosphoproteome, worker

MP05-06

CHARACTRIZATION OF THE SIGNALING MECHANISMS IN PANCREATIC ISLETS ISOLATED FROM NORMAL AND OBESE DIABETIC db/db MICE

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Introduction and Objectives

Type 2 diabetes (T2D) is caused by pathophysiological abnormalities and dysfunctional response to glucose in pancreatic islets. Its prevalence is estimated to increase to 69% of adults in developing countries and 20% in developed countries between 2010 and 2030, in parallel with the increasing incidences of obesity. The db/db mouse is a mouse model of obesity-induced diabetes type 2, which has a mutation in the Leptin receptor, which regulates adipose-tissue mass through the action of the hormone Leptin. Mature insulin secretion has been shown to be decreased in pancreatic islets of the db/db mice, and over time they developed insulin resistance. In addition, the morphology of the pancreatic islets from db/db mice is characterized by large and diffuse compartments (ER and Golgi) in comparison with normal islets. Interestingly, incubation with low level of glucose in vitro reverted the change in morphology of the db/db islets in addition to an increase in mature insulin secretion. In this study we tried to investigate this beta-cell adaptive plasticity in isolated islets from healthy and db/db mice using quantitative proteomics and phosphoproteomics.

Methods

We performed iTRAQ-based quantitative proteomics and phosphoproteomics with highly sensitive mass spectrometry of islets obtained from obesity-linked diabetic db/db mice and healthy controls (800-1000 islets per condition), either freshly isolated or after incubation for 12 hours with basal 5.6 mM glucose. We applied our TiSH protocol for sensitive phosphopeptide enrichment and evaluated various pathways using different bioinformatics tools.

Results and Discussion

We identified a total of 35,996 unique peptides and 7,864 phosphopeptides originating from 8,156 and 4,994 proteins, respectively. We elucidated the signaling mechanisms that are involved in the putative kinase-substrate-phosphatase interaction signaling, corresponding regulatory motif substrate, kinases, and significant phosphatase level changes in db/db mice and furthermore, we found novel regulated proteins and phosphosites within five overrepresented canonical pathways (aldosterone signaling, CREB signaling, p70S60K signaling, insulin receptor signaling, and type 2 diabetes mellitus), especially in the db/db group, that were modulated after incubation with low concentration of glucose in the islet culture, correlating with the reversion to normal morphology. Interestingly, we found a clear association of the Golgi serine/threonine protein kinase Fam20C, which is a kinase that phosphorylates secretory pathway proteins, with db/db islets morphology.

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Conclusion

The present results for the first time identified clear molecular pathways associated with the dysfunctional mechanism observed with morphology and beta-cell adaptive plasticity in an obesity associated diabetes type 2 model system.

Keywords: Phosphoproteomics, diabetes, db/db , beta-cell adaptive plasticity, signaling pathways

MP05-07

Phosphate-affinity chromatographic micro-tip technology for enrichment of phosphopeptides towards phosphoproteomic study

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Introduction and Objectives

Protein phosphorylation is a fundamental covalent post-translational modification that is closely involved in complex intracellular regulatory mechanisms and in the onset of various diseases, such as cancers or neurodegenerative disorders. As a result, the phosphorylated forms of proteins have become major targets for clinical proteome analysis aimed at drug discovery and the production of customized medicines. Accordingly, the development and application of specific and stable phosphoproteomic methods that can provide an exhaustive overview of phosphorylated proteins has become an urgent necessity. Here, we describe the design and development of a micropipette tip (called "Phos-tag tip") containing 10 µL of swelled Phos-tag agarose beads, a novel phosphate-affinity tool for the simple and convenient enrichment of phosphopeptides produced by enzymatic digestion from a complex sample of cell lysate.

Methods

The phosphate-binding site in a micropipette tip is an alkoxide-bridged dinuclear zinc(II) complex with 1,3-bis(pyridin-2-ylmethylamino)propan-2-olate (Phos-tag), which is linked to a hydrophilic cross-linked agarose. The Phos-tag tip contains 10 µL of swelled Phos-tag agarose beads and it is used in conjunction with a 1-mL syringe attached with a silicon-tube adaptor. All steps for the phosphate-affinity separation of the binding, washing, and eluting processes are conducted by using aqueous buffers under conditions of neutral pH, in combination with a short operation time, reducing the damage on separated molecules. We applied the method to the separation of phosphopeptides from complex biological samples.

Results and Discussion

Under optimal conditions, we demonstrated enrichment of phosphopeptides in tryptic cellular protein digests. Three independent experiments using this method followed by mass spectrometric analysis permitted us to identify 3,198 total phosphopeptides and 668 unique phosphopeptides from lysates of human embryonic kidney cells (25 µg proteins per each experiment), indicating that ~80% of identical phosphopeptides can be detected reproducibly in the respective experiments. Furthermore, the ratio of target phosphopeptides to total peptides was 92–95% or more, demonstrating that the specificity is vey well.

Conclusion

We have demonstrated a novel procedure for the simple and comprehensive separation of phosphorylated biomolecules such as nucleotides, phosphorylated amino acids, or phosphopeptides by using Phos-tag tip. The method can be successfully used for the purification and isolation of phosphopeptides form complex cell lysate samples with high

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reproducibilities and high purities. We thus expect that this chromatographic micro-tip technology would be used preferentially as an alternative to existing tools for the reliable enrichment of phosphopeptides in phosphoproteomic study.

Keywords: Micro-tip chromatography, Protein phosphorylation, Phosphopeptide, Phosphoproteomics, Phos-tag

MP05-08

ModProt: A database for integrating laboratory and literature data concerning protein post-translational modifications

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Introduction and Objectives

Protein post-translational modifications (PTMs) play crucial roles in regulating protein function and cell signaling. Abnormalities in protein PTMs are both causes and consequences of disease. Therefore, it is essential to determine how PTMs influence protein functions by comprehensively collecting information concerning them. Mass spectrometry (MS) allows us to obtain a great deal of information regarding the PTM sites on various proteins. However, PTM analysis results have not been utilized efficiently to understand the functional regulation of proteins. To address this deficiency, we have developed an original database, ModProt (Post-Translational Modification Map of Proteome), to integrate PTM data collected with MS-based approaches and from the literature. The ultimate goal of the ModProt database project is to enable PTM-based diagnosis through PTM site map comparisons.

Methods

Experimental information concerning protein PTMs was systematically assembled from our MS analysis data. For MS-based proteomics, PTM-enriched peptides were analyzed with either a LTQ Orbitrap Velos or TripleTOF 5600 system. To identify peptides, peak lists were subsequently compared against human protein sequences in the UniProtKB/Swiss-Prot databases using MASCOT. Additional information was gathered from literature reporting MS analysis of PTM sites, and also from the UniProt and neXtProt databases.

Results and Discussion

To develop the ModProt database, we constructed a web-based laboratory information management system (LIMS). This system allows us to administer the ModProt database and to view PTM site maps and corresponding protein information including amino acid sequences and official gene symbols. Furthermore, several differences in PTM status under different conditions, such as healthy and diseased states, can be detected by comparing PTM data from this database. The ModProt database helps us to manage our own laboratory information obtained through proteome-wide PTM analysis, and to obtain further insights into specific PTM sites that may be important to protein functions. We believe that the ModProt database will serve as a powerful tool for basic biomedical research and advanced clinical investigations based on protein PTM data in the context of personalized medicine.

Conclusion

We developed the ModProt database, which integrates our laboratory data and literature information about PTM sites, and subsequently constructed a web-based LIMS that allows us to administer the ModProt database and view PTM site maps. The ultimate goal of ModProt is to achieve PTM-based diagnosis and personalized medicine using the database.

Keywords: Mass spectrometry, PTM, Proteomics

MP05-09

Phos-tag SDS-PAGE methodology that effectively uses phosphoproteomic data for profiling the phosphorylation dynamics of MEK1

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Introduction and Objectives

The MAPK pathway is one of major signaling pathways that couple cellular responses to the binding of growth factors to cell surface receptors. MEK1, an essential component of the MAPK pathway, is phosphorylated during activation of the pathway; 12 phosphorylation sites have been identified in human MEK1 by MS-based phosphoproteomic methods. Here, we introduce a strategy for Phos-tag SDS-PAGE that effectively utilizes existing information on the location of phosphorylation sites (phosphoproteomic data). Our objective is to show how this strategy can shed new light on the phosphorylation dynamics of a typical signaling protein, MEK1, in the process of activation and deactivation of the MAPK pathway.

Methods

Phos-tag SDS-PAGE was performed for profiling the phosphorylation dynamics of MEK1. By subsequent immunoblotting with an anti-HaloTag antibody, we analyzed a HaloTag-fused MEK1 protein and 12 potential phosphorylation-site-directed mutants of the protein transiently expressed in HEK 293 cells. Furthermore, we demonstrated inhibitor-specific profiling of MEK1 phosphospecies by using three MEK inhibitors: TAK-733, PD98059, and U0126.

Results and Discussion

In this study using Phos-tag SDS-PAGE, we found that multiple variants of MEK1 in differing phosphorylation states are present in typical human cells. The Phos-tag-based strategy, which makes effective use of information on the location of phosphorylation sites, demonstrated that MEK1 is constitutively and mainly phosphorylated at the Thr-292, Ser-298, Thr-386, and Thr-388 residues in vivo, and that combinations of phosphorylations at these four residues produce at least six phosphorylated variants of MEK1. Like the levels of phosphorylation of the Ser-218 and Ser-222 residues by RAF1, which have been well studied, the phosphorylation statuses of Thr-292, Ser-298, Thr-386, and Thr-388 residues vary widely during activation and deactivation of the MAPK pathway. As a result of these changes in phosphorylation states, the abundance ratios of multiple endogenous MEK1 species, including constitutive phosphorylated variants, also varied greatly. The Phos-tag-based strategy permits qualitative and quantitative time-course profiling of intact MEK1 phosphospecies molecules in their respective phosphorylation states without loss of information about the molecular masses of the protein species.

Conclusion

This study contains important findings obtained by an application of the Phos-tag methodology. In particular, the data on inhibitor-specific profiling of MEK1

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phosphospecies demonstrate that our Phos-tag-based strategy needs to be applied in the course of the development of drugs based on inhibitors of protein kinases that could provide significant new therapies. In the era of rapidly increasing quantities of phosphoproteomic data, the solid Phos-tag-based strategy should permit the identification of novel phosphoprotein species and the elucidation of their functions in a broad range of human proteomes.

Keywords: Phos-tag, MAPK pathway, MEK1, MEK inhibitors

MP05-10

Dissection of protein kinase D signaling during thymocyte development using various phosphoproteomic strategies

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Introduction and Objectives

Thymocyte development is tightly regulated by T cell antigen receptor (TCR) signaling. Although several tyrosine kinases are well known to orchestrate TCR signaling, the roles of serine/threonine kinases have yet to be fully defined. Protein kinase D (PKD) is a serine/threonine kinase family within the CaMK group, and one PKD isoform is highly expressed in T cells and is phosphorylated upon TCR stimulation. We have found that PKD is crucial for thymocyte development and therefore searched for downstream substrates of PKD by combining various phosphoproteomic approaches.

Methods

Phosphoproteins were enriched by IMAC from wild-type and PKD2/3-deficient thymocytes and were subjected to 2D-DIGE analysis. Several protein spots showing TCR stimulation- and PKD2/3-dependent increase in intensity were identified by mass spectrometry. The identified proteins were validated by Phos-tag Western blotting and in vitro phosphorylation by recombinant active PKD2 and PKD3. Furthermore, changes in phosphorylation of specific sites were examined by TiO2-based phosphopeptide enrichment from wild-type and PKD2/3-deficient thymocytes followed by LC-MS/MS analysis.

Results and Discussion

We identified several candidates for PKD substrates, which have been previously reported to be involved in T cell development. Phos-tag Western blotting suggested TCR stimulation- and PKD2/3-dependent phosphorylation of these proteins. Both PKD2 and PKD3 could directly phosphorylate these proteins in vitro, and the phosphorylation sites were identified by mass spectrometry and site-directed mutagenesis. Finally, LC-MS/MS analysis of TiO2-enriched phosphopeptides indicated PKD2/3-mediated phosphorylation of these substrates at specific sites upon TCR engagement.

Conclusion

These results suggest that PKD governs T cell development through a signaling pathway including new PKD substrates.

Keywords: protein kinase D, T cell development, 2D-DIGE, IMAC, TiO2, LC-MS/MS, Phos-tag

MP05-11

Early phosphoproteomic dynamics for potential growth-regulating mechanisms in G-1 treated renal cell carcinoma cell line

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Introduction and Objectives

Renal cell carcinoma (RCC) is emerging as a major cause of death in kidney diseases, and is the cause of ~1% cancer cases in Taiwan. At present, several targeted therapeutic drugs have improved the overall survival for patients with metastatic RCC, but a high recurrence is usually observed. Therefore, there is a need to develop other method for managing RCC. Recent studies have shown that estrogen may be an alternative approach for the RCC treatment. In our preliminary data as well as other observations, it was found that estrogen may activate estrogen receptor (ER) to interfere with the growth factor-related signaling pathways, resulting in RCC growth inhibition. A newly found membrane-bound ER GPR30 is emerging as another cellular regulator in response to estrogen. Several studies show GPR30 can perturb the growth factor-related ERK signaling pathway. In this study, we aim to investigate the possible role of G-1 in regulating the RCC growth and cellular responses. The temporal phosphorylation changes in response to GPR30 will be deduced by quantitative phosphoproteomics approach. This study will provide the cellular functions of G-1 in RCC and facilitate the future development of future RCC management.

Methods

The RCC cell line 786-O was treated with 2 μM G-1 for 1, 5, 10, 15, and 30 min. At each time point, DMSO-treated cells were also prepared as control group. Two biological batches were prepared. To harvest the cell lysate, the cells were washed three times with ice-cold PBS and lyzed in a lysis buffer containing 100 mM TEAB, 4% SDS, protease inhibitors, and phosphatase inhibitors. After acetone precipitation, the protein pellet were resuspended in 100 mM TEAB and 8 M urea. The protein solution were reduced, alkylated, and digested in trypsin. The peptides were further dimethyl labeled and desalted. The phosphopeptides were enriched by sequential IMAC method, which Ga and Fe ions were used. The enriched phosphopeptides were analyzed by LTQ-Orbitrap XL and the raw data were analyzed by MaxQuant software. Downstream bioinformatics analyses were performed in Perseus and GproX software.

Results and Discussion

In our preliminary data, we have shown that G-1, which is a GPR30 agonist, can inhibit RCC growth. In addition, G-1 can induce intracellular phosphorylation change, especially in ERK kinase. It is therefore necessary to study the possible effect of G-1 in RCC growth regulation and the phosphorylation changes in RCC. We next performed temporal, quantitative phosphoprotoemics study using a RCC cell line 786-O as a model system. We have found several pathways in response to G-1 treatment. We are now validating our results by other biochemical methods.

Conclusion

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These protein candidates in G-1-related signaling pathway are expected provide a clear picture on the future treatment of RCC.

Keywords: G-1, renal cell carcinoma, quantitative phosphoproteomics

MP05-12

Carbon source dependent phosphoproteomic analysis of Methanosarcina mazei N2M9705

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Introduction and Objectives

Global warming is recognized as an important environmental problem which resulted in the climate change and extreme weather. The major goal of United Nations Climate Change Conference in 2015 is to constraint and control the global greenhouse gas emission. Methane is the second most prevalent greenhouse gas but also considered as a renewable energy which emission from human activities and environmental source via anaerobic digestion by methanogenic archaea. Methanosarcina mazei is one of the species in Methanosarcinales order that possess ability to produce methane through methylogenotrophic-, hydrogenotrophic-, and acetoclastic-methanogenesis process from precursor trimethylamine, carbon dioxide coupled with hydrogen, or acetate, respectively.

Methods

In this study, the M. mazei N2M9705 was respectively cultured with three kinds of carbon sources for further phosphoproteomic analysis. According to the Gel-free and Gel-based tryptic peptide preparation, the phosphopeptides were enriched via HAMMOC TiO2 tips for nano-LC-MS/MS analysis. The phosphoproteomic dataset was organized through both MaxQuant 1.5.1.2 and Proteome Discovery 1.4.

Results and Discussion

Among identified 528 unique phosphoproteins, the ratio of phosphorylated-Ser, Thr, Tyr, Asp, and His were 38.6%, 24.5%, 12.1%, 17.6%, and 7.1%, respectively. Only five phosphoproteins were conserved among three kinds of growth conditions indicated that the phosphorylation mediated regulation was dependent on the supplied carbon source. According to the phosphoproteomic dataset, we hypothesized that methanogenesis process may be regulated through protein phosphorylation modification. To focus on the phosphorylation mediated regulation in methanogenesis pathway, methyl-CoM reductase subunit B (McrB) and acetyl-CoA decarbonylase/synthase (ACDS) protein complex will be the candidates for further functional validation.

Conclusion

In this study, we uncovered a new possible perspective way to control the methane production from methanogenic archaea. This information may improve owners' willingness to apply in livestock farm to obtain sustainable energy, methane, from biowaste through systematic anaerobic digester to reduce the greenhouse gas emission.

Keywords: Carbon metabolism, Methanogenesis, phosphorylation regulation

MP05-13

Ultra-sensitive motif-targeting approach for stoichiometry measurement of drug-responsive tyrosine phosphorylation dynamics in EGFR

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Introduction and Objectives

Phosphorylation stoichiometry, defined by the ratio of the total amount of protein with a site-specific phosphorylation to the total amount of protein, is a critical parameter on the onset of a given signaling transduction pathway. Compared to the conventional quantitative phosphoproteomic analysis, measurements of the phosphorylation stoichiometry may delineate whether the stimulus-induced alteration is regulated by upstream kinase activity to change degree of phosphorylation or by transcriptional regulation to affect protein abundance. Using EGFR as study model, we integrated the immunoprecipitation with motif-targeting kinase reaction and quantitative phosphoproteomics as a sensitive method for detection and quantification of the phosphotyrosine. The result on the TKI-responsive tyrosine phosphorylation dynamics of EGFR may shed light on how does the site-specific changes initiate downstream signaling.

Methods

EGFR complex from the SILAC (light and heavy) labeled lung cancer cell PC9 were pull-down with cetuximab followed by trypsin digestion. After phosphatase treatment of heavy-labeled EGFR, equal amount of light-labeled EGFR tryptic peptide are mixed together for IMAC purification. The flow-through of IMAC is subjected to the motif-targeting phosphorylation via EGFR kinase reaction. Upon the second IMAC purification, the ratio of light/heavy from EGFR-derived phosphopeptides represent the fractions of initially un-phosphorylated amount and then the phosphorylation stoichiometry can be further calculated

Results and Discussion

Here, we developed an IP-based motif-targeting quantitative approach which integrates protein immunoprecipitation (IP), metabolic labeling, dephosphorylation by phosphatase, and tyrosine kinase reaction for stoichiometry measurement. This strategy greatly increases the sensitivity to detect tyrosine phosphorylation; 14 phosphotyrosine sites can be identified in NSCLC cell lysate. Among these identified phosphotyrosine sites, Tyr869 was reported to regulate cell cycle, while Tyr1110 and Tyr1172 in cytoplasmic domain were related to cell mobility and cell growth inhibition, respectively. Interestingly, the result show five novel phosphotyrosine sites located in extracellular domain, which have not been reported. We apply this IP-based motif-targeting approach to analyze the tyrosine phosphorylation dynamics in response to geftinib (a TKI drug) treatment in PC9 cells (EGFR with exon 19 deletion mutation) within 6 mins. The temporal profile shows that

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Tyr1110 and Tyr1172 sites have high stoichiometry at basal level and drop to 50% at 5 minutes. These results are consistent with western blotting. Application of this approach on comparison of wild type EGFR and its mutation subtypes is on-going.

Conclusion

We anticipate this motif-targeting quantitative analysis may allow us to delineate the TKI-responsive tyrosine phosphorylation profile in EGFR, leading to new perspectives to overcome TKI drug resistance in NSCLC

Keywords: EGFR, tyrosine kinase inhibitor, NSCLC, motif-targeting

MP05-14

Deep (Phospho)Proteome coverage of the archaea P. furiosus reveals t he broad existence of fascinating ancestral protein kinase activity

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Introduction and Objectives

P. furiosus , a hyper-thermophilic archaeon is a model microorganism of the third domain of life, halfway between two well-known bacteria and eukarya domains. Systematic proteome studies allow us to gain insight into the evolution of important biological processes. Protein phosphorylation is one of the most common post-translational modifications, possibly even in all living organisms, as it regulates virtually every cellular process. However, the study of phosphorylation events in archaea is scarce due to its substoichiometric nature and the difficulties in sample preparation. Here we present in-depth proteome and phosphoproteome analyses of the archaea P. furiosus with the aim to unravel conservation and/or evolution in protein phosphorylation.

Methods

Having an optimum growth temperature of 100 °C, P. furiosus is an extremely stable organism. Therefore, we first refined sample preparation by integrating precipitation and liquid/liquid partition for proteome analysis. We next used Fe-immobilized metal ion affinity chromatography (Fe-IMAC) for our phosphoproteome analyses. The MS raw files were processed by Maxquant for peptides and phosphosites identifications. Gene ontology and motifs were analyzed by DAVID and Motif-x, respectively.

Results and Discussion

A high proteome coverage was achieved (64% of predicted proteins) by triplicate 2h LC-MS analyses, without the need of any fractionation. This fast and comprehensive identification demonstrated the success of our sample preparation protocol. A total of 1, 073 phosphosites from 451 proteins were confidently identified by triplicate Fe-IMAC enrichments followed by 1h LC-MS analyses. Consistent with previous archaea phosphoproteomics, a high phosphotyrosine prevalence was detected with an exceptional percentage ratio of 60:20:20 Ser/Thr/Tyr phosphorylation. Gene Ontology analysis indicated that those phosphoproteins are involved in cellular biosynthetic (n=132), nucleobase, nucleoside, nucleotide and nucleic acid metabolic (n=92), and cellular nitrogen compound metabolic (n=138) processes suggesting an important regulatory role of phosphoproteins in P. furiosus. In addition, 19 phosphoproteins are annotated to have kinase activity in terms of molecular function. Moreover, both eukaryote- and bacteria-type phospho-motifs were enriched implying the possibility of co-existence of eukaryote- and bacteria-like phosphorylation machineries in archaea. In agreement with our results, homology-based searches of gene sequences revealed the presence of eukaryotic-like protein kinases and phosphatases.

Conclusion

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We established a simple method that is suitable for proteome and phosphoproteome analyses of extremophiles. Using this method, we unraveled that archaea might possess both bacterial and eukaryotic-like phosphorylation traits. Further gene sequence and protein domain blasting will provide clues on how protein kinases evolve along the phylogenic tree that ultimately connects all life on Earth.

Keywords: Phosphoproteomics, archaea, kinase mapping, evolution

MP05-15

Super-SILAC Mix Coupled with SIM/AIMS Assays for Targeted Verification of Phosphopeptides Discovered in a Large-Scale Phosphoproteome Analysis of Hepatocellular Carcinoma

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Introduction and Objectives

Numerous literates have established the close association between aberrant phosphorylation and many cancer types, including hepatocellular carcinoma (HCC), which is a common and poor prognostic cancer worldwide. However, the efforts to globally discover and verify deregulated phosphoproteins of HCC specimens are limited, which are important for unraveling HCC pathogenesis and therapeutic targets.

Methods

In this study, we developed a quantitative phosphoproteomics platform combining stable isotope dimethylation labeling and online 3D SCX-TiO2/RP-LTQ-Orbitrap to compare phosphoproteome between three pairs of HCC tissues and adjacent non-tumor counterparts. To expand our verification capability, we evaluated the use of LTQ-Orbitrap run in SIM/AIMS mode with super-SILAC mixture as internal standard to quantify a subset of phosphopeptide candidates in HCC tissue samples

Results and Discussion

The large scale phosphoproteome analysis yielded 3100-4700 quantifiable phosphopeptides with 57 up-regulated and 73 down-regulated (> 2-fold change) in all three paired specimens. We confirmed the increased phosphorylation of two proteins (LMNA and NIPA) by Western blotting using phospho-specific antibodies. Among 253 selected phosphopeptides, 36 in SIM mode and 34 in AIMS mode with median CV of 8. 5% and 8.8% were successfully quantified and showed good correlation with discovery phase.

Conclusion

Collectively, these results indicate the feasibility of the super-SILAC mix-SIM/AIMS assay for targeted verification of phosphopeptides discovered from large scale phosphoproteome analysis of hepatocellular carcinoma.

Keywords: Phosphoproteome, super-SILAC mix, SIM, HCC

MP05-16

Integrated analysis of genomics, proteomics, and phosphoproteomics in cells and tumor samples

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Introduction and Objectives

As part of the Clinical Proteomic Tumor Analysis Consortium (CPTAC), we have recently published the first large-scale proteomic and phosphoproteomic analysis of high-grade serous ovarian tumors. We observed that phosphorylation status was an excellent indicator of pathway activity and could discriminate between patient survival times. In the current work we have combined this data with comparable data from breast cancer tumors and cancer cell lines treated with kinase inhibitors, to answer several fundamental questions about the role of phosphorylation in cellular processes and cancer.

Methods

All samples were analyzed using an iTRAQ approach with enrichment for phosphorylated ser/thr residues. The total dataset comprised over 150 samples with very deep proteomic coverage (>20,000 phosphopeptides confidently identified).

Results and Discussion

We first found that the correlation between kinase protein abundance and abundance of phosphorylated target peptides was very low, indicating that kinase abundance is not a good proxy for phosphorylation status overall. However, highly correlated kinase-substrate pairs were significantly more likely to be true relationships (from existing knowledge), demonstrating that this method could be used to identify kinase targets in some cases. We found that, compared to transcript and protein levels, phosphorylation status was a more effective discriminator between tumor groups (survival time or subtype). We used this analysis to identify several novel kinase-substrate relationships that were differential between tumor subtypes, and that correlated with pathways where phosphorylation was affected by drug treatment. These relationships are currently under investigations as potential novel targets for therapeutic intervention.

Conclusion

This study represents the first phosphoproteomic pan-cancer study on tumor samples.

Keywords: cancer, data integration, phosphoproteomics, drug repurposing

MP05-17

Analysis of the phosphorylation of actinin-4 involved in cancer metastasis

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Introduction and Objectives

Actinin-4 (ACTN4), an actin-bundling protein previously identified by our laboratory, is closely associated with cell motility, cancer metastasis, and a variety of cellular processes. Three mutations in the ACTN4 gene encoding ACTN4 are associated with the dysfunction of podocytes in familial focal segmental glomerulosclerosis. We recently identified a novel alternative splice variant of ACTN4 (ACTN4-sp) that was specifically expressed in high-grade neuroendocrine tumors (HGNT) of the lung. The expression of ACTN4-sp, which differs from ACTN4 by three amino acids, was correlated with a poor prognosis in HGNT patients. Reconfiguration of the actin filaments is necessary for changes in cellular structure, such as for cell surface protrusion and focal adhesion. These processes have been reported to involve 1) epidermal growth factor (EGF)-induced tyrosine phosphorylation of ACTN4, and 2) calpain-dependent cleavage of the phosphorylated ACTN4. We postulated that the tyrosine kinase that phosphorylates ACTN4 is an epidermal growth factor receptor (EGFR). Thus, in this study, we aimed to validate the enzyme responsible for the phosphorylation and to identify the phosphorylated tyrosine residues.

Methods

Using an in vitro kinase assay, we confirmed the tyrosine phosphorylation of ACTN4 by EGFR. We determined the phosphorylation sites of trypsin digestion of the phosphorylated ACTN4 using TripleTOF5600 (AB SCIEX, Framingham, MA) and 2-dimensional image converted analysis of LC-MS (2DICAL).

Results and Discussion

ACTN4 consists of four spectrin repeats, a pair of C-terminal EF-hands, and an actin-binding domain (ABD), which itself consists of a tandem pair of calponin homology (CH) subdomains (CH1 and CH2). All of the mutations in three familial mutations and ACTN4-sp were restricted to the CH2 subdomain of the ABD. Increased actin binding was previously observed upon phosphorylation at residue Y265 in CH2. In this study, we found that the mutations and the phosphorylated residue were located on the side of the helix, facing the helix-helix interface between CH1 and CH2. In addition, A549 cell lines overexpressing ACTN4 Y265 mutations showed decreased cell motility.The mass spectroscopy sequence coverage for the ACTN4 peptide was 77.6%, and six phosphorylation sites were identified.

Conclusion

Our findings suggest that the three-dimensional structures of the mutated peptides and ACTN4-sp differed from that of ACTN4, and that these changes were responsible for the differences in the biological characteristics of the cancer metastasis. Currently, we are developing an assay to further investigate the phosphorylation sites in the ACTN4 peptide by multiple reaction monitoring.

Keywords: Actinin-4, Phosphorylation, Cancer metastasis

MP05-18

Delineating mechanisms of Englerin A-induced cell death in renal cancer using multiple proteomics strategies

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Introduction and Objectives

Englerin A (EA) is a small molecule natural product with selective cytotoxicity against renal cancer cells. Though EA was shown to induce cell death through cell cycle arrest and/ or inhibition of insulin signaling pathway in renal cancer cells, its molecular mechanisms specific to renal cancer remain unclear. Here we employed mass spectrometry-based proteomics approaches in combination with bioinformatics analysis to understand pathways modulated by EA in renal cancer cells.

Methods

A498 renal cancer cells were used for all proteomics experiments. SILAC based liquid-chromatography mass spectrometry (LC-MS/MS) approach was used for proteome and phosphoproteome (through TiO2-based phosphopeptide enrichment) analyses. Tandem mass tag labeling–based approach was applied for thermal shift profiling experiments. For SILAC, cells were treated with 100 nM EA for 24 hours and for thermal profiling cell lysates were incubated with 1µM EA for 30 minutes. All LC-MS/MS analyses were performed using standard bottom-up proteomics protocols and software with default settings.

Results and Discussion

Proteome profiling experiments showed no significant EA-responsive changes. Phosphoproteome profiling displayed marked changes in phosphorylation status, suggesting that EA-mediated effects are post-translational rather than transcriptional/ translational. Bioinformatics analyses highlighted activation of stress and apoptosis, and deregulation of mitotic functions upon EA treatment. Kinase prediction together with comprehensive network analysis showed EA hyperphosphorylates heat shock protein 27 (HSP27) via p38 signaling network specifically in renal cancer cells. Preliminary data from thermal shift profiling showed possible upstream EA binding targets in renal cancer cells.

Conclusions

Our comprehensive proteomics analyses provide a system-wide view of EA-specific mode-of-action that is mediated by a cascade of phosphorylation events, inducing stress-mediated cell death via HSP27 deregulation in renal cancer. Ongoing analysis with our thermal shift data and functional validation experiments will shed light onto renal cancer-specific mechanisms of EA.

Keywords: Englerin A, renal cancer, phosphoproteome, HSP27

MP05-19

Phospho-proteomics on pathways studies

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Introduction and Objectives

Protein phosphorylation is one of the most important post-translational modifications (PTM). Phosphorylation plays an important role in cell growth, adhesion, proliferation and differentiation via a variety of signaling pathways. In living cells, phosphorylation controls spatio-temporal forms of cytoskeletal components and temporal activities of protein kinases. We have focused our research in the phospho- proteome of mice brains to study the activity of Jun N-terminal protein kinases (JNKs) in relation to other protein kinases involved in neurogenesis of mice brains. JNKs belong to the mitogen-activated protein kinase family (MAP kinases family) and consists of 10 different isoforms of JNKs, which are coming from 3 genes: jnk1, jnk 2 and jnk 3. Jnk 1 and 2 are ubiquitous expressed in all body while Jnk3 is mainly expressed in brain, testis and heart. All of JNKs contains a Thr-Pro-Tyr motif and their activation consists in the phosphorylation of threonine and tyrosine in this domain. Once JNKs are activated by other MAPKs, which act upstream, they can act downstream in the modulation of genes and/or proteins, respectively in nucleus and/or cytoplasm.

Methods

Our approach consists in a label free phospho-proteomic enrichment of proteins extracted from mice brains, using TiO2 beads. We have been also looking at combining different technics to improve the phospho-site coverage such as combination of TiO2 and IMAC (SIMAC).

Results and Discussion

Our aim has been to identifying the activation/deactivation through phosphorylation/de-phosphorylation mechanisms of proteins involved in JNK pathway. TiO2 enrichment seems to be a very robust technique for phospho-sites enrichment and it gave us a good possibility for looking into the pathways that we are interested in. We think that our study and our findings are helpful in the field of phospho-proteomics as well as pathway discovery even though there is still a lot more to improve regarding the technique (phospho-peptide enrichment).

Conclusion

Since pathways are the basics of system biology, it becomes very important to know more about them especially because most of the proteins involved in a pathway are related each other in many different ways that need to be known to address biological questions. So it results very important to keep on studying pathways to better understand the connection within proteins and thanks to the always more competitive technologies (mass spectrometry tools are evolving faster and faster over time) we hope to be more precise in this broad field of pathway analysis and bio-marker discovery. The use of phospho-proteomics has been really important for us to improve the knowledge on the

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field of pathway analysis, in particular in the JNK family pathway.

Keywords: Post-translational modification, phoshorylation, signalling pathway, MAP kinases.

MP05-20

Phosphoproteomics-based prediction of cellular protein kinome profiles

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Introduction and Objectives

Protein phosphorylation, caused by protein kinases, is one of the most ubiquitous post-translational modifications. Phosphorylation is well known to regulate various biological functions and abnormal protein phosphorylation is closely related to many diseases including cancer. Therefore, it is very important for the understanding of disease mechanisms to find the kinase that causes characteristic protein phosphorylation to a specific disease. Recent development of liquid chromatography-tandem mass spectrometry (LC-MS/MS) with effective enrichment methods for phosphopeptides has enabled to identify thousands of phosphosites on substrates at one analysis. However, this method never reveal responsible kinases, which actually work on those substrates. The whole picture of signaling network essentially consisting of cascades of phosphorylation reactions cannot be revealed without kinase-substrate information. In previous studies, large scale kinase-substrate relationships were obtained through in vitro kinase reaction with dephosphorylated peptides from cell lysates, analyzed by LC-MS/MS. In this study, using the information of these kinases-substrates pairs, we converted the phosphoproteome data to the upstream kinase profiles.

Methods

In our experimental workflow, proteins were extracted from HeLa cells treated with molecular targeting drugs, and digested by lysyl-endopeptidase (LysC) and trypsin. The digested peptides were enriched by aliphatic hydroxy acid modified metal oxide chromatography (HAMMOC) and analyzed by LC-MS/MS. We utilized two independent measures to quantify kinase-substrate sequence preference: position weight matrices (PWMs) and fold enrichment (FINC) scores of motif sequences. To evaluate this kinase prediction method, we performed SRM (selected reaction monitoring) analysis using synthetic peptides designed from protein kinase sequences and built kinase-activity assay systems by targeted phosphoproteomics.

Results and Discussion

For each drug, around 8,000~10,000 phosphopeptides were identified. From these identified phosphopeptides, we evaluated the kinases that were thought to be responsible for characteristic phosphorylation of each drug treated cells. We found that these two metrics were useful to predict kinases. In addition, the kinase-activity assay system is able to quantify the kinase activity directly and we could confirm our kinase prediction method utility. Therefore, we applied this method to predict responsible kinases of 64 cancer cell lines derived from three different tissues (endometrium, ovary, and breast). More than 45,000 phosphopeptides and 20,000 phosphosites were identified by LC-MS/MS. We could profile these cancer cell lines using upstream kinases predicted

from phosphoproteome data.

Conclusion

We successfully predicted the kinase activity profiles, based on the profiles of phosphorylated peptides identified by LC-MS/MS, and this method will be useful to unveil signaling network.

Keywords: phosphorylation, protein kinase, LC-MS/MS

MP05-21

Phosphoproteome Analysis of the Pathogenic Helicobacter Pylori 26695

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Introduction and Objectives

Gram-negative, spiral-shaped Helicobacter pylori colonizes the human stomach and is frequently associated with atrophic gastritis, peptic ulcer disease, functional dyspepsia and gastric carcinomas. H. pylori has the small genome size (about 1.7 Mb) with three sigma factors (σ80, σ54, σ28), four complete two-component systems (ArsRS, CheAY, FlgRS and CrdRS), two atypical orphan response regulators (HsrA and HP1021) and four transcriptional regulators HspR/HrcA and Fur/NikR to form a simple transcriptional regulatory network. It suggests that the post-translation modification such as phosphorylation is required for H. pylori to respond extracellular stimulation and against harsh acid environment.

Methods

The cells were collected for proteins extraction. The total proteins were separated and collected from protein gel fractionation. Four protein fractions were digested to peptides by trypsin. Then peptides were enriched with titanium dioxide (TiO2) separately. Phosphopeptides were analyzed using LC-MS/MS on a high-accuracy LTQ-Orbitrap mass spectrometer. The identified phosphoproteins were analysed by MaxQuant software.

Results and Discussion

In total, 168 unique phosphopeptides from 131 phosphoproteins are identified from the late-log growth stage of H. pylori and have a distribution of 33.6:30.5:12.6:9.8:13.3 % for the Ser/Thr/Tyr/His/Asp phosphorylation. Among these phosphoproteins, the serine phosphorylation is found on response regulator HsrA and proximity to helix-turn-helix domain, implying that phosphorylation might affect the DNA binding ability of HsrA. Further validation will be done through electrophoresis mobility shift assay (EMSA).

Conclusions

HsrA is essential for the growth of *H. pylori*. Thus, the serine phosphorylation on HsrA might alter the promoter-binding affinity and further affect the expression of downstream target genes.

Keywords: *Helicobacter pylori*, Phosphoproteome, HsrA

MP05-22

Autophagy induces changes in the Sarcoma kinome and phosphoproteome in response to arginine starvation

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Introduction and Objectives

88% of sarcomas lack expression of argininosuccinate synthase (ASS1), rendering them susceptible to treatment by arginine deprivation strategies such as pegylated arginine deiminase (ADI-PEG20). The net result of arginine depletion is the induction of a prosurvival autophagy process with dramatic alterations in the proteome. We used both a leimyosarcoma and angiosarcoma cell line to find common changes in the mesenchymal tumor lineage. We are using the novel methods of kinomic and phopshoprotoemic mass spectrometry workflows to map and identify changes that can be used for drug development.Aim of the study: we hope to identify targets for synthetic lethality in sarcoma by mapping changes in the kinome and the phosphoproteome in response to arginine starvation that are induced by arginine deiminase.

Methods

Utilized “multi-omic” approach to study kinome and phosphoproteome changes in leimyosarcoma (SL) and angiosarcoma (PCB) cell line to find common targets in response to arginine starvation induced by ADI.

Results and Discussion

We quantified more than 1900 proteins and 4400 phosphorylation sites with high precision in SL and PCB cell line. Changes of individual protein were subtle as less than 1% of protein showed significant changes upon ADI treatment. However, pathway enrichment analysis revealed distinct protein clusters regulated by ADI including cellular metabolism and cell cycle in both cell lines. Proteins involved in several metabolic pathways, including amino acids, carbohydrate and certain glycolytic metabolites, were significantly up-regulated. In contrast, pathways involved in cell proliferation such as DNA replication, RNA transport and ribosome, were significant down-regulated. The result demonstrated that the sarcoma proteome was selectively altered by ADI intervention. In respect of protein kinases, despite the expression profiles were distinctly different between SL and PCB. The expression patterns of AGC and TKL kinase family members were down-regulated in response to ADI in both cell lines. We are currently constructing a kinase-substrate network to decipher kinase signaling networks altered by ADI treatment in order to identify targets for validation and functional studies.

Conclusion

We utilized an “-omics” approach to quantify more than 1900 proteins and 4400 phosphorylation sites in the kinome and phosphoproteome of sarcoma. Metabolic, cell cycle proteins and certain group of kinases are both regulated by ADI in the SL and PCB cell lines. Further investigation of underlying kinase-substrate signaling network is ongoing.

Keywords: Sarcoma, kinome, phosphoproteome, autophagy, argininosuccinate synthase

MP05-23

Deep coverage phosphoproteome characterization of human colorectal cancer cell secreted exosomes and new phosphosite discovery

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Introduction and Objectives

Identification of all phosphorylation forms of known proteins is a major goal of chromosome-centric human proteome project (C-HPP). Recent studies have found that certain phosphoproteins can be encapsulated in exosomes and function as key regulators in tumor microenvironment. But, no deep coverage phosphoproteome of human exosomes has been reported to date, which makes the exosome a potential source for the new phosphosite discovery.

Methods

In this study, we performed highly optimized MS analyses on the exosomal and cellular proteins isolated from human colorectal cancer SW620 cells. Phosphoproteome of exosomes were systematically analyzed with multiple bioinformatics tools and computational algorithms.

Results and Discussion

With stringent data quality control, 313 phosphoproteins with 1091 phosphosites (localization probability > 99%) were confidently identified from the SW620 exosome, from which 202 new phosphosites were detected. The criteria of such new phosphoprotein discovery included, peptide residue length >= 9, exclusive unique peptide(s) >= 1, as well as no peptide matches found in the dbPTM, PhosphoSitePlus or SubPhosDB databases, even 1 amino acid mismatch was allowed upon alignments. Interestingly, we found that 142 phosphoproteins were identified only in the exosomal fraction, but not in the cellular fraction. Moreover, only 3 new phosphosites were identified in overlap from both exosome and cell fractions. These results justify that the exosome is a unique source of finding new phosphoproteins for C-HPP. Furthermore, we found that exosomal phosphoproteins tended to be enriched on chromosome 11. The exosome was found to have a remarkably high level of tyrosine-phosphorylated (pY) proteins (6.4%), which was considerably different from the known ~1% level in human cells. These pY-proteins were functionally relevant to ephrin signaling pathway-directed cytoskeleton remodeling, according to the Ingenuity Pathway Analysis, ClueGO and PhosphoPath analyses.

Conclusion

In conclusion, we here report the first deep coverage phosphoproteome of human cell-secreted exosomes, which leads to the identification of new phosphosites for C-HPP. Our findings provide insights into the exosomal phosphoprotein systems that helps to understand the signaling language being delivered by exosomes in cell-cell communications.

Keywords: Exosome, phosphoproteome, new phosphosites, C-HPP, signaling pathway

MP05-24

Quantitative Phosphoproteomic Analysis Reveal Cyclic Stretch-induced Pathways in Human Lung Cancer and Normal Cells.

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Introduction and Objectives

Lung cancer is one of the leading causes of death in the world and many lung cancer related literatures had reported since 19 century. Lung cells are subjected to the mechanical forces periodically while breathing. Whereas most of the researches of lung cancer were based on the traditional cell-culturing method in which cells were seeded on a static surface. Moreover, over 7,000 reports about cyclic stretch have been published in the past 50 years, however, the study in cyclic stretched-induced phosphoproteome remains unclear. In this study, we applied a time-dependent quantitative phosphoproteomic approach to reveal cyclic stretch-induced pathways in adenocarcinomic human alveolar basal epithelial cells A549 and normal lung fibroblast IMR-90.

Methods

We stretched lung cancer cell line A549 and normal fibroblast IMR-90 for 15, 30 minutes and 1 hour and extracted the proteins, and then labeled the control and the treatment samples with CH3 and CHD2, respectively. Furthermore, we applied hydroxy acid-modified metal oxide chromatography (HAAMOC) to enrich the phosphopeptides, used LC-MS/MS and Maxquant software to identify and quantify them.

Results and Discussion

In this study, we applied a time-dependent quantitative phosphoproteomic approach to reveal cyclic stretch-induced pathways in adenocarcinomic human alveolar basal epithelial cells A549 and normal lung fibroblast IMR-90. In the time-dependent phosphoproteome data, there are 350 phosphosites corresponding to 912 phosphorylated proteins and 138 phosphosites correspond to 452 phosphorylated proteins were significantly regulated in A549 and IMR-90 cell lines, respectively. Using bioinformatics approaches including functional enrichment, network analysis and phosphorylation motif analysis, we found that stretching might regulate several important cellular processes in two cell lines. In cyclic stretched A549 data analysis, we found that the functions of protein ubiquitination, protein localization and membrane organization have been enriched. On the other hand, phosphoproteins that are significant regulated in IMR-90 cells mainly participating cell cycle regulation, organelle localization and cell junction.

Conclusion

The stretched-induced phosphoproteins are significantly different in lung cancer A549 and fibroblast IMR-90 cell lines. This study not only gives new information about the cyclic stretch-induced pathways, but also provides a resource for the study of lung cancer therapy.

Keywords: cyclic stretch, phosphoproteomic analysis

MP05-25

Mining drug resistant targets from tyrosine kinase inhibitor-responsive phosphoproteome in non-small cell lung cancer

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Introduction and Objectives

EGFR tyrosine kinase inhibitor (TKI) has demonstrated improvement in progression-free survival of patients with EGFR mutation including exon19 deletions (del19), and L858R within exon21 in non-small cell lung cancer (NSCLC). However, all patients with initial response will ultimately acquire resistance and suffer from tumor recurrence. The drug resistance is generally triggered by secondary mutation (T790M) of EGFR, MET and HER2 amplification. However, clear mechanisms of drug resistance are yet to be discovered. The main goal is to identify the mechanism and targets contributing to EGFR-TKI sensitivity and resistance in NSCLC by a quantitative phosphoproteomic approach to obtain the dynamic phosphosiganture during response to TKI and after resistance to TKI.

Methods

We have used a clinically relevant model of acquired TKI resistance in NSCLC. PC9 is a gefitinib-sensitive cell line harboring an EGFR exon 19 deletion, and its derivative PC9/gef is a resistant cell line that was selected from parental PC9 cells after continuous exposure to low doses of gefitinib. Therefore, we used this model to study acquired resistance to the EGFR inhibitor gefitinib. The NSCLC cells were treated with gefitinib, lysed, digested by tryptic digestion, and labeled by iTRAQ-8plex. After mixing these labeled peptides, the phosphopeptides were enriched by IMAC stage-tip and analyzed by LTQ Orbitrap Fusion. The data were processed by PD2.1 and phosphorylation sites were assigned by ptmRS. The differential phosphoproteins were validated by Western blot and functional assay.

Results and Discussion

Upon gefitinib treatment of the TKI-sensitive PC9 cells for 1 and 3 days, significant deactivation of pY1068-EGFR and reduced phosphorylation of its downstream ERK were detected by western blot. In the resistant PC9/gef cells, however, we observed persistent phospho-ERK signal upon gefitinib treatment, suggesting that TKI resistance is EGFR-independent in the gefitinib-resistant cells. As expected, exposure to gefitinib led to down regulation of the EGFR(Y1197), ERK1/2 (T202/Y187) within the known TKI-responsive canonical NSCLC pathway. Among the 5968 phosphopeptides from 2059 proteins identified with a protein and peptide FDR <= 1%, 142 phosphopeptides from 132 proteins show down-regulated phosphorylation in gefitinib-treated sensitive cells, while no change in resistant cells. These include known resistant targets, such as PXN which has been reported to confer resistance to gefitinib in NSCLC. These potential drug resistant phosphoproteins will be evaluated their potential roles in regulating TKI resistance in lung cancer therapy.

Conclusion

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In summary, the quantitative phosphoproteomic approach reveals the gefitinib-responsive and resistant phosphorylation network, which may shed light on the resistant mechanism for NSCLC.

Keywords: NSCLC, phosphoproteome, TKI-resistance

MP05-26

Phosphoproteome of Spirulina platensis C1 reveals the relevance of photosynthesis and chlorophyll biosynthesis in response to high-temperature stress

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Introduction and Objectives

The cyanobacterium Spirulina platensis C1 is commercially exploited for production of human food supplements, fodder, and pharmaceuticals. The large-scale culture of this organism is usually grown in open systems for 3,000 tonnes of the total industrial production. Therefore, temperature elevation and reduction are important environmental factors affecting the productivity of the photosynthetic performance of outdoor Spirulina cultures.

Methods

In this study, we attempt to elucidate the regulatory mechanisms responding to low (22°C) and high (40°C) temperature stress involving in cellular signal transduction networks by using high accuracy LTQ-Orbitrap mass to analyze Spirulina phosphoproteome.

Results and Discussion

We identified a total of 202 phosphoproteins in S. platensis originating from 455, 290, and 114 unique phosphopeptides from 40°C, 35°C, and 22°C, respectively. Notably, so many phosphoproteins identified in the cells after immediate temperature elevation (40°C). Among them, the numbers of phosphoproteins were involved in the photosynthesis system and the protein turnover and chaperonin system. Especially, 34 phosphoproteins participating in the photosynthetic light reaction machinery containing contains photosystem I (PSI) and photosystem II (PSII) were found under high temperature stress. Furthermore, through site-directed mutagenesis of double mutation Tyr253/Ser255 on the light-dependent protochlorophyllide reductase (Por, SPLC1_S531380) caused the reduction in the amount of chlorophyll a biosynthesis in response to 40°C.

Conclusion

Our results constitute an important advance in understanding the physiological functions of Spirulina in response to temperature changes underlying protein phosphorylation and facilitate the elucidation of the entire photosynthesis signaling pathways for improving industrial use in future prospects.

Keywords: Spirulina, photosynthesis, temperature stress, phosphoproteomic analysis

MP05-27

Use of multiplexed kinase biosensor technology and SWATH-MS for monitoring chronic myelogenous leukemia (CML) signaling

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Introduction and Objectives

The advances in precision medicine and the development of tyrosine kinase inhibitors (TKIs) have revolutionized strategies for treating cancer patients. TKIs have benefited numerous cancer patients; however, problems including drug resistance and residual disease have emerged over time. Preliminary evidence suggests that individual variability in response to drugs is due to distinct pharmacodynamics among patients. In treating CML, it has been shown that decreased Bcr-Abl substrate phosphorylation in mononuclear cells may be an indicator of initial TKI response and could even predict longer-term prognostics. Such 'real-time' monitoring of drug response during treatment is not widely performed, because technical hurdles present a challenge to efficient analysis of patient material. The goal of this project is to develop a technique for measuring patients' response to TKI treatments by investigating kinase signaling profiles in their tumor cells.

Methods

Artificial peptide biosensors that are specific substrates of Abl, Syk, JAK2, and Src family kinases were used to probe kinase activity in leukemic K562 cells and CML patients' mononuclear cells. The biosensors are comprised of peptide sequences that serve as surrogate kinase substrates and a cell penetrating sequence that drives the biosensors into cells. Phosphorylation levels of the peptide biosensors were quantified using multiple reaction monitoring (MRM.) The biosensor technique is antibody independent, and can be multiplexed to detect real time activity of multiple kinases. Additionally, we examined protein fold change by comparing Abl or Syk kinase activated/inhibited samples versus control. Human peripheral blood mononuclear cells were treated with phytohemagglutinin and ionomycin to enhance Abl expression. Cell lysate were digested and analyzed using SWATH-MS.

Results and Discussion

We are able to detect Bcr-Abl activity and inhibition by imatinib in the human CML cell line K562. MRM enabled reproducible, selective detection of the peptide biosensor at fmol levels from aliquots of cell lysate equivalent to ~15,000 cells. From the SWATH data, we identified 1051 proteins. Nine proteins were found to exhibit significant fold changes (95% confidence, at least two folds.)

Conclusion

Our data suggested that multiplexed biosensors can be used for monitoring activity of kinases in primary samples. The biosensor technique has the potential to provide complementary information about intracellular signaling that is not revealed by other techniques. While reproducibility and precision need to be further optimized, our data suggested that SWATH-MS has the potential of providing a comprehensive way of

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studying the machineries involved in various biological processes. Future directions will be to profile proteome signatures for CML patients pre- and post- treated with imatinib mesylate (Gleevec) using SWATH-MS.

Keywords: SWATH, DIA, chronic myelogenous leukemia, MRM, kinase

MP05-28

The ability to attack host iron acquisition pathways in Vibrio vulnificus YJ016 by phosphoproteomic analysis

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Introduction and Objectives

Vibrio vulnificus is an opportunistic human pathogen that is highly lethal and is responsible for the overwhelming majority of reported seafood-related deaths in Taiwan. The rate of progression to this bacterium infection could be very rapid with mortality up to 50% especially when the patients had some preexisting diseases such as liver diseases cirrhosis or hepatitis. Previous studies reveals that the high serum iron levels and the decline in immunity are two of the important susceptible factors resulted in human infection. Otherwise, iron plays a crucial role in infection rate of V. vulnificus, which the infection mechanism is still unknown.

Methods

In this study, the phosphoproteome of V. vulnificus YJ016 was analyzed via a shotgun approach and high-accuracy mass spectrometry.

Results and Discussion

Totally, 214 unique phosphopeptides and 205 phosphoproteins were identified. The distribution of His/Asp/Ser/Thr/Tyr phosphorylation sites were 11%/18%/31%/26%/14%. We further focus on two virulence associated phosphoprotrins, hemolysin and RTX repeat-containing cytotoxin protein.

Conclusion

Furthermore, ferrochelatase is extremely critical enzyme in heme synthesis pathway which may modulate phosphorylation.

Keywords: Vibrio vulnificus, phosphoproteome, hemolysin

MO07-01

Investigating the basic assumptions in protein abundance estimation using SWATH-MS data

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Introduction and Objectives

In bottom-up quantitative proteomics, peptides are usually measured in the mass spectrometer as surrogates for the respective proteins. With different underlying models and implied assumptions, a variety of approaches have been proposed to estimate protein abundance using peptide intensities. Recently, SWATH-MS, a highly reproducible and quantitatively accurate technology, has been developed to potentially fragment and record all precursors as digital maps. By using SWATH-MS data, we set out to investigate the basic assumptions in protein quantity inference.

Methods

We first investigated several basic assumptions by analyzing SWATH-MS datasets from an inter-laboratory study. This study includes more than 200 injections of the same sample, carried out in 11 participating labs and measured over one week. It provides a rich and solid foundation to test the assumptions under different conditions (e.g. technical replicates; replicates injected within a single day / within a site / across sites). We further evaluated the robustness of peptide intensity signals in terms of coefficient of variance (CV) and missing values. The observed variability was attributed to specific features in the peptide sequences. Last, a novel “voting” strategy, that empirically assesses the consistency of peptide intensities by multiple measurements, was developed and used to select an optimal set of peptides for protein quantification. Protein abundance estimation by this set of peptides was evaluated and compared with the naive top 3 method, using another benchmarking dataset consisting of human, yeast, and E. coli proteomes mixed in defined proportions.

Results and Discussion

We demonstrated that SWATH-MS generated a comprehensive set of peptides with conserved relative intensity ranks between replicates. After normalization, to eliminate the effects of inter-run variations, median CVs of 9.8% (within a site) and 20% (across sites) were observed at the peptide level. This variability was recapitulated in terms of peptide features (e.g. peptide responses in MS; PTMs; sequence motifs). Furthermore, the simple voting strategy was shown to be effective at selecting a set of quantitatively consistent peptides. Unsurprisingly, this set contained a smaller proportion of the above listed features while still covering the majority (80.6%) of the proteins in the inter-laboratory study. Applying this set of peptides to a dataset with known protein fold changes, we observed a higher number of proteins (23%) with smaller quantification estimation errors, compared with the top 3 approach.

Conclusion

By investigating the basic assumptions in protein abundance estimation, we demonstrated that SWATH-MS is a highly reproducible and quantitatively precise

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technique that allows quantifying a comprehensive set of proteins. In this preliminary study, a simple and general method was developed to select, from multiple repeated measurements, a potentially optimal set of peptides for protein quantification.

Keywords: Quantitative proteomics; SWATH-MS

MO07-02

Reactome - Interactive Pathway Analysis for Proteomics

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Introduction and Objectives

Reactome is a free, open-source, curated and peer-reviewed knowledgebase of biomolecular pathways. Its aim is to provide intuitive bioinformatics tools for visualisation, interpretation and analysis of pathway knowledge to support basic research, genome analysis, modeling, systems biology and education.

Methods

Pathways are built from connected reactions that encompass many types of biochemical events. Reactions are derived from literature and must cite a publication that experimentally validates them. Pathways are authored by expert biologists and peer reviewed before incorporation into the database. 9,584 reactions in Reactome cover 9, 238 human gene products (12,527 including IntAct interactors), supported by 22,838 literature references. The Reactome web interface has been completely redeveloped over the last two years, providing a highly interactive, visually attractive interface to Reactome content, as well as integrated widgets to display molecular structures, expression data, and interactions from other resources in their pathway context.

Results and Discussion

Users can search for proteins or compounds and see details of the complexes, reactions and pathways they participate in. Pathway diagrams allow users to examine the molecular events that constitute the steps in pathways and to view details of the proteins, complexes and compounds involved.Different forms of pathways analysis can be performed with the Reactome analysis tools. Users can submit a list of identifiers for overrepresentation analysis or submit quantitative datasets, such as microarray data, for expression analysis. Results of these analyses are overlaid onto the Pathways Overview and Diagram Viewer for easy navigation and interpretation. The pathway analysis is highly interactive, genome-scale datasets can be analysed within seconds.Interaction data from multiple resources can be used to expand pathways. Interactors from IntAct are included by default in the search feature and can be taken into account in the analysis service. Reactome is open source, open data, and Pathways, subsets, or all Reactome content can be downloaded in many formats including TSV, CSV, PDF, SBML, BioPax and PSI-MITAB. The Reactome visualisation components have been developed for efficient integration into third party websites.

Conclusion

Reactome provides a high quality, open access, manually curated resource for human pathways, as well as efficient software interfaces for pathway analysis and exploration. Reactome is accessible at http://www.reactome.org, as well as its asian mirror at http://reactome.ncpsb.org

Keywords: Pathway analysis, Visualisation, Protein Networks, Protein Interaction

MO07-03

MSCypher: A High-Throughput Peptide Identification Strategy for Complex Mixtures

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Introduction and Objectives

Shotgun proteomics based on high-resolution mass spectrometry (MS) is transforming the post-genomic era and offers an unbiased systems-wide analytical method that compliments existing targeted MS and antibody-based approaches. However, using current technology only about 15-30% of the >100,000 observable individual peptide isotopic patterns1 are ever identified. During traditional data-dependant MS acquisition (DDA) of complex samples, nearly all MS/MS spectra generated contain contaminating fragment ions that arise from isolation and fragmentation of multiple co-elution peptides. These chimeric spectra are largely penalised based on the 'one MS/MS spectrum–one peptide' strategy enforced by nearly all available MS/MS search algorithms.

Methods

This high-resolution MS approach takes advantage of co-eluting peptides and uses a dynamic isolation window to evenly distribute the number of co-fragmented species during acquisition. Processing these multiplexed spectra datasets has required a redesign of the current analysis workflow, and now includes retention time prediction, dynamic probabilistic fragment ion matching and a supervised ensemble learning method for peptide feature identification.

Results and Discussion

Our initial results indicate that this multiplexed MS/MS approach provides both enhanced sensitivity and a gain in coverage proportional to the number of peptides simultaneously analysed. We also maintain high confidence levels for peptides and modified peptides identified. In comparison to the MaxQuant workflow, we obtain 100% more PSMs, 40% more unique peptide identifications and >5600 proteins (in a 90min gradient and across replicates). Implementing the common quantitative cut-off of two or more peptides per protein, we still maintain >5300 quantifiable protein groups.

Conclusion

Here we report MSCypher, a new workflow that combines a modified tandem MS acquisition method with a novel searching strategy that results in significant improvements in speed and sensitivity.

Keywords: Search EngineMachine LearningShotgun Proteomics

MO07-04

neXtProt in the context of human proteomics projects

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Introduction and Objectives

Focused solely on human proteins, neXtProt (www.nextprot.org) is built on top of the corpus of sequences and annotations provided by UniProtKB/Swiss-Prot and provides a wide range of expert-curated supplementary information at genomic, transcriptomic and proteomic levels. As a reference resource for the C-HPP project, neXtProt works in close collaboration with other major resources to integrate relevant data, build tools to exploit them, and provide adequate metrics.

Methods

Currently, neXtProt integrates tissular and subcellular localization data from the Human Protein Atlas, as well as peptide and phosphorylation site identifications submitted through ProteomeXchange and reprocessed through Peptide Atlas. In addition to proteomics data generated by the HPP consortium, neXtProt curates post-translational modifications such as ubiquitinations, glycosylations, acetylations, from carefully selected proteomics datasets. neXtProt also integrates the sequences of synthetic peptides for which data is available in SRMAtlas in order to help investigators choose proper reagents for quantitative proteomics. Besides proteomics information, neXtProt integrates useful information at genomic and transcriptomic levels, including nearly 2 million single amino acid variations.

Results and Discussion

To help the HPP community to better exploit the integrated data, the neXtProt team recently developed three tools : - a specific “peptide viewer”, showing detailed information on all the matching peptides that were identified in biological samples, including their trypticity, N-/C-terminality, and PTM(s). - a “peptide unicity checker” allowing to assess the unicity of each peptide at the level of entries or at the level of isoforms, taking single amino acid variations into account or not. - a dedicated “protein existence viewer”, which summarizes the validation status of entries for each chromosome. In order to be compatible with the most up to date semantics technologies, neXtProt’s relational database has been recently converted into a RDF-based (Resource Description Framework) graph representation. This representation is extremely powerful to navigate through the richness of the neXtProt data and to search it using the SPARQL query language. The advanced search is accessible at http://search.nextprot.org.

Conclusion

We are keen to collect feedback concerning our proteomics tools and advanced search capabilities, and making them evolve to better serve the proteomics community.

Keywords: protein knowledgebase, human, biocuration, proteomics, semantics, HPP, RDF

MO07-05

A genetic algorithm to locate responsive subpathways for time-course proteomic data

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Introduction and Objectives

The analysis methods designed for gene expression data produced by microarray become well-established. However, these tools are hardly applicable on proteomic data. Proteomic data usually have larger variation, smaller sample size, smaller identified entities, and the results are sensitive to experimental conditions and instruments. The incomplete nature of proteomic data makes the investigation of underlying mechanisms hardly achieved. Despite the issues originated from incomplete data, using a protein-protein interaction (PPI) network as the reference of biological interactions may over simplify the problem. First, most of PPI networks do not provide the information of regulation types. Second, the metabolites and other small chemicals also play important roles in biological regulations, eliminate them from the reference network may remove some key information as well. Third, directly using a PPI network as reference also ignore the fact that the subunits of a protein complex may not be functional alone. Another issue rises from the experimental design. Proteomic studies usually focus on specific proteome. Proteins do not belong the specific proteome are inaccessible under current experimental condition

Methods

In this study, we introduce a genetic algorithm to locate the responsive subpathways for time-course proteomic data. We use the pathways provided by the Kyoto Encyclopedia of Genes and Genomes (KEGG) as the reference of biological regulations. The KEGG pathways provide us information of regulation types, metabolites and complex composition, by which we build a comprehensive network to address the issues mentioned above. We also retrieve the keywords annotation from Uniprot to select accessible proteins in specific proteome. In order to locate the responsive subpathways on the KEGG pathways, we design a genetic algorithm to detect subpathways of higher expression ratio with coherent relationship. We also take protein accessibility and the composition of protein complex into consideration in our algorithm.

Results and Discussion

The performance of the proposed algorithm is demonstrated using three public experimental datasets with different levels of biological complexity: the acetylation in response to carbon overflow, the phosphorylation via cAMP/PKA signaling, and the cellular proteomic data during differentiation. We compared the proposed algorithm with jActiveModules and BioNet. Generally, the subpathways provided by BioNet and jActiveModules are highly-connected networks, and the performance shrinks as long as the data are dispersed; whereas the proposed algorithm is more tolerated to missing or inaccessible proteins, hence we are able to provide candidate responsive subpathways

when the data are incomplete.

Conclusion

The design of our algorithm take many aspects of proteomic data into considerations, and the algorithm yielded possible subpathways in agreement with the discussion of the associated publication.

Keywords: time-course data; functional annotation; computational proteomics

MP06-01

Omics Discovery Index - Discovering and Linking Public Omics Datasets

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Introduction and Objectives

Biomedical data, in particular omics datasets are being generated at an unprecedented rate. As a result, the number of deposited datasets in public repositories originating from various omics approaches has increased dramatically in recent years. However, this also means that discovery of all relevant datasets for a given scientific question is non-trivial. Here, we introduce the Omics Discovery Index (OmicsDI - http://www.ebi.ac.uk/Tools/omicsdi), an integrated and open source platform facilitating the discovery of omics datasets. OmicsDI provides a unique infrastructure to integrate datasets coming from multiple omics studies, including at present proteomics, genomics and metabolomics, as a globally distributed resource.

Methods

Building on the experience of the ProteomeXchange consortium, we have developed a lightweight central metadata-based portal for the efficient discovery of omics datasets. Each participating omics data resource provides a minimal metadata description for each relevant dataset, and these are then indexed by Lucene, an industry standard document indexing system. The resulting resource can be accessed for search and browsing through a web interface, as well as through computational web services. As only data descriptions (metadata) is indexed, OmicsDi is resource-efficient and relatively easy to maintain up to date. For actual data access all dataset descriptions reference the source repository, avoiding the replication of often very large raw data sets.

Results and Discussion

As of June 2016, OmicsDI provides a lightweight discovery tool including more than 70,000 omics datasets from ten different repositories, three different omics types, and three continents. While advanced metadata-based browsing and indexing supports dataset findability, the lightweight approach avoids the development of redundant concepts and infrastructure. The original datasets are not replicated, but referenced. In the interest of sustainability, the responsibility for provision of a well-formatted metadata records lies with the original data providers, similarly to the concept of publisher data provision to PubMed or EuroPMC. OmicsDI metadata records will also contribute to the broader-scope bioCADDIE index currently under development. OmicsDI supports full text search as well as ontology-based search extensions. In addition, we use the concept of biological dataset similarity, based on the number of shared biological entities, for example protein identifications, among datasets. This allows us to suggest potential

relationships among datasets even if they don't share sufficient metadata annotation.

Conclusion

OmicsDI provides a central, lightweight index for currently more than 70,000 omics datasets from ten different repositories, supporting one-stop discovery of relevant, potentially multi-omics, datasets. OmicsDI is accessible at http://www.ebi.ac.uk/Tools/omicsdi/

Keywords: proteomics, metabolomics, multi-omics, data discovery

MP06-02

DAPAR & ProStaR: software to perform statistical analyses in quantitative discovery proteomics

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Introduction and Objectives

The objectives of quantitative discovery proteomics are to identify proteins in several biological samples that separate into at least two different biological conditions, and to perform a relative quantification, so as to discriminate between the proteins which are significantly differentially abundant, and those which are not. Once the outcome of the analytical pipeline is available, the quantitative analysis may start. Its objective is to rely on an efficient and reproducible statistical pipeline to isolate the subset of proteins that are characteristic of the differences between the biological conditions, on which further more exhaustive wet-lab experiments will be performed.

Methods

In general, quantitative analysis is composed of the following processing steps: 1. Filtering: Some peptides or proteins may be discarded, on the basis of several user-defined criteria (number of missing values, contaminant database, decoy sequences, etc.). 2. Normalization: The protein abundances are rescaled to account for the variability between the analyses. 3. Imputation: To maximize the power of the statistical analysis, the missing values are imputed. 4. Aggregation: The peptide intensities are aggregated together so as to infer back the abundances of the proteins originally present in the samples.5. Differential analysis: Finally, null hypothesis significance testing, as well as p-value adjustment are conducted.DAPAR (Differential Analysis of Protein Abundance with R) is an R package that either proposes new algorithms for these five computational steps, or simply binds the R packages implementing pre-existing state-of-the-art methods. The main feature of DAPAR is to gather in a single package, all the necessary statistical routines for quantitative analysis.

Results and Discussion

However, as is, its use requires being comfortable with R programming, which is not the case for all proteomics practitioners. This is why, DAPAR is accompanied by ProStaR, a package that relies on Shiny technology to dynamically build web-based GUI to DAPAR functionalities. Moreover, ProStaR is also available in server mode: a single (server) machine is installed and maintained with R, DAPAR and ProStaR, on which each practitioner connects through a given URL. This makes ProStaR particularly suited for proteomics labs where a single bioinformatician deploys and maintains the tools that are used by the proteomicians for their data analyses.

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Conclusion

In addition to providing menus devoted to each of the five processing steps (filtering, normalization, imputation, aggregation and differential analysis), ProStaR provides import/export functionalities, as well as a “descriptive statistics” menu where it is possible to visualize the dataset in hands, so as to best understand it, or to produce display elements for publications. DAPAR and ProStaR are actively maintained. It can be directly tested through a demo mode on the following website: www.prostar-proteomics.org.

Keywords: statistical analysis of quantitative label-free experiments

MP06-03

Combining Proteomics and RNASeq data for Biological Evaluation.

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Introduction and Objectives

It is recognized that a single analytical techniques are unlikely to provide all of the evidence required for confirming biological hypotheses. This increases the desire to investigate larger result sets with varied data sources each requiring complex data manipulation and analysis, on top of complex analysis of the individual analytical set. Standardization is difficult and sometimes impossible to achieve as methods for statistically evaluation are also very diverse—each providing a subtly different result, which may alter the biological conclusion. Cluster analysis is commonly used as an unbiased strategy for evaluating the results from large omics experiments, however this is not trivial and consideration of data type and experimental design are needed. Here we present a novel method for simplifying and integrating cluster analysis which also provides unique overviews of the data for visualization accelerating hypothesis development.

Methods

RNASeq and SWATH-MS data were generated on a series of samples from the K562 human chronic myeloid leukemia cell line and three drug-resistant derivatives. RNASeq was processed using a Galaxy workflow with TopHat and Cufflinks. SWATH-MS data were generated using a TripleTof® 5600 instrument and processed using DIAUmpire, Protein Pilot™ 5.0, and the OneOmics™ Software pipeline for quantitation. Results were imported directly into an application for alignment and statistical processing of the results which utilized multiple methods for cluster analysis.

Results and Discussion

765 proteins and 10026 genes were profiled in the experiment. SWATH data processing results were included if the confidence of the result was greater than 75%. RNASeq results were filtered by requiring at least one experimental contrast (control vs. drug-treated) exhibited a significant result. This resulted in a 92.9% alignment of the proteomics data with the RNASeq data and a 94.3% intersection of the individual RNASeq results. From this alignment a series of clusterings of the feature data was performed to identify groups of proteins and genes which showed similar trends within the data. These individual results were then combined to identify proteins and genes which clustered together within the different methods. These groupings were then subjected to GO analysis providing and insight into a series of different biological processes which were enriched in the combined cluster results.

Conclusion

Single cluster methods indicated trends in the data which may include a number of false positives. By using multiple methods the false positives can be reduced by investigating those which co-cluster. By using GO terms an insight into the biological processes which are being modulated can be identified. Using multidimensional visualizations of the

MP06-03

results, a series of clustered protein and gene expression profiles indicated novel biological interactions leading to novel biological hypotheses to be concluded

Keywords: MultiOmics visualization, SWATH-MS, RNASeq

MP06-04

iTop-Q: an intelligent top-down proteomics quantification tool using the DYAMOND algorithm for charge state deconvolution

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Introduction and Objectives

Liquid chromatogram coupled with mass spectrometry (LC-MS) is a pre-dominant platform for proteomics research. Top-down proteomics has increasingly become the method of choice for analyzing intact proteins to study genetic variation, alternative splicing, and post-translational modifications (PTMs) of the proteins. Several algorithms and tools have been proposed for intact protein identification using LC-MS/MS spectra, such as ProSightPC, and TopPIC. However, very few tools have been developed to perform intact protein quantification from LC-MS spectra. Though recently MASH Suite Pro has been available to provide per-spectrum quantification analysis, manual processing is still required to quantify proteins based on abundances obtained from multiple spectra. To the best of our knowledge, currently there is no fully automated tool for large-scale protein quantification in top-down proteomics. Thus, in this study, we present a fully automated tool, called iTop-Q (intelligent Top-down proteomics Quantification), for top-down proteomics quantification.

Methods

Instead of utilizing single spectrum for protein quantification, iTop-Q constructs the extracted ion chromatograms (XICs) of possible protein peaks across multiple spectra to provide accurate quantification. Without using THRASH to characterize proteins with many different charge states, iTop-Q is implemented with a new algorithm, called DYAMOND, to determine protein abundances based on XICs. Furthermore, iTop-Q performs a complete procedure from protein detection to protein alignment to determine protein abundances based on XICs across replicates/samples.

Results and Discussion

To evaluate the performance of iTop-Q, a standard protein dataset and a public large-scale yeast lysate dataset obtained from Joe R. Cannon et al. (Analytical Chemistry, 2014) were applied. The standard dataset includes a standard protein (i.e., Carbonic Anhydrase) in three technical replicates with the same concentration, and the public yeast lysate protein dataset includes 7 fractions, each with at least 3 technical replicates. A total of 292 proteins were identified by the authors from these 7 fractions using ProSightPC 3. 0. Applying iTop-Q on the standard protein dataset, Carbonic Anhydrase was detected with mass error <0.0028%, which implies the correctness of the protein charge states determined by the DYAMOND algorithm. Furthermore, the average protein abundance ratios calculated by iTop-Q was 1±0.07, compared to 1±0.21 calculated by MASH Suite Pro, showing that iTop-Q achieved a smaller standard deviation than MASH Suite Pro. In the yeast lysate dataset, excluding 74 proteins undetected by iTop-Q due to low signal-to-noise ratios, 218 identified proteins were successfully detected with accurately

MP06-04

calculated protein masses (mass error in average < 0.09%). Meanwhile, the median protein abundance ratios for the 218 detected proteins among replicates were equal to 1. 0.

Conclusion

Keywords: Top-down proteomics, LC-MS, protein quantification, charge state deconvolution

MP06-05

Sensitive and Fast Identification of Bacteria by MALDI-TOF MS fingerprints

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Introduction and Objectives

Matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF MS) has been widely used for bacteria identification at the genus, species and even strain levels. With this concept, commercial MALDI-TOF MS systems, including Vitek MS (bioMérieux) and Biotyper (Bruker Daltonics), have been adopted for the identification of a variety of bacteria from blood samples, and received the clearance from US Food and Drug Administration (FDA). In this work, we developed a sensitive computation method to quickly and accurately identify bacteria by calculating spectral similarity score and validating the highest similarity score with statistical hypothesis test.

Methods

A library of 307 bacteria reference spectra was firstly built by collecting MALDI-TOF MS fingerprints of different bacteria at different cells numbers. With this library, bacteria were identified \by spectra pattern matching. Three scoring methods were used to calculate spectral similarity score. The identification was reached based on the highest similarity score, and validated with statistical confidence.

Results and Discussion

As a proof of concept, bacteria were successfully identified with series of math models and statistics methods inferred from experiment spectral data.

Conclusion

Therefore, an entire MALDI-TOF MS fingerprints identification process could be accurately accomplished within 20 second by the newly developed method, which could facilitate timely determination of appropriate anti-bacteria therapy and decrease the risk of mortality from bloodstream infections.

Keywords: Algorithm / MS / MALDI-TOF/Bacteria/Spectra Similarity/ Protein

MP06-06

A statistical method of automatically selecting optimal software package for detecting differential abundance in proteomics studies

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Introduction and Objectives

As tools for quantitative label-free mass spectrometry (MS) rapidly develop, a fundamental research problem in many proteomics studies is the identification of reliable biological markers that show differential expression between distinct sample groups. A number of software packages have already been developed for this task. However, there is no clear consensus about the best practices yet. A few comparative studies have pointed out that the choice of the packages can greatly affect the outcome of the analysis, and no single tool is likely to be optimal under all circumstances. Therefore, we argue that an automated statistical tool which is able to not only choose the optimal package but perform the test of differential abundance accordingly is of best interest.

Methods

We include potential state-of-the-art software packages for detecting differential abundance into our pipeline, such as LIMMA, Rank Product (RP), Reproducibility-optimized test statistic (ROTS), t-test, Significance analysis of microarray (SAM), MaxQuant, and MSstats. As this field is under heavy development, we could easily include more software packages into our pipeline in the future if necessary. The input is the data to be analyzed. Then the area under the true-discovery plot can be regarded as an assessment of which software packages is optimal for each particular data set. True-discovery plot consists of estimated number of true positive markers on the y-axis and number of significant markers on the x-axis. The estimated number of true positive markers is derived by estimating the number of false positive markers, which could be attained by either constructing artificial two-group comparisons within each same group or performing a permutation test. The software package with the most area under true-discover plot is chosen as optimal and used for performing the test of differential abundance. The output is the significance of each marker after adjusted for multiplicity.

Results and Discussion

We use synthetic, simulated, and biological data with variety of sample sizes for assessing the performance of our pipeline. We have spike-in data sets from various publications, including the ones with OpenSWATH under water, yeast, and human background. Synthetic and simulated data are utilized for evaluating sensitivity and specificity. In addition, we use the data from a study of plasma samples aimed at evaluating the ability of the method to handle complex biological variation. Currently about 80% chance the, data-driven, optimal package chosen by our pipeline is verified as the best one, while with 96% chance one of the first 2 choice recommended by our pipeline provides the best results, according to the area under ROC curve.

Conclusion

We conclude that our statistical pipeline can ensure the identification of biological markers

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with high confidence, which is a valuable guidance for validation process.

Keywords: label-free mass spectrometry; quantitative proteomics; model selection; automated pipeline; statistical methods

MP06-07

Updated Scores of Immunohistochemistry-based Expression Profiles in the Human Protein Atlas for Prioritizing Cancer Marker Candidates

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Introduction and Objectives

Levels of protein expression on tissues are considered pivotal in cancer marker discovery, especially while narrowing down a list of protein candidates generated by high-throughput technologies. Comprehensively, the Human Protein Atlas (HPA) provides tissue-based protein expression profiles using tissue microarray technology for immunohistochemistry (IHC) staining, and continuously updates the antibodies and expression profiles. The accumulated IHC images and annotations are valuable resources for reference in selecting marker candidates.

Methods

We have previously published a method to summarize annotations of IHC staining images retrieved from the HPA. The annotations were semi-quantitatively calculated into scores for prioritizing cancer marker candidates. The score is a relative indicator considering significance (among antibodies) and specificity (among cancers) of the expression level for a given antibody in a specific cancer.

Results and Discussion

Currently, we updated the scores according to the latest version of HPA release, and compared the results to the previous data. There were in a total of 6,644,000 annotations of IHC staining images, which involved 22,000 antibodies covering 16,333 genes (Ensemble id), been processed for the update.

Conclusion

The updated scores are available at http://bal.ym.edu.tw/hpa/.

Keywords: Cancer Biomarker, Candidate Prioritization, the Human Protein Atlas

MP06-08

Epsilon-Q: Improved proteomic analysis tool based on extracted ion chromatogram and Combo-Spec search method

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Introduction and Objectives

Label-free quantitation approach has recently been used as an alternative strategy to minimize such limitations incurred during labeling process. Currently available MS system and bioinformatics tools can also support this label-free technique with high resolution and generate huge high quality mass spectral data. Here we report development of analysis software, which can estimate protein abundance ratio between different samples using extract ion chromatogram with various MS/MS data analysis techniques to comparative quantitative proteomics research.

Methods

This software supports two types of MS/MS data analysis method (Combo-Spec search method and single reference peptide database or library search)1,2. After obtaining MS/MS data sets, all peptides are mapped through m/z and retention time axis to align across different samples. At the same time, sequence database search engines or SpectraST identify peptides and proteins. After peptide and protein identification, Epsilon-Q apply appropriate FDR control strategy to remain user specified FDR threshold in protein level. All those identified peptides can be used as landmarks to correct peptide map alignment.

Results and Discussion

Before protein quantitation, Epsilon-Q processes peptide and protein identification task. In this step, Epsilon-Q supports multiple sequence database search and spectral library search. Especially, using the Combo-Spec search method improved protein sequence coverages and it shows improved performance to comparative proteomic analysis. For each protein, Epsilon-Q estimates all peptide abundance ratios in samples and removes outlier to make an accurate protein abundance prediction.

Conclusion

We developed the Epsilon-Q, which supports Combo-Spec search method and estimates protein abundances by extracting ion chromatograms for comparative proteomic analysis.

Using the Combo-Spec search method and extracted ion chromatogram, Epsilon-Q shows better performance compared to label-free protein quantitative analysis. We anticipate that this software would be useful for identifying proteins based on Combo-Spec search method and label-free based comparative proteomic analysis.

Keywords: Proteomics, Mass Spectrometry, Label-free Quantitation, Combo-Spec Search

MP06-09

PECA2: new developments in the statistical modeling of protein concentration regulation in dynamic systems

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Introduction and Objectives

Dynamic regulation of protein concentration can be deciphered from time series data sets of mRNA and protein expression. Protein Expression Control Analysis (PECA, http: //sourceforge.net/projects/pecanalysis/) was one of the first methods that quantitatively dissect protein expression variation into the contributions of mRNA synthesis/degradation and protein synthesis/degradation. The novelty of PECA is in the calculation of the probability score for each protein that the ratio of synthesis rate to degradation rate changed between adjacent time intervals, indicating protein expression regulation changes at a specific time point considering the fluctuations in the mRNA concentration. The resulting probability scores lead to automatic calculation of the false discovery rates, enabling error control in the detection of regulatory changes in the entire data set.

Methods

While PECA was able to compute useful statistical summaries such as the rate ratios of synthesis versus degradation, there was room for improvement in terms of data preprocessing, missing data treatment, and pathway-level interpretation of the dynamic output summary. Here we have further developed the software (PECA2) with three new features. First, PECA2 performs a data smoothing step for the time series data for mRNAs and proteins before fitting the rate ratio model. It uses a flexible curve fitting algorithm called Gaussian process modeling and automatically selects optimal kernel parameters to yield reasonable smoothness in the fitted curve. This step not only removes noisy observations, it also allows for imputation of missing data in the time series. Second, PECA2 provides Gene Ontology (GO) enrichment analysis of the proteins subject to protein-level up- and down-regulation at each time point, with a user specified probability score threshold. From the new GO analysis table, the user can examine whether the member genes of a GO term were translationally up- or down-regulated across the time points. Lastly, we have implemented an additional statistical model to estimate the absolute rates of protein synthesis and degradation given appropriate proteomic data. Note that estimation of absolute rate parameters is impossible because the same change in the protein concentration can explained by multiple reconfiguration of different rate parameters (increased synthesis versus decreased degradation). The new model in PECA2 allows for estimation of the absolute rates when pulsed stable isotope labeling data is available, which enables PECA to directly model the absolute rates of protein synthesis and degradation.

Results and Discussion

We demonstrate the new features of PECA2 using the data set from Jovanovic et al (Science 2015), where murine dendritic cells alter the transcriptome and the proteome in response to lipopolysaccharide stimulation.

MP06-09

Conclusion

Keywords: Dynamic systems, regulation of protein expression, synthesis and degradation rates, statistical model

MP06-10

An Information System Enabling the Organization and Automatic Annotation of Proteomic Experiments with User-specific Proteins

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Introduction and Objectives

ProteinCenter is an annotation framework allowing the interpretation of proteomics results in a biological context using established protein databases and annotated sequence variants, including proteoforms. However, researchers often utilize engineered proteins such as fusion proteins and introduce amino acid sequence mutations to test their hypotheses. To extend the benefits of ProteinCenter beyond currently published protein sequences we have enabled users to store their protein sequences of interest within the system thereby allowing quantitative comparison in proteomes where non-canonical protein sequences are involved. To demonstrate the new functionalities of ProteinCenter we show a differential analysis from an interactomics study using a proximity-dependent strategy where a point-mutation is expected to interfere with biologically relevant interactions.

Methods

The ProteinCenter database schema was extended to store unique protein sequences associated with a user of the system. A naming convention was established to allow a user to associate annotations from a public protein accession with each Experimentally Observable Protein [EOP] that they define. Quantitative results from peptides derived from the non-public sequences were thereby incorporated.The new functionalities were demonstrated by using a dataset generated in an interactomic study of genetically engineered cells expressing fusion and mutated forms of the user specific protein.Cells were processed in biological triplicates and analyzed via a mass spectrometry based workflow using data-dependent acquisition. Proteome Discoverer 2.2 was used to process and quantify identified peptide features in a label-free fashion. A pre-release version of ProteinCenter was used for profiling, differential analyses, and result interpretation in the human proteome background biological context.

Results and Discussion

Peptide search and quantification of user defined proteins was made possible by the new software workflow. All experimentally observed peak quantities from identified peptides introduced by molecular cloning were included in the quantitative results, including profiling, pathway analysis and ontology statistics. Additionally, the more accurate values for EOP abundance were available for normalization steps in alternative analysis workflows.The workflow enabled novel data normalization approaches including scaling to fusion protein abundance across distinct primary sequences. The interpreted context of the study shows relevant pathway over-representation for the wild type samples and typical abundant and irrelevant representation for the mutant.

MP06-10

Conclusion

We demonstrate a system for organization, annotation and analysis of user-defined protein sequences in their proteome-wide biological context.

Keywords: ProteinCenter, data annotation, automation, interaction proteomics, observable proteins

MP06-11

Entrapment sequences method for the evaluation of database search engines and quality control methods in shotgun proteomics

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Introduction and Objectives

With the advance of mass spectrometry and experimental techniques,proteome research has broken through the bottleneck of data generation,and a huge amount of mass spectrometry (MS) data has been accumulated rapidly in the past few years.Meanwhile, the lack of efficient data analysis and quality control methods has greatly blocked the way of proteome development.Target-decoy searching strategy has become one of the most popular strategies to control the false identifications in MS/MS data analysis.However,the target-decoy strategy can estimate the false discovery rate (FDR) within an dataset,it cannot directly evaluate the false positive matches in target identifications.In this study,we introduce the entrapment sequences method to set up an objective standard to evaluate the performance of quality control methods and database search engines.

Methods

In the entrapment sequences method,the target sequences are comprised of sample sequences (A) and entrapment sequences (B),which are of low homology with the sample sequences.Then the combined target sequences (A+B) are reversed to construct decoy sequences (A'+B').By using different labels,we can separate the PSMs into different kinds and calculate the actual false identifications in target PSMs,peptides and proteins.Using standard datasets and human sample datasets,we first explored the best proportion of entrapment and sample sequences.Then we gave definition and equation of Estimated FDR and Actual FDR.At last we evaluated the performance of five database search engines (Mascot, X!Tandem, Comet, MS-GF+ and Tide) and five quality control methods (BuildSummary, PepDistiller, PeptideProphet, FDRAnalysis and ScoreBased Method).We proved entrapment sequences method could be an excellent strategy to assess each step of the mass spectrometry data analysis process.

Results and Discussion

For small entrapment/sample proportion,there were very few entrapment PSMs identified. As the proportion grew,more entrapment PSMs were identified.When the size of entrapment sequences increased to ten times of that of sample sequences,the amount of entrapment PSMs was roughly equal to that of decoy PSMs without losing too many sample identifications.Thus we can calculate Actual FDR using entrapment sequences PSMs at a given Estimated FDR.We evaluated the performance of quality control methods and database search engines in two aspects:1) Quantity.The MS-GF+ and PepDistiller identified the most number of PSMs,peptides and proteins at different FDR level.2) Reliablity.The MS-GF+ and PepDistiller had Estimated FDRs more closer to Actual FDRs.

Conclusion

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1) Ten times could be a proper proportion for entrapment and sample sequences.2) Estimated FDR=Ndecoy / Ntarget; Actual FDR=Ntrap / Ntarget.3) PepDistiller has a better performance than other four quality control methods.4) MS-GF+ has a better performance than other four database search engines.

Keywords: entrapment sequence,protein identification,quality control,tandem mass spectrum,target-decoy

MP06-12

Firmiana: A One-stop Proteome Data Processing and Integrated Omics Analysis Cloud Platform

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Introduction and Objectives

The big bang of the data produced by the next generation proteomics requests a powerful, integrated data processing and analyzing online platform, which can host millions of datasets and analyze hundreds and thousands of experiments at a time. However, such platform is still not available to date. Here, we launched FIRMIANA (V1.0) (www.firmiana.org), an integrated cloud Proteome platform that allows proteome identification, quantification, bioinformatics analysis, and knowledge mining in an automated, high-throughput fashion. Up to now, FIRMIANA 1.0 hosted and processed over 2000 experimental proteome datasets (>400M MS spectra) from 20 laboratories around the world, facilitating real-time parallel proteome comparison and bioinformatics analysis for hundreds of datasets, simultaneously. As a big data platform, FIRMIANA 1.0 is connecting to other databases to achieve multi-omics integration of life sciences, bridging bench side to knowledge. We envision that FIRMIANA 1.0 and its subsequential high-performance-computing (HPC) version will play central roles in Proteome study in big data era of lifeomics.

Methods

Data management covers raw data management and metadata management. Core workflow automatically execute a series of operations when integrated MS files are scanned. Quality control is to acquire protein list in specific FDR with selected filters Quantification is to gain quantification area processed on a homemade algorithm. Comparison search can impute missing protein via RT-regression on comparing experiments and then obtain quantitative protein matrix by QC for further analysis. Knowledge extraction from Big Proteome Data

Results and Discussion

We launched the Firmiana to host raw MS files, execute proteome identification/quantification, facilitate bioinformatics analysis, and refine biology knowledge from big proteome data.

Conclusion

The Firmiana has bridged the thorough procedures in proteomics, from wet experiment design to knowledge mining. The whole system was developed with the principles: high capability, big data, extensible, and user-friendly, which largely increases the usability, while decreased the requirement of bioinformatics expertise for users.

Keywords: Proteomics, Cloud platform, Big data, Galaxy

MP06-13

Analysis of the specific molecular networks of cancer stem cells using a novel data integration tool iPEACH

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Introduction and Objectives

Recently, the integration of multiple omics has been expected to be a powerful method for understanding the global biological systems and giving important information of clinical application. However, processing the voluminous data which are described with different formats arose from each analysis with different systems, highlighted the need for a mining system for data integration. Previously, we created the iPEACH (Integrated Protein Expression Analysis Chart, PCT/JP2011/58366, Mol Cell Proteomics 2013) to integrate information from several analysis types into a useful data file. In this study, iPEACH was used for the study of the cancer stem cells (CSC) to understand the cancer regulation mechanism and network involved in CSC maintenance/ differentiation.

Methods

To study the dynamic changes at the molecular level in glioma stem cell clones (GSC) upon their differentiations, protein and mRNA extracted from GSC clones cultured in the stemness-sustaining or differentiation-inducible medium. Using these extracts, proteome differential analyses were performed with combinational methods by the iTRAQ (8 Plex) and 2D-DIGE (PI 4-7 and 3-10). For mRNA differential analysis, DNA microarray (affimetrix human U133.2) was used. All the data were integrated into one chart by iPEACH, and the molecular list rearranged by the iPEACH score was subjected to GO and network analyses using KeyMolnet and KEGG.

Results and Discussion

Proteome differential analysis with the iTRAQ and 2D-DIGE methods identified 5028 (TripleTOF 5600), 3164 (TOFTOF 5800), 4332 (QSTAR Elite), and 190 (2D-DIGE PI4-7/3-11) proteins, respectively. mRNA differential analysis quantitatively identified 21857 genes. After the integration of all data into one chart by iPEACH, the molecular list was rearranged by the iPEACH score. The top 200 molecules in up or down regulated were subjected to GO and network analyses. The results revealed that during the GSC differentiation, GSCs specifically upregulate the molecular signals related to cell motility, neuronal differentiation and cell death such as MAPK, PI3K-mTOR signal, CD44 signal and integrin signal, and downregulated cell cycle, energy production, DNA metabolism and translational controls such as RB-E2F signal, ATM-ATR signal, TCA cycle and glycolytic pathway. These networks identified in this study were mostly unique in the GSC so far reported and may be ideal candidates for targeting gliomas.

Conclusion

These results suggest that the iPEACH, which integrates all of the molecular information

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from omics, is greatly useful to identify new therapeutic targets not only in cancer stem cells but also in any other disease models.

Keywords: glioma stem cells, data mining, mRNA, proteomics, differential analysis, DNA array, 2D-DIGE, network analyses

MP06-14

Workflows for improving mass measurement accuracy in mass spectrometry

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Introduction and Objectives

Scientific workflows are important tools for automation and excellently suited for integrative mass spectrometry and proteomics data analyses. Connecting inputs and outputs of different modules is not sufficient for complete automation of an analysis. Most multi-step processes require decisions to be made by an agent and is often data-dependent. Knowledge level information processing is one approach for automating data analyses with conditional constructs in one or more steps. On a lower level, there should be actions/parameters to be done/used for the conditions defined within a particular domain. Formalized knowledge, ontologies and controlled vocabularies, make such condition-decision making feasible within scientific workflows. We here demonstrate a workflow which combines MS and MS/MS data in order to improve mass measurement accuracy.

Methods

The workflow is created in Taverna Workbench, a scientific workflow management system. The mass spectrometry data are first converted to mzXML. One advantage is that the built-in services of Taverna works well with XML. Secondly, the Proteomics Standards Initiative has produced a controlled vocabulary (PSI-MS CV), which is used to embed metadata on how the datasets were acquired. This information is later used for selecting the correct parameters at each step. Another type of “formalized knowledge” is used for visualization. If datasets are encoded with anatomical (tissue/cell type) origin, the spatial distribution of the protein and gene expression could be brought together and compared visually using an anatomical ontology.

Results and Discussion

The workflow reads the metadata and selects the most optimal parameters for the analysis. The expert user can also apply specific parameters, overriding the defaults. The first module checks all necessary inputs that will be necessary for the analysis and whether the user-specified parameters are valid. If the latter is not the case, the most optimal parameters are chosen for the data based on the metadata. The next module executes an X!Tandem database search on the MS/MS data. An alignment module then aligns MS-only and MS/MS data using a genetic algorithm to fit a piecewise function mapping accurate masses and identified peptides. The retention times are then modified accordingly in the pepXML file. PeptideProphet is then run and a probability value assigned to each peptide-spectrum match. Each MS-only mzXML file is then calibrated using identified peptides in the pepXML file as internal calibrants. Finally, each identified peptide is quantified in each sample/file. When the sample information is provided, the results can be visualized spatially to reveal biologically interesting differences in abundance levels, or unexpected correlation between gene and protein expression.

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Conclusion

Using the ontological hierarchy, we can automate decision making for parameter selection as well as visualization of the results for different levels of abstraction.

Keywords: Mass spectrometry, scientific workflows, ontology

MP06-15

An expert system for evaluating the validity of protein quantification in individual samples analyzed by high-sample-throughput SWATH-MS

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Introduction and Objectives

Protein quantification is a central task in life science research and disease management. This is usually accomplished by the use assays for a specific analyte such an affinity reagent – or mass spectrometry-based assays. Even well established assay may fail in certain samples due to unpredictable biological heterogeneity or sample-specific signal interference. Such instances may lead to incorrect decision making, particularly in clinical settings. The validity of mass spectrometric assays for protein quantification in a particular sample out of large number of samples is often difficult to assess, even though multiple global error models have been developed in computational proteomics. In this study we aimed to develop a computational solution to evaluate the validity of MS-based protein quantification assays for any specific sample, and investigate the causes for failure of such assays.

Methods

We developed a workflow to perform chromatographic extraction using the OpenSWATH/OpenMS pipeline and wrote an artificial intelligence software using expert-system algorithm to evaluate, refine and validate MS assays from high-throughput SWATH/DIA data sets. Its performance was evaluated on data sets containing AQUA peptide mixtures with known concentrations spiked in water, yeast and human cell lysates, respectively (90 SWATH maps), and dataset consisting of NCI-60 SWATH data acquired in duplicate (120 SWATH maps).

Results and Discussion

We defined a set of expert rules that evaluate the validity of peptide quantification in a specific sample by ad hoc examination of all the potential fragments, retention time, peak shape and precursor signals. This was achieved by learning the 90 SWATH maps containing AQUA peptide mixtures with known concentrations spiked in different backgrounds. The software tool demonstrated capability to generate close to 3 million chromatographic plots in fewer than 2 days for both manual and computational inspection of SWATH assay validity.We observed that the same amount of spiked-in peptide generated different signal intensities as a function of changing background. The validity of some assays demonstrated dependency on backgrounds, interfering signals, and the signal-to-noise ratios. We further applied the tool to interpret 120 SWATH maps from the NCI-60 cancer cells, leading to a quantitative data matrix with improved missing value percentage and quantitative accuracy, compared to automated OpenSWATH analyses.

Conclusion

We developed an automated computational tool for evaluating the validity of the SWATH/DIA-based assay in any specific sample, which can be applied to large number

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of SWATH maps with improved performance. We uncovered common causes for failures of SWATH assays in clinical specimens, and obtained unprecedented confidence in determining the absence of a peptide in a particular sample. This led to protein quantification in any sample in a large data set, where applicable, with unprecedented precision.

Keywords: analytical assay; SWATH; expert system; clinic

MP06-16

Integration of Differentially Expressed Proteins and mRNAs with Deregulated microRNAs in Human Glioblastoma: 2-Dimensional Molecular Maps

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Introduction and Objectives

Gliomas are primary tumors of the central nervous system with Glioblastoma multiforme (GBM, WHO Grade IV) being the most aggressive and lethal, still waiting for effective treatment options. Integration of molecular changes observed in these tumors and further exploration to identify regulatory cascades involved in gene expression and downstream cellular processes associated with tumor development and progression are therefore important for translational possibilities.

Methods

We have analysed membrane and nuclear proteome of these tumors using iTRAQ based LC-MS/MS proteomics workflow to identify differentially expressed regulatory proteins and integrated the data with transcriptomic and microRNA data on GBM from The Cancer Genome Atlas (TCGA) resource. This integrated analysis revealed regulatory cascades encompassing microRNAs and their mRNA / protein targets associated with altered tumor- related functions and processes.

Results and Discussion

From a total of 767 differentially expressed proteins identified in the membrane proteome of GBM, 115 were found to be associated with proliferation and invasion, two hallmarks of GBM and were concordant at both transcript (TCGA data) and protein levels. We used these paired entities as targets to identify their regulatory miRNAs altered in GBM, using target prediction programs and/or experimentally verified miRNA target information from miRWalk database. Additionally, the analysis of nuclear fraction revealed a repertoire of 120 proteins that included known RNA or DNA-binding proteins involved in transcriptional, post-transcriptional regulation of gene expression that may be involved in these processes. The integrated view at the level of altered microRNAs (miRNAs) and their mRNA and protein targets of GBM is useful to reveal 2-Dimensional molecular maps of miRNA and their targets with linkages in regulatory and functional dimensions. The regulatory cascades as above including regulatory miRNAs may be explored in the context of hallmark processes such as transcription or post transcriptional regulation, cell proliferation and invasion underlying GBM pathogenesis.

Conclusion

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An approach and schema for the integration of proteomics, transcriptomics data and the regulatory microRNAs is presented that generates 2-Dimensional molecular maps of differentially altered entities linking regulatory cascades in specific functional context. The strategy not only adds strength to the individual molecular entities identified in single ome experiments but permits targeted validation of the cascades to allow identification of a panel of regulatory miRNA that may be involved in a specific function. It has broader implications and can be implemented for a multitude of functional networks and pathways not only for GBM but also tumors in general.

Keywords: Glioblastoma, microRNA, Proteomics, Transcriptomics, multi-omics data integration

MP06-17

DynaPho: a web tool for inferring the signaling dynamics from time-series phosphoproteomics data

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Introduction and Objectives

Phosphorylation-dependent cellular signaling is known to play a diverse role in regulating multiple cellular processes. Recent technological advances in mass spectrometry-based phosphoproteomics have enabled us to globally identify and measure site-specific phosphorylations. Novel and improved computational tools and analysis methods are required to transform large-scale phosphoproteomics data into valuable information of biological relevance. In this study, we developed a web-based tool named “DynaPho” for the analysis of time-series phosphoproteomics data. The goal of this system is to assist non-bioinformatics experimentalists to access and interpret their large-scale phosphoproteomics data.

Methods

DynaPho consists of five analysis modules: (1) phosphorylation profile clustering; (2) time-dependent function enrichment; (3) construction of dynamic protein interaction networks; (4) generation of kinase activity profiles; and (5) construction of kinase-substrate interaction networks using correlation analysis.

Results and Discussion

DynaPho is an integrative platform for uncover the signaling dynamics from time-series phosphoproteomics data. DynaPho provides not only the conventional bioinformatics analyses, such as identification of over-represented functions and motifs, as well as clusters with similar profiles; but also novel advanced analysis methods. One of the advanced methods is for generation of the kinase activation profile by integrating flanking residues and phosphorylation levels of phosphorylated sites to infer the status of kinases at each time point. This profile assists users to identify the key kinases under their experimental conditions. Another method is for construction of time-dependent interaction networks by integrating phosphoproteomics data with protein-protein interaction information. Users can investigate the transition of a signaling pathway via its dynamic network viewer. DynaPho can also construct kinase- and phosphatase-substrate interaction networks using correlation analysis.

Conclusion

Overall, DynaPho is user-friendly web server for analyzing, annotating, and visualizing the dynamics of time-series phosphoproteomics data, and is freely available via http: //dynapho.jhlab.tw/.

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Keywords: phosphoproteomics, signaling dynamics, interaction networks, function enrichment

MP06-18

Analysis Workflow for Quantitative Proteomics, Employing Triplex Dimethyl Labelling and Ion Mobility Assisted Data Independent Acquisition

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Introduction and objectives

Stable isotope labelling can be routinely applied in LC-MS proteomics, for accurate and reproducible quantitative profiling on a large scale. Labels are incorporated metabolically, enzymatically, chemically or by stable isotope labelling. Here we demonstrate a novel informatics processing pipeline for data sets generated using dimethyl chemical labelling, applied in triplex. This method has the advantage of being broadly applicable to any sample type, and has quantitative reproducibility close to that achievable with metabolic labelling. We demonstrate the benefits of using software able to incorporate retention time (tr) alignment and profiling, as well as profiling of ion mobility (IM) drift times (td) to increase confidence in peptide quantification and sensitivity.

Methods

High resolution precursor and product ion LC-MS data were acquired in IM-DIA ((H)(U)DMSe) mode on a SYNAPT G2-Si platform. Data were processed with Progenesis QIp to perform feature detection and quantification, LC-MS map (m/z and tr) alignment and peptide identification. Feature data (quantification; tr and drift time profiles) and associated identifications were streamed to the new Proteolabels plug-in for Progenesis QIp. Proteolabels was used to auto-detect the experimental design, and tolerances for matching in the m/z, td and tr dimensions for grouping differentially labelled features derived from the same peptide. Peptide profiles scoring below 50 (recommended setting) were removed from the analysis, prior to protein grouping and quantification by weighted averaging. Quantitative values were exported to statistical software for further processing and analysis.

Results & Conclusions

To assess the performance of the workflow and informatics, three proteomes, E.coli, S. cerevisiae, and HeLa, were post-digestion labelled with light, intermediate and heavy stable isotope dimethyl reagents, respectively. The HeLa digest was retained at a fixed amount, representing a dominant, non-changing background matrix, whist the E.coli and S. cerevisiaedigest amounts were varied, providing test samples with proteome ratios of 25:25:50, 15:35:50, 10:40:50, and 5:45:50. Replicate measurements were included and technical and experiment-wide quantitative FDR and ROC values determined to

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demonstrate quantitative performance. Moreover, Progenesis QIp affords co-detection across samples and data sets, including both technical and biological replicates, as well as time-course samples, which increased on average the number of detected isotopic clusters by 2.1 fold compared to analysis of data on a single run basis. In addition, the number of quantifiable isotopically labelled peptide pairs by Proteolabels was found to be significantly increased by a factor of 1.8, requiring a minimum of two peptides for quantitation, when compared to commercially available software for the analysis of DDA and DIA data, including IM, with subsequent protein amino acid coverage increase. Retention and drift time profiling applied to triplex dimethyl-labelled samples, to detect, identify and score peptide and protein quantification values.

MP06-19

Bioinformatical analysis of depletion effect on SRM plasma profiling

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Introduction and Objectives

Blood plasma is the "proteome collector" from the whole organism and one of the easiest biological samples to access. It contains the large dynamic range of proteins, whereas low abundant proteins are considered to be clinically valuable. That's why the depletion of highly abundant proteins is a popular method in plasma proteome research. But depletion of abundant proteins can result in concomitant removal of low abundant proteins, as it was shown by A. Yadav et al.. In this study, we were encouraged to estimate depletion effect on chromosome 18 blood plasma proteome while using MARS Hu-14depletion system.

Methods

We used SRM data obtained within targeted quantitative screening of Chromosome 18 encoded proteome in plasma samples of astronaut candidates. For each chromosome 18 protein we analized its concentration in both whole and depleted plasma (in 54 samples). The comparison of the data on protein identification in whole and depleted plasma revealed 59 and 56 proteins identified in the whole and depleted plasma samples, respectively, with 84 proteins identified in total. 31 proteins were common for both types of plasma samples. 19 proteins (from 31) were identified in both, whole and depleted plasma in at least 1 sample. They could be diveded into 3 groups: 1) proteins with slight and unchanged enrichment,2) proteins with apparent enrichment by 1 order of concentrations3) proteins whose concentration decreased by more than an order of magnitude.

Results and Discussion

Lowering the dynamic range of plasma samples by immunoaffinity removal of 14 high abundant proteins using the MARS® Hu-14 system allowed the identification of 28 proteins. On the other hand, we have identified 26 unique proteins in whole plasma. The absence of those 26 proteins from the list of registered in depleted plasma is obviously a result of nonspecific adsorption under immunoaffinity removal of major proteins, such as serum albumin, antibodies, etc. For those 26 proteins and for 19 proteins with depletion effect we analyzed their protein-protein interactions profiles using interactomic resources (STR!NG, Intact etc.).

Conclusion

Bioinformatical analysis of protein interactomic profiles demonstrated, that 16 proteins of chr 18 could interact with abundant proteins from MARS-14. Using GO annotation, we further characterized the subcellular locations of the proteins, quantified with depletion effect, but now significant correlation between protein abundance, its location and depletion effect coefficient was obtained.

Keywords: Bioinformatics, proteomics, SRM, mass-spectrometry, blood plasma

MP06-20

MIAPE-QC: a standard document for quality control of MS data

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Introduction and Objectives

Quality control (QC) is increasingly recognized as a crucial aspect of mass spectrometry based proteomics. As a result, software tools for computing quality control metrics for mass spectrometry data started to proliferate in recent years. However, as the different tools that compute the diverse metrics from experimental LC-MS/MS data can extract only a specific set of implemented QC features, it is a challenging task to compare and integratively analyze the outputs of these tools.To meet this urgent need, the newly launched HUPO-PSI QC standard initiative develops the MIAPE-QC document as one of its key modules.

Methods

As the first step, we have collected and classified a set of LC-MS/MS QC metadata based on the existing works of QC metrics, qcML and QC ontology, as well as additional metrics collected from experimentalists.In addition, a new “wish list” mechanism will be developed to capture richer QC information for possible future induction of new consensus terms to the minimum list, and stronger semantic links of meta terms with QC controlled vocabularies and ontology modules.

Results and Discussion

Similar to the other sister MIAPE guidelines, a minimum set of common denominator QC metadata will be defined in MIAPE-QC for easy communityadoption.As more and more MS data produced by proteogenomics experiments, the scope of metadata will be extended to cover this new approach. The HUPO-PSI QC standard initiative plans to submit the draft MIAPE-QC document to the 2017 PSI spring workshop for discussion and approval.To assist the check of minimum-required QC metadata during data submission, we also plan to develop tools for automated compliance checks in collaboration with the ProteoRed team.

Conclusion

Quality control is playing a growing role in mass spectrometry based proteomics. A standard documentation of QC-specific metadata is required not only to unify the features from different QC metrics, but also to facilitate data exchange and make sensible decisions by using this common set of QC metrics.As one of its key modules, MIAPE-QC

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document will facilitate the use of QC metrics more broadly in the proteomics community and to enable the data exchange and archiving of mass-spectrometry-derived QC metrics together with the community standard format for QC data and its association controlled vocabulary(CV).

Keywords: Quality control, QC metrics, Metadata, MIAPE-compliant

MP06-21

Evaluation of Search Engines for Phosphopeptide Identification and Quantitation

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Introduction and Objectives

Phosphorylation regulation is vital for cellular activity and biological function. However, the identification and quantitation of biological post-translational modifications are challenging for both mass spectrometer (MS) acquisition and proteomics search engines. Fragmentation of phosphopeptides often results in spectra that are dominated by the neutral loss of the phosphate group. Even with new advances in complementary fragmentation techniques, the database search is still accompanied with incorrect assignment and missed identifications. In this study, we evaluated multiple search algorithms for phosphopeptide identification, site localization and quantitation accuracy in large phosphoproteomics.

Methods

HeLa cells were lysed, digested, labeled with TMT10plex reagents, and further enriched for phosphopeptides and analyzed on a Thermo Scientific™ Orbitrap Fusion™ mass spectrometer coupled to a Thermo Scientific™ Easy-nLC™ 1000 chromatograph. The data were analyzed using SequestHT and Byonic as part of Thermo Scientific™ Proteome Discoverer™ (PD™) 2.1 and MaxQuant software 1.5.3.34. A FDR of 1% at the peptide level was used to filter the results. PtmRS was used to calculate the site localization probabilities of all the PTMs in PD software.

Results and Discussion

We compared SequestHT, Byonic and MaxQuant search engines for phosphopeptide identifications, site localization, and quantitation. Byonic software provided the most number of phosphopeptide identifications due to the application of protein-aware FDR (2D FDR)[1]. PtmRS node was coupled to SequestHT and Byonic in the PD platform to calculate the PTM site probability score, which added confidence and validation for the true phosphopeptide identifications. The capability of different search engines to handle different fragmentation techniques, including CID, HCD and ETD were also compared. The results showed that HCD type dissociation with OT detection was favored by all search engines. Relative quantitation of phophoproteomics was achieved using isobaric TMT labeling techniques. Due to distinct algorithms used for the post-acquisition processing and the quantity extraction, different quantitation results were returned from multiple search engines. The quantitation outcome was evaluated against the theoretical values.

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Conclusion

A challenge for this workflow is to achieve accurate quantitation on the highest number of phosphopeptide identification. In addition to the improvement on the instrument methods for data acquisition, a novel database searching method was developed in PD. The new workflow was developed for MS3 searching workflow, but was capable of extracting identification from both the MS2 and MS3 spectra. The novel searching workflow improved the phosphopeptide identifications by 30%, while maintaining high quantitation accuracy.

Keywords: SequestHT, Byonic, Maxquant, identification, quantitation, phosphopeptides

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MP06-22

Implementation of flexible search for proteomics metadata

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Introduction and Objectives

The ProteomeXchange (PX) Consortium (http://www.proteomexchange.org) provides a globally coordinated data submission and dissemination platform for mass spectrometry proteomics data in the public domain, involving the main existing proteomics repositories. The members of the Consortium are PRIDE (https://www.ebi.ac.uk/pride), PeptideAtlas/PASSEL (http://www.peptideatlas.org/passel), MassIVE (https://massive.ucsd.edu), and jPOST, which has just joined the Consortium (http://jpost.org). Public datasets from the different members can be accessed into a common interface called ProteomeCentral (http://proteomecentral.proteomexchange.org). A set of technical and biological common metadata about the datasets has been agreed by the PX members. Although the ProteomeCentral web interface provides a state-of-the-art search functionality, it is not well-suited to construct more complex searches. In the context of 'Linked Open Data', a concept about connecting data independently of the involved biological data types, we chose the Resource Description Framework (RDF) data model to achieve this intended more advanced search functionality, to improve dataset discoverability.

Methods

We first designed an RDF schema for the metadata agreed by the PX members. We then implemented a converter from the existing PX XML format (which contains the common metadata) to RDF using Ruby, with the rdf-ruby application programming interface (https://github.com/ruby-rdf). The converted RDF files were loaded into a triplestore, a data management system for RDF. Although well-known ontologies and controlled vocabularies (CV) were reused to describe concepts and relationships (such as Dublin Core and the PSI-MS CV), we have defined additional ontology terms as needed.

Results and Discussion

As of June 2016, there were 2,300 public dataset entries accessible through ProteomeCentral, which were converted to RDF. Once the converted RDF files were loaded into a triplestore, we were able to successfully search PX datasets by using much more complex queries. One concrete example of the queries that are now possible (among many others) is: "list accession numbers of datasets which were measured by "LTQ Orbitrap Velos" instruments, for "Homo sapiens" samples, and published after 2013. Since we employed the RDF data model, which is globally used e.g. in federated queries, we will not only be able to search for proteomics datasets, but also integrate these searches with datasets from other fields such as genomics, transcriptomics and metabolomics.

Conclusion

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The newly available RDF platform enables users to perform much more complex and flexible query searches, which make PX proteomics datasets more “findable”, increasing the potential for data reuse.

Keywords: Metadata, ProteomeCentral, ProteomeXchange, RDF

MP06-23

QC portal for MRM-MS assay : Unified diagnostic assay guidelines for protein biomarkers

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Introduction and Objectives

Multiple reaction monitoring-mass spectrometry (MRM-MS) became mainstream method for quantitative proteomics, which made the validation of a method and the analyzed data important. In this QC portal of MRM-MS assay, we organized all the guidelines from International Organization for Standardization (ISO), European Medicines Agency (EMA), U S Food and Drug Administration (FDA), Korea Food & Drug Administration (kFDA), Clinical Proteomic Tumor Analysis(CPTAC), Clinical & Laboratory Standards Institute (CLSI) and programed the website to validate if the uploaded MRM-MS analyzed data qualifies as a biomarker assay.

Methods

JSP, HTML, XML, and Java Script were used to develop the webpage. A server was composed of Apache Tomcat, Mysql, Rserve, and Linux as operating system. Input files were skyline-derived output files (csv file), and each files were organized by specific columns in order. SQL, JAVA, and R programs were interworked to evaluate all the categories and show the results.

Results and Discussion

When uploaded with Skyline-output file (csv), results of accuracy, precision, recovery, selectivity, matrix effect, dilution, integrity, reproducibility, sensitivity, calibration, carry over, and stability according to the standards of each independent agency were shown with tables and figures.

Conclusion

This QC assay portal can evaluate if the results of method validation by MRM-MS assay have followed the guidelines provided by each agency, proving effectiveness of a biomarker.This QC assay portal can evaluate if the results of method validation by MRM-MS assay have followed the guidelines provided by each agency, proving effectiveness of a biomarker.

Keywords: MRM-MS, Assay Portal, Method validation, diagnostic assay, QC

MP06-24

Proteomic Cinderella: customized analysis of large amount of MS/MS data in one night

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Introduction and Objectives

Proteomic challenges, stirred up by the advent of high-throughput technologies, produce large amount of MS data. Nowadays even a small biology lab equipped with the state-of-art instruments can become a big-data source. The simplicity of the advanced analysis is overlapped by far with the complexity of interpretation and storage of the information obtained. Routine manual search does not satisfy the “speed” of modern science any longer.

Methods

In our work the necessity of single-thread analysis of bulky data emerged during interpretation of HepG2 proteome profiling results. We searched for proteoforms in 2DE gel separated into 96 cells and analyzed obtained MS/MS data (192 raw files of total volume 114 Gb) over and over again – with variable computing machines, settings, databases, and combinations of search engines.Effective solution for customized search strategy, realized in open-source graphical user interface SearchGUI (http://searchgui.googlecode.com), allowed us to solve this rather sophisticated task. We compared ?ontribution of each of seven search engines (X!Tandem, MS-GF+, MS Amanda, MyriMatch, Comet, Tide, Andromeda, and OMSSA) into total result of proteoforms identification and optimized set of engines working simultaneously.

Results and Discussion

We selected couple of X!Tandem and OMMSA as the most time-efficient and productive combination of search engines and complemented it with Mascot results. We added homemade java-script to automatize our pipeline from files’ picking to reports’ generation. All these settings resulted in rise of the efficiency of our customized pipeline unobtainable by manual scouting: the analysis of 192 files searched against up-to-date configuration of human proteome (129411 entries) downloaded from TrEMBL knowledgebase took 11 hours.

Conclusion

Like Cinderella, separating the wheat from the chaff in one night, smart integration of search algorithms tailored for scientific needs can rapidly translate massive data into biological knowledge.

Keywords: Algorithms, Databases, Tandem Mass Spectrometry, Proteoforms, Proteomics

MP06-25

EBprotV2: Statistical analysis of labeling-based quantitative proteomics data with applications to clinical data

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Introduction and Objectives

We had previously developed EBprot, a method for calculating significance scores of differential expression in labeling-based proteomics experiments. EBprot directly models the distribution of peptide-level ratios and scores each protein based on consistent evidence across the peptides. This approach is distinct from the common practice of assigning p-values to protein-level ratios, which loses reproducibility information. However, EBprot relied on inflexible parametric distributions (Gaussian mixtures) and its R implementation was computationally slow when a dataset contained a large number of proteins (e.g. >10,000 proteins) in a dataset. Here, we present EBprotV2 that considerably improves the original version of EBprot and demonstrate the peptide-centric analysis framework using a recently published breast cancer proteomics data (Tyanova et al. 2016).

Methods

EBprot was rewritten into a computationally fast C++ program (EBprotV2), with flexible semi-parametric modelling. The new model is more robust in handling ratio data which are heavily skewed or multi-modal than the previous parametric model. Moreover, we devised a method to calculate weighted average intensity ratios for individual peptides in a data set with multiple samples (e.g. large sample analysis), accounting for the reproduced detection of all peptides in every protein. This workflow renders EBprot applicable to group comparisons based on multiple samples, similar to the analysis of label-free proteomics data. Our analysis method represents the estimation-driven statistical analysis, rather than the conventional hypothesis testing framework that is unsuitable for untargeted proteomics data with a large number of missing data and notable discrepancies across peptides within the same protein.

Results and Discussion

We applied the tool on a superSILAC data set (Tyanova et al. 2016) with 40 breast cancer tumor samples from three major subtypes (14 ER+/PR+, 15 Her2+, and 11 triple negative (TN)), to identify the proteins that are uniquely expressed in the TN subtype. Using our weighted average ratio derivation, we constructed the peptide ratio data based on peptides with at least 4 samples in all subtypes. For the purpose of comparison, we also derived the protein ratio data by taking the median peptide ratios in each protein. At 5% false discovery rates, we recovered previously characterized TN specific markers such as CMBL and AGR2 as well as HER2 markers, ERBB2 and GRB7, in both protein-level and peptide-level analysis. However, only the peptide-level analysis identified additional negative markers of TN, FOXA1 and INPP4B, in the comparison against HER2.

Conclusion

EBprotV2 is a powerful tool for differential protein expression analysis which utilizes

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peptide-protein hierarchy to score proteins. This tool provides faster computation speed and more flexible nonparametric model than the previous implementation. We also illustrated application to a clinical dataset.

Keywords: EBprot, differential expression, non-parametric, breast cancer

MP06-26

LASH plot: A new tool for clinical biomarker discovery

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Introduction and Objectives

A good graphical display is vital for clear and effective scientific presentation. This is particularly important in metabolome-wide association study (MWAS) or genome-wide association study since they both involve a high volume of data. In GWAS, Manhattan plot is used to present the association between statistical significance i.e., a negative log(p-value) against the genomic position. A similar approach had been used for MWAS in which the genomic position is replaced by m/z ratio for mass spectrometry or proton chemical shift in nuclear magnetic resonance (NMR) spectroscopy. However in clinical laboratory medicine, a significant p-value of a biomarker does not always gurantee its clinical usefulness. Alternatively, the performance of a clinical biomarker is best judged by using the area-under-receiver operating characteristic curve (AUROC). In this work, we will present a novel analysis which provides a 2-dimensional visual display for both the p-value and the AUROC, and we coined it as a “LASH” plot.

Methods

We applied the MWAS data in our previous study of malignant pleural effusion and compared the visual presentation between Manhattan plot and LASH plot. The MWAS data was acquired by LC-MS/MS in both positive and negative ionization mode from 50 patients. 2731 features were detected in positive mode and 3137 features were detected in negative mode. Manhanttan plot was prepared by comparing the -log (p-value) from t-test against the m/z ratio of each feature. Lash plot is prepared by compaing the -log(p-value) againt the AUROC. Both plots were done using Microsoft Excel.

Results and Discussion

Using conventional Manhanttan plot, significant biomarkers can be filtered by using a metabolome-wide significance level (MWSL) at p-value > 2 × 10-5. Using LASH plot, we can filter the data in a 2-dimensional way, (i) according to the MWSL value on the y-axis and (ii) according to the AUROC of each LC-MS/MS feature on the x-axis. Through this method, we can easily identify clincally useful biomarker with a significant p-value and an optimal test performance (usually an AUROC > 0.9). In addition, we observed a "lash-shape" curvilinear relationship between -log(p-value) and the AUROC. Because this graphical presentation had never been presented before and we have coined this novel graphical presentation as “LASH" plot.

Conclusion

We have applied LASH plot in 2 of our studies and successfully identified important clinical biomarkers. We enisage this novel graphical presentation will be very useful in presenting both MWAS and GWAS data.

Keywords: LASH plotMetabolome-wide association study (MWAS)

MP06-27

PPICurator: a System for Extracting Protein-Protein Interaction Information from Literature

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Introduction and Objectives

As an important and challenging issue of biomedical information extraction, protein-protein interaction (PPI) extraction has received lots of attention. Previous researches in this area mainly focused on development of efficient algorithms and great progresses have been made, but there is still much room for improvement. What's more, there is a lack of throughout-assisted curation tool to satisfy the requirement of extracting PPI information and handling large scale corpus. To address these issue, a novel web-based protein-protein interaction information extraction system (PPICurator) is presented here.

Methods

The PPICurator system uses browser/server architecture and consists of four functional modules: Abstract localization & update module, Retrieval & filter module, PPI information extraction module and Output & visualization module. A novel Support Vector Machine(SVM)-based classifier was developed to extract PPI from literature and employed in this system. The SVM-based classifier was trained on four different feature sets - keywords features, POS features, logic features and dependency parsing features and evaluated using standard dataset.

Results and Discussion

The SVM-based classifier reaches a Precision of 81.8%, Recall of 96.4% and F-score of 88.5%, which is the state-of-the-art performance on the LLL05 standard corpus. PPICurator has several outstanding characters different from other systems, such as batch searching, species-based classification of abstracts, PPI scoring, PPI direction & type assignment, PPI annotation extraction and PPI network visualization. It is a complete literature curating platform for PPI information, focusing on usability, simplicity and reliability. Also it offers highly usable interfaces for manual and automatic in-line extraction of PPI information.

Conclusion

The presented result shows that PPICurator is a state-of-the-art solution to assist molecular biologists and biocurators to extract PPI-relevant information from literature: (1) The SVM-based classifier trained on standard dataset and practical data achieved good performance. (2) It offers user-friendly interfaces for manual and automatic extraction of PPI information. (3) It keeps current literature and related resources properly updated in a consistent way. PPICurator is available at http://ppicurator.hupo.org.cn.

Keywords: Protein-Protein Interaction / Literature Mining / PPI Extraction / PPI Annotation Extraction

MP06-28

Critical transition and its regulatory network from chronic inflammation to hepatocellular carcinoma

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Introduction and Objectives

Many recent studies have supported that chronic inflammation contributes to development and progression of various cancers, including hepatocellular carcinoma (HCC). However, molecular mechanisms at a network level from chronic inflammation to HCC have been elusive, especially during the critical transition.

Methods

Here we introduced c-myc tumor-prone transgenic mouse model infected by woodchuck hepatitis virus (WHV). By applying our dynamical network biomarker (DNB) model to analyze the time-series proteomic data of WHV/c-myc mice and age-matched wt-C57BL/6 mice.

Results and Discussion

We identified the 5 month of transgenic mice to be critical period of cancer initiation, which is consistent with clinical symptoms during HCC progression. We also detected the DNB genes, which play the critical role in the malignant transition. Especially, two members of DNB, induce dysfunction of arachidonic acid metabolism, which can further activate the upstream of inflammatory response through the inflammatory mediators of transient receptor potential channels finally lead to impairments of liver detoxification and further malignant transition to cancer.

Conclusion

Our results not only open a new way to elucidate the critical transition of the HCC from dynamical and network perspectives, but also might help us to understand the pathogeneses for c-myc-induced hepatocarcinogenesis even for the human-suffered HBV-associated HCC and provide new insights into intervention strategies for preventing from malignancy of chronic hepatitis.

Keywords: Critical transition; Dynamical network biomarker; WHV/c-myc transgenic mouse model; hepatocellular carcinoma

MP06-29

PTMtopographer: a hidden Markov model-based tool for global post-translational modification prediction with in-protein false discovery rate estimation

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Introduction and Objectives

Mass spectrometry (MS) enables proteome-wide detection of post-translationally modified peptides for well-studied modifications such as phosphorylation. However, many biologically important post-translational modifications (PTMs) such as ubiquitination and SUMOylation remain undetected due to the limited detection coverage of MS. Therefore, computational prediction of PTMs is still valuable for the analysis of a general class of PTMs. The existing prediction algorithms can be grouped into sequence motif-based approaches or machine learning methods that exploit physico-chemical properties of amino acids. While many algorithms allegedly perform well, these methods commonly lack a means to estimate false identification rates when applied in a high-throughput manner. To address the critical gap, we developed a model-based tool for PTM site prediction on sliding windows in the proteins sequences, called PTMtopographer.

Methods

In PTMtopographer, a hidden Markov models (HMM) is trained to compute the probability of harboring a desired type of PTM in a sliding window of amino acids. Specifically, we compute the probability that the window contains a bona fide PTM based on the amino acid composition and the average values of physical-chemical properties. The properties include hydrophobicity, dissociation constant (pKa1), graph shape index, polarizability, volume, a-helix frequency, and b-sheet frequency. The posterior probability scores lend themselves into automatic calculation of Bayesian false discover rates, which are calculated for all the sites within each individual protein separately. This allows for objective selection of score thresholds and robust control of false discoveries in the prediction.

Results and Discussion

We evaluated the performance of PTMtopographer in phosphorylation site prediction for serine/threonine (S/T) and tyrosine (Y) and ubiquitination site prediction for lysine (K). We trained the model on the data from the PhosphoSitePlus database, and performed prediction on the entire human proteome of Uniprot database with a two-fold cross validation scheme. Measuring the overall classification performance by the receiver-operating characteristic (ROC), the HMM approach was better than a naïve Bayes classifier but slightly poorer than or equivalent to a logistic regression model and a support vector machine classifier using the same properties. However, our approach was able to estimate the FDR accurately for both types of PTMs, where the benchmark error rate was constructed by proportionately counting the windows that do not contain a candidate site but score above the threshold.

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Conclusion

PTMtopographer is applicable to generic PTM types with flexible specification of model parameters and fast C++ implementation with multi-threading. It also facilitates the user to reference their own experimental data over the prediction results.

Keywords: Post-translational modification, prediction, hidden Markov model, false discovery rate

MP06-30

Evaluation of false discovery rate-controlling strategies for proteogenomic search

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Introduction and Objectives

In a typical proteogenomic search, customized databases comprised of known sequences as well as evidences from genomic and transcriptomic studies are used. Searches against such inflated databases usually result in decreased numbers of identified peptides due to increased scores of random hits, posing a challenge on accurate false discovery rate (FDR) control. Although separate or multi-stage FDR estimation has been recommended, its performance on novel peptide identification has not been thoroughly evaluated, mainly due to the difficulty in confirming the existence of identified novel peptides. In this study, we used spike-in datasets, containing a standard protein mixture in a yeast background, to simulate the performance of various FDR control strategies for proteogenomic search.

Methods

We used two public spike-in datasets for simulation, where 50 and 25 fmol of 48 human proteins from the Universal Proteomic Standard-1 (UPS1) were respectively spiked in per microgram of a yeast background. In our simulated search, the yeast background was regarded as known proteins and the 48 proteins as novel. We downloaded the two datasets (PXD001819 and PXD002370) from the PRIDE database. They were searched against a merged database comprised of 6,752 UniProt yeast proteins, 172,121 UniProt human sequences, 48 UPS1 protein sequences, and 179 common contaminants. Three search tools were used: Comet, MS-GF+, and X!Tandem. Both target-decoy (TD) and a mixture model-based (MB) methods were applied for FDR estimation. In particular, three approaches to FDR control - global, separate, and multi-stage filtering of known and novel peptides - were tested.

Results and Discussion

When the peptide-spectrum-matches (PSM) were determined using 1% FDR threshold, the true FDR for human sequences was 7.34% (10.72% at peptide-level), meaning that the FDR was highly underestimated for novel peptides. At 1% FDR (PSM-level), the global FDR method showed higher sensitivity than the separate and multi-stage methods and also showed higher true FDR than other strategies. When we compared sensitivity of different FDR control strategies at 10% true FDR, the global MB FDR method yielded the highest sensitivity (11.96%). As for search engines, MS-GF+ showed the highest sensitivity (14.67%).

Conclusion

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An estimated FDR for novel peptides in proteogenomic searches were found highly underestimated, resulting in many false positives. We compared six FDR control strategies on three different search results using spike-in datasets in terms of sensitivity and true FDR. While it is suggested that the separate or multi-stage FDR approaches would yield better performance than the global FDR method, the global FDR estimation using the MB approach showed the best performance according to our experiments, which we believe mimic the proteogenomic search, in terms of the ratio between potential novel sequences and known (or random) sequences in the target database.

Keywords: Novel peptide discovery, Proteogenomics, Spike-in data, FDR control

MP06-31

Development of Universal MS Signal Processor for Improved Data Independent Acquisition Performance

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Introduction and Objectives

The data independent acquisition (DIA) approaches have become major alternatives to data-dependent acquisition (DDA) methods for large-scale proteomic studies. In contrast to the sequential detection, selection, and analysis of individual ions in DDA, DIA systematically parallelizes the fragmentation of all detectable ions within a wide m/z range regardless of precursor signal of the peptide precursors, thereby providing excellent quantification and identification performance for complex proteomics sample. Unlike the DDA approach requires fractionation and multiple replicates to improve the proteome coverage, the DIA can have high proteome coverage in a single LC-MS analysis. This greatly facilitate the proteome analysis using large sample sets. However, although the DIA providing an excellent analytical platform for the proteomics analysis, but it still depends on the comprehensive proteomics analysis using DDA approach. In our previous work, a universal MS spectra/signal processor (UniQua) was developed to optimize analysis performance and can integrate the data from different MS types and proteomics approaches into a unified platform. The dynamic calibration function of UniQua can improve the mass accuracy without routine MS calibration. With the use of UniQua, the identification performance was improved in comparison with the vendor optimized software. Both the MS and MS/MS based quantification performance can also be enhanced by the use of UniQua. In this study, the UniQua was applied to improve the DIA on both of the qualification and quantification performances.

Methods

The DDA analyses were preprocessed with or without UniQua for MS/MS database search using MASCOT algorithm. The DDA database search results were then imported into the Skyline and used as peptide library for DIA analysis. After creating the peptide libraries from different mzXML preprocessing workflows, the DIA raw data was converted into mzXML and imported into the Skyline without or with the UniQua preprocessing.

Results and Discussion

Without UniQua preprocessing, the skyline software can identify and quantify approximately 2800 proteins. However, for the same DDA and DIA datasets preprocessed by UniQua, about 3200 proteins can be identify and quantify.

Conclusion

With the use of UniQua, about ~9% more proteins can be identified in DDA analysis. This will increase the performance for peptide spectra library construction.The UniQua improved the DIA protein identification performance by ~12%.The UniQua slightly improved the quantitation accuracy in DIA analysis.This study highlight the importance of spectra processing in DIA workflow for obtaining more reliable and sensitive proteomics analysis result.

Keywords: UniQua, Data Independent Acquisition, Signal Processor

MP06-32

MAGIC-Web: A Platform for Untargeted and Targeted N-Linked Glycoprotein Identification

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Introduction and Objectives

Aberrant protein glycosylation is associated with cancers and other diseases. However, the diversity in glycan structures and site-specific heterogeneity of glycan occupancy make the interpretation of intact glycopeptide tandem mass (MS2) data challenging. Therefore, we have developed MAGIC-web to provide highly accurate and automated untargeted and targeted N-linked glycoprotein analysis. Notably, MAGIC-web is the first web server having such abilities. To be specific, MAGIC-web contains the following four modules: MAGIC (for untargeted analysis), MAGIC+ (for targeted analysis), Reports Integrator, and Glycan Search. MAGIC-web is accessible from http://ms.iis.sinica.edu.tw/MAGIC-web/index.html.

Methods

MAGIC accepts intact N-linked glycopeptide MS2 spectra as input. Spectrum filtering is first performed to filter out non-glycopeptide spectra. To detect the correct Y1-ions, MAGIC is implemented with a novel Y1-ion pattern matching method, called Trident, that detects a triplet pattern corresponding to [Y0, Y1, Y2] or [Y0-NH3, Y0, Y1] from the fragmentation of the core of N-linked glycopeptides without requiring any prior information. Then it generates in silico MS2 peptide spectra by removing B- and Y-ions and assigning the correct precursor m/z to serve as input to a database search engine to search against a large-scale protein sequence database. In contrast, MAGIC+ assigns peptide sequences using user-provided protein sequences by the target-decoy approach, i.e., searching the in silico spectra against a target-decoy database. Specifically, proteins are in silico digested into peptides with $\leqq 2$ miscleavages and only peptides containing the consensus motif are regarded as target sequences. Decoy sequences are then constructed from target sequences.

Results and Discussion

The performance of MAGIC and MAGIC+ was evaluated using horseradish peroxidase (HRP) and large-scale human HeLa cells. The MAGIC-generated in silico

MS2 spectra were searched against the Swiss-Prot database via Mascot. Total of 81 spectra (7 glycopeptides) were identified in HRP and 112 spectra (36 glycopeptides of 26 glycoproteins) were identified in HeLa cells. In MAGIC+, in silico MS2 spectra were searched against customized target-decoy database. For HRP, MAGIC+ accurately determined the glycopeptides in the 81 Mascot-reported spectra and also assigned these peptides to additional 19 spectra. For HeLa cells, we randomly selected 5 out of 26 Mascot-reported proteins to build the database. MAGIC+ successfully assigned the 45 Mascot-reported spectra with correct peptides and 5 new glycopeptides from 4 selected proteins were observed from the dataset.

Conclusions

MAGIC-web offers a user-friendly platform that can help users to process intact glycopeptide MS2 data in batch and deliver accurate results for both untargeted and targeted analyses. MAGIC-web also offers data visualization to support easy result interpretation.

Keywords:

N-linked glycoproteins, mass spectrometry, Y1-ion detection, glycoproteomics

MP06-33

Characterization of a large-scale phosphorylation motifs in human proteome

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Introduction and Objectives

Protein phosphorylation is a post-translational modification that is essential for a wide range of eukaryotic physiological processes, such as transcription, cytoskeletal regulation, cell metabolism, and signal transduction. Although more than 200,000 phosphorylation sites have been reported in the human genome, the physiological roles mostly remain unknown.

Methods

In a previous study we identified 178 phosphomotifs and the evolutionary conservation patterns are described by comparative genomic analysis for the known phosphosites observed in the human phosphomotifs. Our comparative genomic analysis was performed using genomic data of nine species that span from yeast to humans. The present data provide an overview of evolutionary patterns in acquisition of phosphomotifs and relationships between motif structures. By using these data, we investigated kinase substrates associated with phosphoproteins and the evolutionary conservations of kinase groups, and we also analyzed fractions of kinase groups from worm to human genomes.

Results and Discussion

The substrates of AGC kinases showed higher conservation than those of the CMGC kinase family. In addition, we show the correlation between the evolutionary conservation and the distribution of disease related nonsynonymous mutations on the phosphomotifs. Phosphorylation motif structures and the evolutionary conservation of phosphosites were assessed and characterized, indicating the physiological roles of unreported phosphosites. Thus, it is suggested that interactions between protein groups that share motifs would be helpful for inferring kinase–substrate interaction networks.

Conclusion

Finally, our computational methods can be used to elucidate relationships between phosphorylation signaling and cellular functions.

Keywords: Phosphorylation, Evolution

MP06-34

New functionality for the Trans-Proteomic Pipeline: tools for the analysis of proteomics data

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Introduction and Objectives

High throughput LC-MS/MS is capable of simultaneously identifying and quantifying thousands of proteins in a complex sample; however, consistent and objective analysis of large datasets is challenging and time-consuming. Over the past thirteen years, we have continually developed and provided improvements to the Trans-Proteomic Pipeline (TPP), an open source suite of tools that facilitates and standardizes such analysis. The TPP includes software tools for MS data representation, MS data visualization, peptide identification and validation, protein identification, quantification, and annotation, data storage and mining, all with biological inference. We present an overview of the TPP and describe newly available functionality.

Methods

We introduced several new features in version 5.0 of TPP. We have added new tools that extend the utility of the software suite: StPeter performs label-free quantification based on spectral indices, and Kojak searches and validates peptides from cross-linking experiments. The new package also features a simpler, self-contained, and more flexible installation that now includes required 3rd-party software (Perl and Apache), and allows the user to specify installation and data locations. We also provide new support for Apple Macintosh systems. Updated versions of Comet, Lorikeet, and ProteoWizard are included. The build and deployment system has been simplified, making it easier to build on all platforms. Ongoing bug fixes and improvements to the user interfaces have also been made.

Results and Discussion

All of the TPP software tools are available for download under an open source software license and can be installed on Microsoft Windows, UNIX/Linux, and MacOS X. Free email support for the installation and operation of these tools is also available through a popular, community supported listserver, as is a searchable knowledge base.The TPP is a collection of over 30 tools that can be strung together as a pipeline or run individually as needed. The unique fully open source architecture enables other researchers to build and incorporate additional tools to enhance the functionality of the TPP.Over the past 12 months, there have been over 9,000 downloads of the various versions of the TPP source code (which can be compiled on Windows and Linux/Unix operating systems) and the pre-compiled Windows distribution of the TPP tools from our Sourceforge site. Furthermore, over 750 researchers have been trained in the theory and usage of the TPP at the week-long in-depth course presented semi-annually at the Institute for Systems Biology.An easily searchable email distribution list was established as a means to provide support and build a user community around these tools. This list has been quite active

and useful to the end users; there are currently 1,400 members. Thousands of messages have been posted in topics spanning over 2,900 threads, such as installation problems, usage queries, bug reports, and feature requests, among others.

Conclusion

Keywords:

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jPOST: Current Status in 2016

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Introduction and Objectives

The technologies of the mass spectrometry (MS) for the proteomics have been improved dramatically in recent years, and now a large amount of proteomics data are generated more easily and more rapidly. To integrate proteomics datasets generated from various projects and institutions, we have been developing a proteome database named jPOST (Japan ProteOme STandard Repository/Database, http://jpost.org/) since 2015. The jPOST project consists of three topics, the proteomics data repository, the raw MS data re-analysis protocol, and the curated database. Here, we'll report the current status of the jPOST project.

Methods

The proteomics datasets including raw MS data, peak lists, and identified peptide lists are deposited in the repository via the Internet. The deposited and published raw data will be re-analyzed along our developing protocol, and only the high-quality results will be stored in the database.

Results and Discussion

The repository was opened as the first proteomics data repository in the Asia/Oceania area in this May and has already accepted more than 100 projects including 10,000 files and 1,000 GB data size from 13 species, as of 30 June. Additionally, jPOST will join to the ProteomeXchange (PX) consortium (http://www.proteomexchange.org), which is an issuing institution for the global proteomics dataset identifiers (ID). Therefore, the deposited proteomics datasets to jPOST will be assigned with the global common ID numbers through the PX. As regards the re-analysis protocol, we are developing a protocol to obtain more accurate results under the common conditions against all collected raw data by the combination of multiple peak-picking software and multiple search engines to reduce the biases of each software. The database will be developed based on the Semantic Web technology using Resource Description Framework (RDF) data model to publish data more useful in computing. Therefore, we designed the common RDF schema for describing proteomics metadata with the ProteomeCentral (http://proteomecentral.proteomexchange.org), which stores proteomics metadata from various repositories, and we also designed the jPOST original RDF schema to represents peptide and protein identification results.

Conclusion

The repository was released, and the stored raw MS data are growing. To utilize stored raw data, we are continuing the development of the re-analysis protocol and the high-quality proteome database.

Keywords: jPOST, repository, database, RDF

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A virtual-experimental 2DE together with ESI LC-MS/MS as an efficient approach for study of proteome heterogeneity and dynamics

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Introduction and Objectives

Human cells and plasma proteomes are tremendously complex and composed from diverse and heterogeneous gene products (proteoforms). Recent developments in mass spectrometry and systematic approaches (technology and methodology) promise to bring new insights into this complexity. A combination of mass spectrometry with classical biochemical separation technologies is particularly attractive for this systematic investigation. Among biochemical methods, two-dimensional gel electrophoresis (2-DE) is a most powerful protein separation technique that allows not just separating proteoforms but determining their physic-chemical parameters (pI and Mw). In our study, we performed the panoramic analysis of cellular and plasma proteins using a combination of virtual (in silico) and experimental 2DE with high-resolution nano-liquid chromatography-mass spectrometry. This approach is moving proteomics study on the next level of the acquisition of knowledge about proteomes.

Methods

Separation of proteins by 2DE. 2D images analysis by ImageMaster™ 2D Platinum v 7.0. The tryptic peptides separation by chromatography on Agilent HPLC system 1100 Series with following tandem mass spectrometry on Orbitrap Exactive Plus mass spectrometer. Protein identification and relative quantification using Mascot. Virtual (in silico) construction of 2DE maps.

Results and Discussion

To get better impression about diversity of proteoforms in a particular proteome, the graphs were drawn where experimentally measured physico-chemical parameters of proteoforms were plotted against the theoretical (in silico) parameters of corresponding proteins. This approach was applied on several cell lines and plasma proteomes. Additionally, whole gels (not just spots) were analyzed using this approach. This allowed to detecting in a single proteome more than 20000 proteoforms coded by more than 4000 genes. The 3D-graphs showing distribution of these proteoforms including proteoforms of biomarkers in 2DE map were generated. A comparative analysis of these graphs between normal and cancer cells and between cells and plasma was performed. This analysis showed a high variability and dynamics of proteoforms.

Conclusion

A virtual-experimental 2DE together with ESI LC-MS/MS allows studying heterogeneity and dynamics of proteomes with high efficiency.

Keywords: proteome, inventory, proteoforms, virtual, two-dimensional electrophoresis, mass spectrometry,

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Improved peptide feature detection in the OpenMS software framework.

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Introduction and Objectives

The reliable detection of peptides in liquid chromatography – mass spectrometry (LC-MS) data is a key algorithmic step in the analysis of quantitative proteomics experiments. We present new software tools for the detection and quantification of peptide features in labelled and label-free LC-MS proteomics data, based on vendor-independent data formats. The tools are available as part of the OpenMS software framework and as Proteome Discoverer Community note (Thermo Fisher).

Methods

The FeatureFinderMultiplex tool can detect single peptide features or peptide features multiplets in MS1 spectral data of LC-MS experiments. It is therefore equally suited for the analysis of both label-free and stable isotope labelled experiments. Within an OpenMS analysis workflow, it is often used in combination with two further tools for pre- and post-processing. The SpectraMerger tool applies a simple moving average to subsequent MS1 spectra, suppresses background noise and thereby facilitates the peptide detection. The MultiplexResolver tool is used to post-process the quantitative results. Peptide quantification at MS1 level (using FeatureFinderMultiplex) and peptide sequence identifications at MS2 level (using common search engines such as Mascot, X!Tandem or MS-GF+) are performed independently. Only in a second step are the identifications mapped to the quantified features. The MultiplexResolver tool checks and if necessary resolves any conflicts.

Results and Discussion

The flexibility and modularity of OpenMS workflows allows the user to combine our new tools with powerful algorithms such as MS-GF+, Percolator and the protein inference engine Fido. We present the analysis of both labeled and label-free experimental data from different types of mass spectrometry machines.

Conclusion

By combining the presented new tools with well established algorithms for peptide and protein identification, we designed reliable OpenMS workflows for the analysis of labelled and label-free quantitative proteomics experiments. A single software framework allows the bioinformatics analysis of data from all major mass spectrometer vendors.

Keywords: quantification, peptides, label-free, labelled, SILAC, software

MP06-38

jPOST: repository opened

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Introduction and Objectives

Recent improvements of mass spectrometry have been causing a flood of data in proteomics. In order to facilitate sharing and reuse of the promising data sets, it is important to construct a public data repository being accessible and high quality. Recently, the ProteomeXchange consortium has been set up to provide a coordinated submission of mass spectrometry data set for proteomics to the major proteomics repositories. However, the data transfer to current submission points of ProteomeXchange such as PRIDE in Europe and MassIVE/PASSEL in US via the Internet from Asia/Oceania is usually very slow and highly troublesome. In order to clear these problems, we decided to develop a new proteome repository called jPOST (Japanese ProteOme Standard) repository.

Methods

We have developed the jPOST repository, which consists of a newly-developed, high-speed file upload process, flexible file management system and easy-to-use interfaces. Users can release their “raw/processed” data via this site with a unique identifier number for the paper publication. Users also can suspend (or “embargo”) their data until their paper is published. The file transfer from users’ computer to the jPOST server is very fast (roughly ten times faster than usual file transfer) and uses only web browsers – it does not require installing any additional software.

Results and Discussion

The data repository accepts mass spectrometry raw and processed data for proteomes from all over the world. The function for the assignment of the global common accession number to deposited data will be added in jPOST to join the ProteomeXchange consortium. The repository also stores detailed metadata such as samples, instruments, tools, and their settings into the repository. It also provides peptide and protein views for data sets deposited as a complete submission. Current system has already accepted more than 100 projects including 10,000 files and 1,000 GB data size after the jPOST repository was launched.

Conclusion

We have developed and launched the jPOST repository to share and store proteome data sets for world-wide researchers, and it should be also established as a hub repository in the Asia/Oceania area. The jPOST repository is available at https://repository.jpostdb.org/.

Keywords: jPOST, repository, database

MP06-39

Recognizing millions of consistently unidentified spectra across hundreds of shotgun proteomics datasets available in the PRIDE Archive database

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Introduction and Objectives

On average, 75% of spectra analysed in a mass spectrometry (MS) experiment remain unidentified. We use spectrum clustering at a large-scale to shed a light on these unidentified spectra. The likelihood that sequence variants and peptides with unexpected PTMs have been observed grows with increasing numbers of datasets coming from different origins and experimental settings. The PRIDE Archive database at the European Bioinformatics Institute (EMBL-EBI, http://www.ebi.ac.uk/pride/archive/) is one of the largest MS proteomics public data repositories worldwide. Due to the success of the ProteomeXchange Consortium, the amount of data deposited to PRIDE Archive is growing exponentially.

Methods

The new open-source spectra-cluster algorithm (https://github.com/spectra-cluster) was designed to run in the Hadoop open-source software framework, taking advantage of its parallelisation capabilities. We clustered all identified and unidentified spectra from all publicly available “complete” datasets in PRIDE Archive by April 2015 (256 million spectra, 190 million unidentified and 66 million identified spectra).

Results and Discussion

By clustering all tandem MS spectra publicly available in PRIDE Archive, coming from hundreds of datasets, we were able to consistently characterize three distinct groups of spectra: 1) incorrectly identified spectra, 2) spectra correctly identified but below the set scoring threshold, and 3) truly unidentified spectra. The complete spectrum clustering results are made available through the new version of the PRIDE Cluster resource (http://www.ebi.ac.uk/pride/cluster). Additionally, spectral libraries for 16 species are made available. Thereby, for the very first time, we were able to recognize millions of spectra that are commonly observed in hundreds of proteomics experiments but consistently remain unidentified. Using a multitude of complementary analysis approaches, we were able to identify less than 20% of the consistently unidentified spectra. In addition, we were able to infer identifications for originally unidentified spectra through spectrum clustering.

Conclusion

The redeveloped PRIDE Cluster resource is intended, among other aims, to encourage and simplify further investigation into these unidentified spectra. To our knowledge, this is the first time that the clustered version of a complete proteomics repository is made

available. Thereby, the described three fractions of spectra can be readily recognized and investigated at a repository scale.

Keywords: Computational proteomics, spectrum clustering, unidentified spectra, data repositories

MP06-40

Proteoforms space odyssey: strategy of scouting

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Introduction and Objectives

An important factor that limits the study of the proteome is its heterogeneity i.e. an abundance of various nsSNP, splice-forms and post-translational modifications (including modifications activating and inactivating a protein) of proteins. Necessity for identification and investigation of alternative splicing (AS), single amino acid polymprphisms (SAP) and post-translational modifications (PTM) at the protein level is determined by the influence of these processes on the level of expression and functional properties of proteins.

Methods

To perform the study on assessment of human proteins (proteoforms) species diversity it was carried out a comprehensive research of gene expression for HepG2 cell line based on transcriptomic data and proteoforms read from these genes using proteomic methods (2DE and mass spectrometry). Classic 2DE analysis was improved via separating gel into a grid with many cells containing the corresponding protein spots and further MS analysis of each cell.

Results and Discussion

Transcriptomic analysis of HepG2 cell line resulted in exome-specific proteoform catalogues, containing 52 thousand amino acid sequences, encoded by 12 thousand genes. 2DE proteomic profiling of HepG2 cell line with further mass spectrometry analysis allowed to discover over 30 thousand proteoforms encoded by 4 thousand genes. Herewith only 18 thousand different proteoforms are available to be visualized by means of gel electrophoresis. Primary analysis of the combined transcriptoproteomic approach results revealed its promising outlook. As exemplified by human chromosome 18 we managed to describe 38 of 228 detected proteoforms encoded by 50 genes of this chromosome.

Conclusion

Obtained results consist not only of evaluating (the prediction of existence, descriptive characteristics and selective experimental validation) of proteoforms implemented at the protein level, but also of improving experimental approaches to cells and tissues proteotyping. For example, our approach gives opportunity to identify unique proteins from the proteins group detected with the same peptides set.

Keywords: Proteoforms, Transcriptome, Proteome, Human, RNA-Seq, 2DE, MS

MP06-41

Targeting Peptidoforms via SWATH-MS: Quantification of PTM Variability in Human Blood Plasma

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Introduction and Objectives

A large fraction of the proteins that constitute a proteome are modified and their characterization has illustrated the association between post-translational modifications (PTMs) and their biological functions, signaling mechanisms or diseases. Mass spectrometry (MS), more specifically tandem mass spectrometry of (tryptic) peptides of proteins is the technology of choice for the systematic detection and quantification of PTMs. Recent developments in data-independent acquisition (DIA) and peptide-centric data analysis strategies enable more consistent peptide quantification but cannot distinguish closely related peptide species (or peptidoforms) without manual validation. Here we present IPF (Inference of PeptidoForms), a novel algorithm for the automated confidence assessment of peptidoform detection for DIA using the peptide-centric approach. We demonstrate the application of the algorithm to detect and analyze the biological importance of human blood plasma protein PTMs based on a longitudinal study of pairs of monozygotic and dizygotic twins.

Methods

IPF generates and tests different hypotheses based on low- to high-confidence annotated spectral libraries from spectrum-centric analyses. Using unique and shared transitions, the confidence of the modification type and site-localization for each candidate peptide signal is propagated by a Bayesian hierarchical model from individual transitions to peptidoforms.

Results and Discussion

We applied IPF to study the quantitative heritable and environmental effects on 4,322 peptidoforms carrying ten different plasma-associated modification types in a longitudinal study of pairs of monozygotic and dizygotic twins. This provided insight into the heritable components of ApoE SNPs and the longitudinal components of fibronectin asparagine deamidation and ApoA1 tryptophan oxidation. IPF was further validated and benchmarked using a synthetic gold standard dataset consisting of biologically representative phosphorylated peptides. We found the error-rate control to be accurate, providing sensitive detection and site-localization for the phosphorylated peptides, reaching a sensitivity of 70% at 5% peptidoform-level FDR.

Conclusion

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Mediated by SWATH-MS and IPF, the consistent quantitative data on peptidoform-level enabled us to assess the effects of the variance components on specific modified peptides in human blood plasma proteins on a large scale for the first time. The generic applicability and scalability of the approach will enable adaptation for a wide range of studies requiring quantitative peptidoform assessment.

Keywords: DIA, SWATH-MS, OpenSWATH, PTM, peptidoform, quantitative variance decomposition

MP06-42

Reproducible Protein Quantification with TRIC: An Automated Alignment Strategy for Comprehensive Data Matrices in Targeted Proteomics

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Introduction and Objectives

Large scale, quantitative proteomic studies have become essential for the analysis of clinical cohorts, large perturbation experiments and systems biology studies. While next-generation mass spectrometric techniques such as SWATH-MS have substantially increased throughput and reproducibility, ensuring consistent quantification of thousands of peptide analytes across multiple LC-MS/MS runs remains a challenging and laborious manual process. We present a novel software that can align hundreds of SWATH-MS runs and apply it to over 200 blood plasma samples, quantifying more than 580 proteins.

Methods

To produce highly consistent and quantitatively accurate proteomics data matrices in an automated fashion, we have developed the novel TRIC software which utilizes fragment ion data to perform cross-run alignment, consistent peak-picking and quantification for high throughput targeted proteomics using SWATH-MS. TRIC uses a graph-based alignment strategy based on non-linear retention time correction to integrate peak elution information from all LC-MS/MS runs acquired in a study. The chosen alignment strategy uses a globally optimal guidance tree which results in minimal alignment error since every alignment step is local and performed between two highly similar runs (as opposed to aligning all runs against a more distant reference run).

Results and Discussion

When compared to state-of-the-art SWATH-MS data analysis (OpenSWATH), the algorithm was able to reduce the identification error by more than 3-fold at increased recall, while correcting for highly non-linear chromatographic effects. In a direct comparison, TRIC achieves higher recall (85 % versus 59 %) at lower error rate (0.3 % versus 3.8 %) than DIA-Umpire on a manually curated SWATH-MS dataset, thus highlighting the benefits of using targeted fragment ion information for identification and alignment. TRIC is scalable to a large number of samples, tolerant to outlier runs and applicable to heterogeneous experimental conditions. We apply TRIC to a set of

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232 unfractionated blood plasma LC-MS/MS runs in order to quantify the variability of proteins in blood plasma in a human twin population. Using TRIC, the data matrix occupancy increased by 42%, achieving over 75% quantified values, enabling us to quantify over 580 proteins (up from 342 proteins using a naive approach) across all samples and decomposing the variability of plasma protein protein abundance into heritable, longitudinal and environmental components.

Conclusion

The TRIC algorithm is the first of its kind, performing alignment on targeted proteomics datasets using the fragment ion data directly. This study demonstrates the importance of consistent quantification in highly challenging experimental setups. TRIC is the last missing piece in a pipeline for automated analysis of SWATH-MS datasets and displays consistent performance and scalability across small and large scale data sets (hundreds of injections).

Keywords: SWATH-MS, DIA, Targeted Proteomics, Software, Alignment

MP06-43

Proteomic Data Sharing by the "ProteoMap Online"

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Introduction and Objectives

Proteomic data sharing is useful among users who handle similar type of samples such as serum or urine. We have developed a web based tool for sharing basic LC-MS/M data for an exchange and standardization of proteomic data. The software can visualize the LC-MS/MS data as 2D image. The MS/MS spectrum data and information of protein identification will be displayed on the 2D image.

Methods

User can upload their LC-MS/MS data (.raw file), MS/MS spectrum as .mgf file, and list of identified proteins as xmal file format by the desk top version of ProteoMap software. Then ProteoMap Online will display integrated MS data which can be shared on Web.

Results and Discussion

This online tool enables a comparison of personal proteome data with the standard reference data and provide an information on protein identification with MS/MS spectrum. We will provide our proteomic data through the ProteoMap online and hope to collect data from users to facilitate proteomic data sharing online. Users can access to the "ProteomMap Omnline" web site and download local tool for data visualization.

Conclusion

The "ProteoMap Online" enables proteomic data collection and presentation on Web. The collected data can serve a reference for peptide elution profile and obtained MS/MS spectra in 2D-image.

Keywords:

MP06-44

A protein quantification modified algorithm for the improvement of tandem mass spectra usage

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Introduction and Objectives

Resolution of the mass spectrometer has been improved with the development of mass spectrometry technology which dramatically enhance the detection of proteins qualitatively and quantitatively. Currently, more than hundreds of thousands of peptide fingerprints can be detected from one sample. However, a great part of mass spectra without the corresponding tandem mass (MS/MS) spectra is ignored in the traditional qualitative tandem mass spectra analysis which significantly affect the quantitative detection of proteins.To improve the usage of mass spectra, we developed a systematic method called Label-free Protein quantification Modified Algorithm (LPMA).

Methods

The workflow of LPMA is as follows: Firstly, LPMA generates a corresponding theoretical peptide library with theoretical digestion of the protein library. Secondly, LPMA obtains all the Extracted ion chromatogram (XIC) in RT (retention time) axis and m/z (mass to charge ratio) axis from the mass spectra. LPMA then establishes mapping pattern between the peptide library and XIC vector by isotopic distribution and retention time. At last, an optimization model is proposed to obtain the best quantitative results under the mapping relationship above.

Results and Discussion

Our result shows that about 10% more peptide segments can be identified for subsequently quantification by LPMA than traditional quantitative methods.

Conclusion

Our method uses the rest undetectable Mass Spectrometry (MS) spectra without MS/MS spectra. It is not necessary for LPMA to build its own AMT (accurate mass and time tag) database that AMT approach requires for. In contrast, LPMA only needs MS spectra and standard reference protein sequence database, eg Refseq, Uniprot, Ensembl.LPMA can complement the existing quantitative approaches and is based on the qualitative result of traditional MS/MS spectrometry search engine such as Mascot, Tandem and SEQUEST.

Keywords:

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Rapid and Efficient Quantification of Proteins Using Routine Proteomics Identification Workflows

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Introduction and Objectives

Techniques to quantify peptides for proteomics workflows span a range of stringencies from simple spectral counting through to clinically acceptable isotope dilution SRM assays. Both ends of this range suffer from problems due to either lack of precision and reproducibility for spectral counting methods or technical complexity due to SRM assay development time, validation and quality assurance.Simple, facile and automated methods now exist to quantify hundreds of proteins in complex mixtures by extracting high resolution MS1 precursor (120K FWHM) extracted ion scan chromatograms that are then automatically correlated with high or low resolution MS/MS data across multiple sample sets.

Methods

Using the software package Pinnacle™ (Optys Tech, Brighton MA) LC-MS/MS data are automatically time aligned and XIC's for ^{12}C, ^{13}C & double ^{13}C isotopes for each identified peptide's precursor ion are automatically extracted and quantified using multiple normalization schemes. Furthermore multiple proteomics data types can be accommodated including SRM, PRM, HR/AM MS, DIA or mixed data from multiple vendors. Scoring for MS and MS/MS accuracy, reproducibility, MS/MS quality, MS1 isotope ratios of experimental vs. theoretical, %CV and t-tests between groups are all automated but can be changed for optimization by the user if required.

Results and Discussion

We will present the applications of Pinnacle to quantify many hundreds of proteins from various enrichment schemes (co-IP, BioID). We have found that this method approaches the precision of SRM (if not the sensitivity) but with the ease of spectral counting methods. An important intermediate step in the Pinnacle workflow is the ability for the analyst to examine thousands of XIC's in a graphical context that allows for stringent quality control over the resulting SRM-like data. Furthermore, we will compare and contrast the precision of XIC based quantification with spectral counting especially with the lower end of the count range.

Conclusion

Using the software package Pinnacle has allowed us to quantify several hundred proteins from moderately complex biological samples such as BioID and co-IP of protein complexes. The simplicity, automation and precision of the workflow allows for us to quantify proteins using qualitative methods data (DDA, IDA) with much better precision and dynamic range than spectral counting and with results similar to SRM albeit without the absolute sensitivity.

Keywords: peptide, protein, quantitation, SRM, MRM, XIC, precursor ion, spectral counting, qualitative, automation, MS1

MP06-46

High quantification efficiency for targeted proteomics using Q-TOF instruments and sophisticated data processing

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Introduction and Objectives

Understanding the dynamic of the proteome requires confident identification of proteins, but primarily depends on very accurate detection of differences between samples using quantitative proteomics. Quantification strongly relies on accurate and reproducible measurements of peptide and protein ratios, especially in the case of label-free proteomics. Data-independent acquisition (DIA) mass spectrometry workflows are gaining increasing interest especially for targeted quantification workflows as they overcome the stochastic selection of peptide precursors in typical data-dependent approaches (DDA). We here present the application of DIA approach on a Q-TOF instrument for accurate quantification and improved identification in a complex sample.

Methods

Protein digest from human HeLa cell line cultures (Pierce, 200ng) were spiked with the Biognosys HRM Kit and measured on an impact II Q-TOF instrument (Bruker Daltonics). All samples were measured using a 90min gradient. Targeted analysis of DIA data was performed using Spectronaut (v.9, Biognosys, Switzerland). Spectral libraries were generated out of six DDA runs (Top20 method) with MaxQuant (v 1.5.3.30) and Spectronaut using default settings. False discovery rates are estimated using the mProphet method (Reiter et al. 2011). To increase the precision of quantification the interference detection algorithm and cross run normalization as implemented in Spectronaut was used.

Results and Discussion

In a preliminary experiment we acquired six DDA runs using relatively low sample amounts for generation of a spectral library. In total, build library consisted of ~20,000 unique peptide precursors resulting in ~3000 protein groups. Library content will be steadily increased by acquiring additional DDA runs in future experiments. Furthermore we will investigate the applicability of public available libraries. For DIA runs mass spectrometer cycled between a MS1 full scan and a number of consecutive MS2 scans covering the full precursor range of m/z 400 – 1200. Resolution of investigated Q-TOF instruments is independent of acquisition speed and thus constant for MS and MS/MS acquisition. On average 95% of the peptides present in the library could be identified in single DIA runs. Quantification resulted in on average log2 ratio of 0 which is expected as non-changing populations were analyzed. All peptides showed a median CV of below 16% for the six runs analyzed.

Conclusion

Results clearly display suitability of DIA approach for in-depth quantitative proteomics on

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Q-TOF instrument in combination with sophisticated data processing using Spectronaut software.

Keywords: DIA, Q-TOF, label-free, impact II, Spectronaut

MP06-47

FeatureFinderIdentification: Targeted feature detection for data-dependent shotgun proteomics

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Introduction and Objectives

Label-free quantification of shotgun LC-MS/MS data is a primary approach in quantitative proteomics, but computationally non-trivial. Given a set of confident peptide identifications ("IDs") derived from MS2 spectra, the aim is to accurately quantify as many of the peptides as possible. The central data analysis step is the detection of peptide-specific signal patterns, called features. Missing values due to imperfect feature detection are a common problem, as are false-positives caused by overcompensating imputation (since not all peptides may be present in all samples of an experiment). Therefore, a feature detection approach that targets identified peptides (minimizing missing values), and that also offers robustness against false-positive features (by assigning meaningful confidence scores), would be highly desirable.

Methods

We developed a new feature detection algorithm within the OpenMS software framework, leveraging ideas and algorithms from the OpenSWATH toolset for DIA/SRM data analysis.We further generated a validation dataset: UPS2 standard spiked into E. coli protein digest in several concentrations, analyzed on an Orbitrap Velos.

Results and Discussion

Our algorithm, FeatureFinderIdentification ("FFId"), implements a targeted approach to feature detection based on information from identified peptides. Significantly, it distinguishes between "intrinsic" and "inferred" IDs when analyzing data from each particular sample of an experiment. Based on the overlap between both groups, two sets of (presumed) true-positive and true-negative feature candidates are defined. An SVM classifier is then trained to discriminate between true and false candidates, and is subsequently applied to the "uncertain" feature candidates from "inferred" IDs, facilitating selection and confidence scoring of the best feature candidate for each peptide.In our validation, FFId reached almost complete (99.9%) quantification coverage of "intrinsic" IDs at competitive accuracy. The evaluation of the feature classification strategy for "inferred" IDs is currently on-going, with promising preliminary results.

Conclusion

We present a novel feature detection algorithm that targets identified peptides and provides statistically meaningful confidence scores. Based on preliminary results, we believe that our approach constitutes an important step towards tackling the "missing value problem" that is often associated with label-free quantification of shotgun proteomics data.

Keywords: bioinformatics, machine learning, shotgun proteomics, label-free quantification, feature detection

MP06-48

An efficient and accurate feature-based label-free quantification software tool for SWATH MS data

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Introduction and Objectives

Data independent acquisition (DIA) mass spectrometry is an emerging method for label-free quantitative proteomics. Intensity-based and spectral counting are two approaches in label-free quantification and the former is usually superior in terms of accuracy. Intensity-based label-free quantification relies on accurate retention time alignment across multiple runs. Here we propose a novel label-free quantification tool for SWATH (a type of DIA) data to obtain fold changes of feature pairs.

Methods

In this new method, the first step is retention time alignment of multiple SWATH runs, using a novel hybrid (profile-based and feature-based) retention time alignment algorithm we have developed for SWATH data. Alignment of MS2 spectra, which is uniquely possible with DIA data, is performed to validate the alignment, using an optimized scheme to combine significant peaks from MS2 spectra of different acquisition windows. Next, an accurate and efficient feature detection method is adopted to extract feature pairs, without peptide identification. Finally, the quantification results of feature pairs are exported, and follow-up analysis can be focused on the significantly changed features.

Results and Discussion

Unlike existing targeted quantification methods such as OpenSWATH (Röst et al., Nat. Biotechnol. 2014, 32, 219-23), our method achieves quantification of feature pairs without prior identification, whereas existing methods require the pre-determination and identification of targets. There is also no need to spike in standard peptides for retention time normalization. We validated our method by comparing our quantification accuracy against OpenSWATH using a “gold standard” dataset acquired on samples of synthetic peptides spiked into various background at known concentration ratios.

Conclusion

Our proposed method can generate fold changes of feature pairs efficiently and accurately without the prior knowledge of peptide identification. This untargeted approach is useful for large scale screening of potential biomarkers from biological samples. Our method is implemented in Python and will be released as a free and open-source software tool.

Keywords: mass spectrometry, label-free quantification, data independent acquisition, SWATH MS, feature detection, retention time alignment

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jPOST: Re-analysis Protocol

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Introduction and Objectives

For the development of a comprehensive database, such as a proteome database, it is necessary to analyze very large scale datasets. It is thus inevitable to collect datasets from multiple resources and to perform multiple testing to reduce the false discovery rate (FDR). Hence the proteome integrated database jPOST project, initialized in 2015, independently analyze published mass spectrometry datasets (so called “raw data”) and store the analysis results in its database. The purpose of “re-analysis protocol,” this developing protocol for our own analysis, is to remove false positive assignments by analyzing all datasets with unified methods and criteria.

Methods

The developing protocol has two processes; one is to increase the obtained number of search assignments, and the other is to improve the quality of assignments, namely, to reduce the false positive assignments. For the former, which is mainly presented in this presentation, multiple peak picking software (Proteome Discoverer, ProteoWizard, MaxQuant) and database search engines (Mascot, X!Tandem, Comet, MaxQuant) are used. These assignments of mass peaks to peptides can be examined by the correspondence of amino acids in assigned peptides and mass peaks in MS/MS spectra; this examination can be utilized for the latter process of the developing protocol.

Results and Discussion

We examined the search results with multiple peak picking software and multiple search engines, and confirmed multiple examples that enough strong and clear mass peaks are assigned to peptides only by one software, of which assignments are confirmed by the correspondences of peaks and amino acids in peptides. This may occur in the results of the difference of algorithms implemented search engines, thus more assignments can be achieved with high possibility based on the characteristics of such differences. We also confirmed that some validation processes equivalent to the manual examination of peak-amino acid correspondences can be utilized for the quantitative quality evaluation of the assignments to amino acids; e.g. the assignment of the adjacent amino acid residues in a peptide by b-ions and/or y-ions, which are originally considered as the estimation method of false positive assignments.

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Conclusion

The quantitative evaluation of the correspondences of mass peaks and amino acids in peptides can be utilized as the validation method of the assignment results by the search engines. This validation method can similarly evaluate the characteristics of peptide identification software, consequently both the improvement of the search result confidence and the increase of the identified peptide number can be expected.

Keywords: jPOST, database, reanalysis, FDR

MP06-50

Label Free Quantitation using Peptide Isotope Peak Intensities in Mass Spectrometry

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Introduction and Objectives

Quantitative proteomics requires measurements of protein abundance among multiple samples in a high-throughput manner. Results from such measurements provide information on how biological systems respond to environmental perturbations at proteomics scale. A number of methods have been developed for quantitative proteomics to obtain high proteome coverage, accurate quantitation, and wide applicability to different types of samples. Here, we report rapid and simple method of quantitative proteomics with focus on label-free quantitation, namely, three isotope quantitation (TIQ) combining intensity of three isotope peaks (M, M+1, and M+2) at the three highest MS1 spectral points[1]. We compared TIQ and other label free approaches using spectral counts or chromatographic peak area. As well as accuracy, precision, and reproducibility, quantification performances including dynamic range were tested in the NanoLC-MS/MS analysis of standard proteins. Moreover this method was applied to discover proteins showing abundance changes in T-cell of patient with atopic dermatitis. [1] Gun Wook Park., et. al; Scientific Reports (2016) 6, 21175.

Methods

All protein samples including T-cell were digested with trypsin. Then, peptides were analyzed by reversed-phase liquid chromatography (RPLC) coupled to Orbitrap MS with CID-MS/MS fragmentation. Peptides were identified against the human database including thirteen standard proteins by using IP2, integrated proteomic pipeline. Identified peptides were automatically quantified by TIQ using the combined intensity of three isotope peaks at three highest MS spectral points.

Results and Discussion

: Label free quantitation by TIQ was validated with standard protein, Enolase, spiked at different concentrations in protein standard mixture solution. In comparisons to quantitation using chromatographic peak area, TIQ exhibited good linearity (R^2 >= 0.99). However, it allows high-speed and fully automated quantitation because TIQ requires no peak area generation from the extracted ion chromatogram(XIC). TIQ also showed better R^2 >= 0.95 and wider dynamic range (10f-1600fmol) compared to the spectral counts method. We applied TIQ for the quantitative proteomic analysis of T-cell enriched from patient with atopic dermatitis. As a result, we found that the 141 and 86 proteins were increased in IAD and EAD, respectively.

Conclusion

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For automated label-free quantitation of the identified peptides, we developed TIQ method using combined intensity of three isotope peaks (M, M+1, and M+2) at the three highest MS1 spectral points. We have highlighted that TIQ allows high-speed quantitation showing similar accuracy with results obtained from chromatographic peak area method.

Keywords: Label free Quantitation, Atopic dermatitis.

MP06-51

Diffacto: a robust and accurate quantification-centered proteomics method for large-scale differential analysis

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Introduction and Objectives

In comparative shotgun proteomics, the objects of measurements are proteolytic peptides, while the objects of interest are proteins. The transition from the measured peptide abundances to the presumed protein expression levels is nontrivial and has not been sufficiently studied. With our new approach named Diffacto, we aim to improve peptide-to-protein transition utilizing the high statistical power of signal correlations in large-scale proteomics datasets. Diffacto detects and removes unreliably measured quantities while aggregating peptide-level information, which makes protein inference and quantification more accurate and robust.

Methods

Peptide ion signals should respond linearly to protein concentration changes, but while the majority behave as expected, there are many outliers present in proteomics data. These deviating peptide signals, if used for estimation of the protein expression level, will reduce the accuracy of analysis, and thus are better disregarded. To this end, a Bioconductor factor analysis package FARMS designed for microarray data was implemented in Diffacto within the proteomics context. The covariations of peptide signals in multiple experiments were estimated, and factor loadings were used as quality thresholds as well as weighting factors in linear regression for estimation of protein abundances.

Results and Discussion

Diffacto was applied to a published dataset acquired from two groups of bladder cancer patients by two complementary quantification strategies, iTRAQ and LFQ. Compared to the original analysis, Diffacto yielded greater overlap and fewer conflicts between the two approaches, which enabled a pathway analysis revealing significant differences in the ribosome and spliceosome machinery as well as in glutathione metabolism.For a full-scale test, we generated a set of 63 high-resolution LC-MS/MS analyses of 20 different mixtures of BSA, human and yeast proteome digests, quantified over 38,000 peptides belonging to more than 4000 protein groups using our DeMix-Q workflow. Diffacto rejected 13% of unreliable peptides with weak correlation to protein concentration (average R=0.31). Aggregating signals from covariating peptides, we obtained a highly accurate protein-level summarization with average R=0.98 between the quantification and the actual protein concentrations. Diffacto represents a further development of the quantification-centered proteomics approach embedded in the DeMix-Q workflow. The central tenet of this approach is that peptide abundances, rather than their sequence identities determined via MS/MS, are the cornerstones of the proteomics analysis.

Conclusion

The emergence of large-scale proteomics data unlocked the power of correlation between

MP06-51

the peptides from the same protein. Adapting the transcriptomics factor analysis for proteomics data, Diffacto identifies and rejects poorly correlated peptides, yielding accurate, sensitive and robust protein quantification.

Keywords: Bioinformatics, Protein quantification, Covariation, Factor analysis

MP06-52

Optimal integration strategy of multi-engine MS spectra search results

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Introduction and Objectives

Multiple search engines based on various models have been developed to search MS/MS spectra against a reference database, providing different results for the same dataset. How to integrate these results efficiently with minimal compromise on false discoveries is an open question due to the lack of an independent, reliable and highly sensitive standard.

Methods

Here, we took the advantage of the translating mRNA sequencing (RNC-seq) result as a standard to evaluate the integration strategies of the protein identifications from various search engines. We used 7 mainstream search engines (MaxQuant, Mascot, OMSSA, X!Tandem, pFind, InsPecT and ProVerB) to search the same label-free MS datasets of human cell lines Hep3B, MHCCLM3 and MHCC97H.

Results and Discussion

These algorithms exhibited similar identification power alone. As expected, the union of 7 engines resulted in a boosted false identification, while the intersection of 7 engines remarkably decreased the identification power. We found that identifications of more than 3 out of 7 engines resulted in increased true positives while effectively reduced false identification. Furthermore, this strategy also significantly improves the peptides coverage in the protein amino acid sequence.

Conclusion

In sum, we demonstrated a simple strategy to significantly improve the performance for shotgun mass spectrometry by optimally integrating multiple search engines, maximizing the utilization of the current MS spectra without additional experimental work.

Keywords: MS/MS spectra search engines, translating mRNA sequencing, true positives, false identification, peptide coverage

MP06-53

CNHPP Data Portal: an integrated framework for proteome-centric pan-omics data

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Introduction and Objectives

Mass-spectrometry (MS)-based high-throughput proteomics technologies have enabled the large-scale examination of proteomes. Proteogenomics studies provide powerful tools not only in facilitating and validating MS-based proteomic discovery, but also offer new functional annotations and evidences to genome and transcriptome. This proteome-centric pan-omics approach is the research strategy taken by Chinese Human Proteome Project (CNHPP). Correspondingly, the project has produced large volumes of omics data, which require a central information infrastructure for them to be properly managed, integrated, annotated, and, more importantly, to be queried and analyzed. We report here the CNHPP Data Portal (http://cnhpp.ncpsb.org), an integrated proteome-centric omics data system, which provides these data services for the CNHPP. The Portal uses modular and advanced big data architecture in the management and integration of ever-increasing data volume and complexity; it employs ontology and rules to strengthen data semantics, and offers feature-rich yet friendly web interfaces for data browsing, searching, and analyses. The CNHPP Data Portal is committed to work closely with ProteomeXchange (PX) consortium and Proteome Standardization Initiative (PSI) in adopting standards and good practices to streamline data flow and sharing. While it is a system primarily developed for CNHPP, it is the key data service component of the Phoenix bioinformatics infrastructure Firmiana, the advanced and integrated open proteomics information system developed and deployed at Bioinformatics Platform of the Chinese National Center for Protein Sciences – Beijing (Phoenix Center).

Methods

Results and Discussion

Conclusion

Keywords: mass spectrometry, data service framework, proteome-centric pan-omics data

TO10-03

The HUPO-PSI Quality Control Working Group: making QC more accessible for biological mass spectrometry

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Introduction and Objectives

Software packages for computing quality control (QC) metrics for mass spectrometry data (e.g. LC-MS/MS data sets) have proliferated in recent years; the challenge in this field is shifting toward informed decision-making based on QC metrics rather than the computation of these metrics. Increasingly common multi-tool analysis pipelines have greatly benefited from the existence of standardized data exchange formats coordinated by the HUPO Proteomics Standards Initiative. To leverage the benefits granted by a community-driven standard format for quality data, and to make QC more accessible for biological mass spectrometry, the Quality Control Working Group was established at the HUPO-PSI meeting in April of 2016.

Methods

The PSI Quality Control Working Group seeks to establish a community-driven standard file format supported by robust APIs with downstream processing and visualization tools. The group hopes to leverage the qcML file format originally proposed by WG co-Chair and OpenMS team member Mathias Walzer (Mol. Cell. Proteomics 2014, 13: 1905). The format received considerable feedback at the 2016 HUPO-PSI meeting, particularly in linking it to HUPO-PSI controlled vocabulary terms. Storage and data access for quality control metrics will be aided through the jqcML library, published by WG Secretary Wout Bittremieux (J. Proteome Res. 2014, 13: 3484). The group intends to work with software teams for existing quality metric tools to support direct output of the draft qcML standard to provide a prototype for its production.

Results and Discussion

Although “shotgun” LC-MS/MS proteomics can assuredly benefit from wider adoption and automation of quality control, other classes of data in proteomics and metabolomics can also benefit from these tools. The working group particularly emphasizes the use of quality control in quantitative proteomics methods, whether by iTRAQ or Data-Independent Acquisition methods, and in metabolomics studies. Producing a standard that is flexible enough to support metabolomics mass spectrometry as well as MALDI / imaging data will require a broader perspective than has dominated quality metric software to date. This WG has members from both communities, ensuring wide applicability of qcML open data standards in a variety of mass spectrometry-based settings.

Conclusion

TO10-03

Quality control will play a growing role in aiding the maturation of biological mass spectrometry as a field. HUPO-PSI seeks to broaden the conversation surrounding quality control in this community. This effort will provide a format definition together with examples and software infrastructure that will enable new research in interpretation of quality control metrics.

Keywords: Quality control, HUPO-PSI, Bioinformatics, Standardization

WO07-04

ProDiGy: a Proteome Knowledge Discovery Gateway

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Introduction and Objectives

The ProDiGy is an online tool for exploring, visualizing, and analyzing proteomic profile datasets from organ, tissue or cell lines.

Methods

Galaxy, JavaScript and HTML5 are used to develop this system.

Results and Discussion

A friendly interface enables researchers to interactively search, filter and download datasets across samples. And several browsers are integrated to provide data visualization, including CAPER, Pathview, SuperHeatmap, Network-view and Hierarchical network. And a galaxy system is used to integrate the database, data analyses and data presentation seamlessly. A canvas is designed for the user customized workflow and three workflow systems are developed for the disease subtyping based on multi-omics datasets, integrative pathway analyses and also the discovery of driven genes. A canvas is designed for the user customized workflow and three workflow systems are developed for the disease subtyping based on multi-omics datasets, integrative pathway analyses and also the discovery of driven genes. Users are allowed to create, edit, share and delete their personalized workflows.

Conclusion

The intuitive Web interface of the portal not only makes CNHPP proteomics and genomics profiles accessible to researchers and clinicians freely and handily, and also thus facilitate biological discoveries.

Keywords: Proteome/ Bioinformatics/ Knowledge Discovery/ Workflow/Visualization

MO06-01

The human immunopeptidome: can big data improve the precision of immunotherapy?

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Introduction and Objectives

The immune system constantly surveys protein expression within a cell through the adaptive immune pathways. This involves the selective binding and presentation on the cell surface of peptides derived from extra and intracellular protein degradation. These peptides are found on the cell surface in complex with molecules encoded by the major histocompatibility complex, a highly polymorphic region of the human genome. In humans these molecules are known as the human leukocyte antigens (HLA). This complex of peptide cargo and HLA molecules is then recognised by clonally distributed T cell receptors expressed on the surface of T lymphocytes. T lymphocytes carry an arsenal of effector mechanisms that can direct both antibody and cytotoxic responses.Co-evolution of the human population with pathogens has lead to the genetic diversification of the HLA molecules such that nearly every individual expresses a unique array of HLA molecules. The study of the peptide cargo of these molecules (termed the immunopeptidome) results in a personalised record of the cellular proteome that can be targeted by immunotherapy in a variety of disease settings.

Methods

We have used both global and targeted mass spectrometry (MRM and SWATH) to study the immunopeptidome of a large number of HLA allotypes. This information can be used to enhance prediction of T cell epitopes and tot tailor immunotherapy in cancer, infectious disease and autoimmunity.

Results and Discussion

The development of the haplodome a large database (>500,000 non redundant peptides) of naturally processed and presented peptides from a variety of HLA molecules will be presented. A general approach to use this information for personalised immunotherapy will be discussed.

Conclusion

Empirical determination of HLA allotype specific peptidomes facilitates future interrogation of patient proteomes to deliver personalised immunotherapy. Mass spectrometry will play a key role in this process in a variety of disease settings.

Keywords: ImmunopeptidomeHLAAutoimmunityallergyCancerVaccines

MO06-02

Quantitative host-pathogen protein network analysis using data-independent acquisition mass spectrometry analysis

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Introduction and Objectives

Many pathogenic bacteria form complex extracellular host-pathogen, protein interaction networks. These networks help the bacteria to evade, exploit and modulate the host's immune system via a wide range of virulence factors. An important challenge in infection medicine is to clarify how these protein interaction networks are formed, organized and how they dynamically change upon changing microenvironments, to develop future treatment strategies.

Methods

Here we present a quantitative targeted mass spectrometry based proteomics strategy to determine the organization and dynamics of host-pathogen protein interaction networks, using the important human pathogen Streptococcus pyogenes as a model system. The strategy relies on both intact bacteria and recombinant affinity tagged proteins as affinity probes followed by quantitative data-independent acquisition mass spectrometry analysis (DIA-MS).

Results and Discussion

The analysis of large cohorts of invasive strains, non-invasive strains and isogenic mutants reveal how S. pyogenes form complex protein interaction networks with human and mouse host proteins and how these interactions are altered between invasive and non-invasive strains. Based on these findings, we selected over twenty bacterial proteins to further increase the resolution of the protein interaction network using affinity purification mass spectrometry analysis, to define a highly interconnected protein-protein interaction network between S. pyogenes virulence factors and human host proteins. We further used the quantitative abilities of DIA-MS to explore interdependencies within the network topology by chemical and genetic perturbation of the network. Finally, the correlation between network organization and virulence was assessed using directed evolution experiments where non-virulent S. pyogenes mouse strains were repeatedly injected in animal models to increase degree of virulence followed by quantitative DIA-MS analysis of the protein interaction network. The results reveal how increased host-adaptation alters the network topology indicating that the organization of the surface associated host-pathogen protein networks can be linked to increased ability to cause severe invasive disease.

Conclusion

The quantitative accuracy accomplished with DIA-MS supports the generation of comprehensive models of the organization and dynamics of host-pathogen protein interaction networks. Follow up experiments based on these models will add knowledge regarding the relationship between bacterial pathogens and protective or pathological immune responses.

MO06-02

Keywords: host-pathogen interaction, bacteria data-independent acquisition mass spectrometry analysis, AP-MS, Streptococcus pyogenes

MO06-03

Proteomic of host-microbiome interactions in a pediatric inflammatory bowel disease inception cohort to identify protein biomarkers

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Introduction and Objectives

Crohn's disease (CD) is one of the two main subtypes (the other being ulcerative colitis (UC)) of inflammatory bowel disease (IBD), a chronic and relapsing inflammatory condition of the gastrointestinal (GI) tract. The cause of CD is unknown, but it is thought to occur through a combination of genetic susceptibility, altered intestinal barrier, dysregulated immune responses and microbial dysbiosis. Pediatric CD represents 25-30% of the CD population, is increasing 5% annually, and Canada has one of the highest rates in the world. Pediatric CD is often more aggressive and extensive than adult CD resulting in long-term health morbidities. There is no cure for CD and treatments are focused on achieving and maintaining remission without relapses. Therapies for CD consist of a combination of corticosteroids, immune modulators, biologics and exclusive enteral nutrition (EEN). Effective therapeutic regiments take time to customize for CD patients. Accurate differentiation between Crohn's disease (CD) and ulcerative colitis (UC) is important to ensure early and appropriate therapeutic intervention. Our objective is to identify proteins that enable differentiation between CD and UC in children with new onset inflammatory bowel disease (IBD).

Methods

Mucosal biopsies and lavages were obtained from children undergoing baseline diagnostic endoscopy prior to therapeutic interventions. Using a super-SILAC-based approach, the proteomes of over 100 pediatric control, CD, and UC patient biopsies were compared. Multivariate analysis of a subset of these (discovery; n=50) was applied to identify novel biomarkers, which were validated in a second subset (validation; n=49).

Results and Discussion

In the discovery cohort, a panel of five proteins was sufficient to distinguish control from IBD biopsies with an AUC of 1.0 (95% CI 0.99 to 1.0); a second panel of 12 proteins segregated CD from UC with in an AUC of 0.95 (95% CI 0.86 to 1.0). Application of the 2 panels to the validation cohort resulted in accurate classification of 96.7% (IBD from control), and 80% (CD from UC) of patients. 116 proteins were identified to have correlation with the severity of disease. I

Conclusion

In summary, this study has identified two panels of candidate biomarkers for the diagnosis of IBD and the differentiation of IBD subtypes to guide appropriate therapeutic interventions in pediatric patients.

Keywords: inflammatory bowel disease, Crohn's disease, ulcerative colitis, host, microbiome

MO06-04

Challenges of biomarker discovery in developing countries: A proteomics investigation to identify unique disease signatures in infectious diseases

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Introduction and Objectives

Biomarkers are useful indicators of specific disease conditions and biological processes. However, their application in clinics is hampered due to several hurdles. These include problems of disease heterogeneity, biological diversity, challenges with validation, clinical trials and many more. Considering over 80% of the global disease burden resides in developing countries, biomarker research should be accelerated to provide improved diagnostics and therapeutics in countries with low-resource settings. Translation of diagnostic biomarkers from bench to bedside requires the establishment of a high-throughput integrated omics platform in combination with quality control procedures in the discovery phase. The storage of biospecimens through biobanking initiatives must also be encouraged. Subsequently, the biomarkers must be validated in large patient cohorts with appropriate controls to evaluate their sensitivity and specificity to a particular disease. Moreover, improvement of biomarker discovery and validation process requires sharing of scientific data among different research groups across the world and advocates the need for non-governmental funds to expedite clinical research, apart from government funding resources. In our study, we have comprehensively investigated the proteome profiles of patients with dengue fever (DF), severe and non-severe vivax (SVM, NSVM) and falciparum malaria (SFM, NSFM) from different endemic regions of India to identify surrogate markers for diagnosis. One of the biggest challenges to effective DF control is the lack of early diagnosis resulting in delayed treatments. Similarly, standard malaria diagnostic techniques such as microscopy and RDT, although robust, fail to easily differentiate VM and FM, obligatory for accurate treatment and control. These factors warrant the need for biomarkers that are unique and specific to these infections for reliable diagnosis.

Methods

Comprehensive quantitative proteomics approaches were used in the discovery-phase; while validation was performed by MRM-based assays, ELISA, and label-free quantification (SPR and octet) in a blinded experiment using samples from a large patient cohort, including SVM patients to identify prognostic markers of severity.

Results and Discussion

A few host proteins such as Serum Amyloid A (SAA) and Haptoglobin (Hp) altered progressively with increase in disease severity and exhibited differential abundance with reverse trends in malaria and dengue fever, while proteins such as Superoxide dismutase, Nebulin, Profilin and Carbonic anhydrase were differentially expressed in SVM patients. To identify unique disease signatures, we also have included a few P. vivax, P. falciparum and dengue viral proteins to the panel of biomarkers for large-scale validation.

MO06-04

Conclusion

This is the first comprehensive proteomics study of multiple infectious diseases for the identification of diagnostic and prognostic markers.

Keywords: Biomarker, diagnosis, MRM assays, infectious diseases

MO06-05

Robust temporal profiling of GRB2 protein complexes in primary T lymphocytes using SWATH mass spectrometry

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Introduction and Objectives

Spatiotemporal organization of protein interactions in cell signaling is a fundamental process that controls the flow of information and drives cellular functions. Although protein-protein interactions play vital roles in living organisms, most systematic exploration of protein interactomes in mammals has been achieved using engineered transformed cell lines. Hence, we still have very little knowledge about the nature and dynamics of such interactions controlling signaling under physiological conditions in primary cells isolated from mammalian tissues. In this study, we assessed whether affinity purification (AP)-SWATH — an advanced mass spectrometry-based workflow — could enable rapid, reliable and precise quantitative analysis of protein interaction dynamics from primary cells. Specifically, we developed mice bearing a genetic tag that permits AP-SWATH analysis of endogenous signaling complexes containing GRB2, a crucial adaptor protein, isolated from primary T lymphocytes.

Methods

We generated a line of gene-targeted mice expressing a One-STrEP-tag (OST) at the carboxyl-terminus of endogenous GRB2 proteins. We isolated CD4+ T cells from GRB2OST mice, lysed the cells before or at various times after activation with anti-CD3 plus anti-CD4 and then isolated protein complexes containing GRB2-OST through the use of Strep-Tactin-Sepharose beads (n=4). Samples were acquired in a SWATH-compatible 5600 Triple-TOF mass spectrometer. AP-SWATH data were analyzed using OpenSWATH and Skyline.

Results and Discussion

The GRB2 interactome identified from primary mouse CD4+ T cells was composed of 79 high-confidence GRB2 interactors, many of them known to be involved in signaling pathways downstream of the TCR. The r value for SWATH-MS quantification was 0.959 from technical replicates using liquid chromatography gradient as short as 30 minute. Notably, 54 GRB2 binders were consistently quantified across the time series in all biological replicates and the median coefficient variation (CV) was ranging from 19.7% to 33.2%. Clustering analysis of the kinetics indicate that ~70% of the quantified interactors were strongly associated with GRB2 ~1 minute post-stimulation. For instance, a subset of 22 proteins involving LAT, VAV, GRAP2, SLP-76 and SOS1 showed maximal association with GRB2 only 30 seconds after stimulation and then disassembled from the bait with different kinetics. Moreover, our AP-SWATH workflow provided a sufficient level of precision and robustness to pinpoint differential GRB2 interaction dynamics between

peripheral T cells and thymocytes.

Conclusion

Our results indicate that AP-SWATH is a precise and reproducible analytical method to quantify high-confidence time-resolved GRB2 protein interactions in primary mouse T cells. This approach could be further applied to expedite the robust profiling of dynamic signaling complexes for key hub proteins in a range of cell and tissue types in vivo to resolve the tissue specific context of cell signaling events.

Keywords: Interactome, primary T cells, SWATH-MS

MP07-01

Extracellular vesicles as potential biomarkers in female patients with suspected adverse effects to the quadrivalent Human Papilloma Virus vaccine

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Introduction and Objectives

In Denmark, around 2000 patients with suspected adverse effects to the quadrivalent Human Papilloma Virus (HPV) vaccine have been referred to 5 hospital centers. In one center, a large group of patients have been investigated for possible biomarkers. Most of these patients were diagnosed with the chronic fatigue syndrome, a syndrome which most clinicians perceive as a functional disease. The aim of this study was to explore the potential of using surface proteins from extracellular vesicles as biomarkers for this illness.

Methods

Plasma from 177 female patients and 96 healthy (vaccinated) female controls were subjected to analysis by a novel protein microarray technology; the extracellular vesicle array (EV Array) (Joergensen et al., 2013, JEV). The array was composed of 29 capturing antibodies targeting well-known EV-associated proteins and proteins found on EVs in various inflammatory and neurological disorders (e.g. Alzheimer's Disease). The capture of vesicles was visualized and recorded using a cocktail of biotin-conjugated antibodies against known EV markers (CD9, CD63 and CD81) combined with fluorescently-labeled streptavidin prior to scanning.

Results and Discussion

The protein profile of the plasma EV´s were analyzed in a multivariate manner with random forests prediction model. The best model, using all markers, resulted in a sensitivity of 0.72 and a specificity of 0.65 (AUC: 0.70). Among the markers with the highest mean importance for the model, we found indicators for thrombocyte activation (CD42b and CD62P) and a marker for neuronal involvement: L1CAM (CD171), a neuronal cell adhesion molecule. These findings suggest an inflammatory neurological disease.

Conclusion

Female patients with suspected adverse effects of vaccination against HPV, have a different composition of EVs in plasma compared with a healthy female control group, of which all subjects were vaccinated. These findings indicate that we are facing a biological state instead of functional disease. Increased incidence of vesicles with thrombocyte activation markers together with an increased incidence of vesicles with the neuronal associated marker L1CAM suggest an ongoing inflammatory neurological disease.

Keywords: Human Papilloma Virus, extracellular vesicles, protein microarray, EV Array, neurology, chronic fatigue syndrome

MP07-02

Digging into meningitis proteome profiles in human cerebrospinal fluid using DIA-MS.

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Introduction and Objectives

Meningitis, the inflammation of the meninges in the brain, is a serious and a life-threatening disease caused by the presence of different microorganisms in the central nervous system (CNS). The severity of the disease, required treatment and disease outcome depend highly on the pathogen in question, and the correct identification of the disease-causing pathogen presents as a critical clinical problem. In this study, our aim was to explore the changes in the cerebrospinal fluid (CSF) proteome in a large cohort of patients with acute bacterial (ABM), viral (VM) or lyme neuroborreliosis (BM) meningitis and to investigate their effect on the host response.

Methods

A total of 137 CSF samples collected by lumbar puncture from patients admitted to the Infectious Diseases clinic at Lund University hospital were subjected to tandem mass spectrometry on Q Exactive Plus (Thermo Scientific) mass spectrometer for generation of a comprehensive spectral library. CSF samples from ABM (n=35), VM (n=21), BM (n=7) and headache controls (n=49) were further quantified using SWATH-like data-independent acquisition (DIA) mass spectrometry analysis. The measured proteins were also assigned to their tissue and cellular origins on the basis of a large human atlas generated in our research group (unpublished).

Results and Discussion

A total of 923 proteins were measured across all samples. Approximately half of all proteins in each disease group were specific to plasma, erythrocytes, lymphocytes and platelets, which can be explained by plasma leakage and host response to an inflammatory environment. Nearly all 82 neutrophil-specific proteins were exclusively found in ABM, showing the critical importance of neutrophil activation in clearance of bacterial infections. The abundance of proteins specific to the brain were decreased in all disease groups, although a few were explicitly found in ABM. This suggests a specific response to bacterial infection from brain resident cells which is further accompanied by markers of cell damage and destruction in the CNS such as 14-3-3 protein gamma, vimentin and cyclophilin A. In the case of VM, an extensive down-regulation can be seen of various proteoglycans, extracellular matrix components and growth factors.

Conclusion

Although it is often difficult in a clinical setting to distinguish between ABM and other types of CNS infections, including those caused by viruses, we have been able to show protein patterns that are specific to disease groups as an effect of the host response. Our findings suggest that by using mass spectrometry, a unique protein profile generated by the

pathogen can be detected and this may lead to early diagnosis and better treatment options. Further investigations are ongoing to elucidate differences in the intra- and inter-species proteome patterns.

Keywords: cerebrospinal fluid, meningitis, mass spectrometry, proteomics, data-independent acquisition

MP07-03

Direct identification of viral epitopes from MHC I molecules by mass spectrometry

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Introduction and Objectives

Persistent infections with viruses such as cytomegalovirus (CMV), human immunodeficiency virus (HIV) or human papillomavirus (HPV) can lead to serious illnesses or cancer development. There are no effective therapies available to permanently eliminate these infections and cure caused diseases. With advances in understanding viral biology and biology of immune responses, one could design therapies by which the immune system is manipulated in order to eradicate the virus or the virus-induced illness. To do so, one potential approach is direct identification of viral antigen-derived epitopes, which are processed and efficiently presented at the surface of infected or diseased cells for immune recognition. These epitopes are, in most cases, of low abundance and therefore difficult to identify. We aimed to develop a nano-UPLC-ESI-MS methodology employing selected reaction monitoring (SRM) for the direct detection of low abundant viral epitopes in the whole cell epitome.

Methods

The methodology was developed first for the detection of human leukocyte antigen (HLA)-A2-restricted HPV16 E6 and E7 epitopes, and then applied to identify HLA-A2 HIV-derived epitopes, and mouse (m)CMV-derived epitopes presented by the mouse major histocompatibility (MHC) I complex H-2D^{b}. First, MHC I-epitope complexes were immunopurified and treated with acid for dissociation of complexes. Next, epitope-containing eluates were subjected to epitope enrichment and purification. Finally, epitopes were analyzed with a targeted highly specific and sensitive nano-LC-MS^{3} approach, where every measured peptide was manually optimized to generate the best possible spectrum.

Results and Discussion

Only the HPV16 E711-19 peptide was previously reported to be presented on HPV16-postive cell lines and tumor samples. We were able to identify the E711-19 peptide and several other HPV16-derived epitopes on the surface of HPV16-transformed cells. Furthermore, the H-2D^{b}-restricted mCMV epitope M45985-993 was successfully detected on the surface of only 1x10^{7} cells, the number of which could be further reduced still allowing reliable identification. Moreover, three low abundant HLA-A2-restricted HIV-derived epitopes were successfully detected on the surface of HIV-transfected cells including, well known highly immunogenic Nef-derived VL10 epitope and newly

discovered yet unreported Gag-p24-derived EY11 epitope.

Conclusion

This work demonstrates that the developed strategy for direct identification of virus-derived epitopes on the cell surface is broadly applicable to various MHC I types and virus-infected target cells. The methodology can be extended to direct identification of any low abundant epitope. In general, directly identified epitopes form a solid base for immunotherapy design.

Keywords: epitope, major histocompatibility (MHC) I complex, human leukocyte antigen (HLA) I complex, cytomegalovirus (CMV), human immunodeficiency virus (HIV), human papillomavirus (HPV), immunopurification, selected reaction monitoring (SRM)

MP07-04

Defective wound healing as observed through clinical and proteome measurements

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Introduction and Objectives

Non-healing wounds are a group of diseases where the skin is refractory to normal wound healing processes. As such, this group of diseases, which includes, venous leg ulcers, diabetic foot ulcers and pressure wounds, are responsible for a significant financial and health burden on patients and healthcare systems worldwide. Critically, the biochemistry that underpins this non-healing state is poorly understood; however, the fluid that exudes from these wounds is rich in proteins that could provide insight into ulcerated skin.

Methods

We have conducted a clinical study that included the collection of biological samples and clinical / psychosocial data over a 24 week period, during which time patients received best-practice care for venous leg ulcers. Proteins within pooled biological samples were first cataloged by shotgun proteomics and then individual samples analysed using SWATH data-independent mass spectrometry to quantify their proteome complement. The resulting data were analyzed within the context of clinical measurements using permutation statistics and receiver operator characteristic analysis as well as a novel greedy aggregate algorithm to identify the impact of individual and specific proteins. Proteins with significant abundance changes were contextualized by gene ontology annotations to enable deeper insight into the key biological processes taking place within non-healing wounds. Candidate prognostic markers were then investigated using ELISA assays.

Results and Discussion

Our investigation identified over 1000 multi-peptide proteins within the wound fluid from chronic wounds. Through SWATH proteome measurements we were able to identify a suite of candidate prognostic markers and key biological processes that may indicate specific underlying issues for a sub-set of wounds and their recalcitrant nature towards clinical care. A number of biological markers that are indicative of the wound healing outcome were also derived from these analyses and their significant changes between healing and non-healing wounds verified by ELISA.

Conclusion

Unravelling the complex biology of non-healing wounds through proteome and clinical data measurements provides some insight into the mechanisms associated with a patient's adverse or positive responses to clinical care. Such information can be developed further to inform clinical practices and enable the meaningful personalisation of wound management.

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Keywords: SWATHWound healingLeg ulcersClinical proteomicsData-independent acquisition

MP07-05

Identifying the endomembrane system and organelle proteomic analysis in protozoan parasite, Trichomonas vaginalis.

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Introduction and Objectives

Protein trafficking to various cellular compartments is one of the major themes in cell biology of the eukaryotes. In the protozoan parasite, Trichomonas vaginalis, only the importation of some metabolic enzymes into hydrogenosomes, the organelle for energy metabolism were studied. Researches on other endomembrane trafficking pathway is rare. In this research, we try to separate crude cytosolic supernatant to multiple fractions by OptiPrep density gradient ultracentrifuge. Isolating the subcellular membrane structures and identify the type of organelle in each density fraction.

Methods

Transmission electron microscopy(TEM), western blotting analysis, LC/MS/MS for proteomics analysis, OptiPrep density gradient ultracentrifuge,immunofluorescence assay(IFA)

Results and Discussion

At first step, scanning all the fractions by western blotting analysis with some organelle marker antibodies for identify specific organelles. We substantially defined the ER, Golgi apparatus and lysosome enriching fractions. Furthermore, two kinds of transcription factor, Myb1 and Myb3 which are involved in gene regulation induced by iron were discovered in some fractions. And TvCyclophilin 1 (TvCyP1), a kind of peptidyl-prolyl cis/trans isomerases (PPIs) that could interact with Myb1 and Myb3 was also found in distinct fractions. This pattern of differential protein enrichment between Myb1, Myb3 and TvCyP1 supposed that they possibly were located at different unknown organelles before interaction induced by iron. Next step, we perform proteomics analysis of fractions by LC/MS/MS in hope of identifying suitable protein markers to define the organelles enriching Myb3. Similar workflows shall be performed for other density fractions Myb1 and TvCyP1. We found most organelle markers that traditionally be used at eukaryotes didn't work on Trichomonas vaginalis. For example, there were numbers of Rab GTPases which can be useful marker of Golgi network exist in these organelles, but no other interacting proteins, like COPI, SNARE, Sec7 domain proteins,… etc. could be found in proteomic data. Finally, we observe the morphology of purified organelle by electron microscopy. One of these fractions was successfully identified as multivesicular bodies (MVBs) enrichment. This was the first time MVBs were purified from Trichomonas vaginalis.

Conclusion

This strategy would prepare the ground for studying trafficking of selected proteins in these compartments under various conditions by western blotting and proteomics analysis. These experiments could be fundamental for further study on endomembrane trafficking of proteins of interests.

Keywords: endomembrane system, Trichomonas vaginalis, density gradient ultracentrifuge, parasite, organelle

MP07-06

In depth proteomic characterization of classical and non-classical monocyte subsets

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Introduction and Objectives

Monocytes are bone marrow–derived leukocytes that are part of the innate immune system. Monocytes are divided in three subsets: classical, intermediate and non-classical that can be differentiated by their expression of some surface antigens, mainly CD14 and CD16. Each subset has a different function and their implication in many of the diseases in which inflammation may play an important role is under very active study. Thus, molecular characterization of these cells may provide very useful to information to understand their biology and pathology.

Methods

We have performed a multicentric proteomic study with pure classical and non-classical populations derived from 12 healthy donors. Classical and non-classical monocytes populations have been isolated by fluorescence-activated cell-sorter from peripheral blood mononuclear cells. classical monocytes were identified as $CD14^{high}/CD16^{-}/CD33^{high}/HLA\text{-}DR^{+}/CD45^{high}/CD3^{-}/CD19^{-}/CD56^{-}$ cells, whereas non-classical PB monocytes were identified as $CD14^{-/low}/CD16^{high}/CD33^{+/low}/$ $HLA\text{-}DR^{high}/CD45^{high}/CD3^{-}/CD19^{-}/CD56^{-}$ cells. Protein extracts were separated on SDS/PAGE and the gel lanes were sliced into 10 pieces. After protein digestion, peptides from each fraction were analyzed by LC-MS/MS in five different laboratories. Protein quantitation was performed using an spectral counting approach.

Results and Discussion

We have identified over 5000 proteins, about half of them in the 5 laboratories, covering 30 % of each chromosome protein content. According to neXtprot database, 100 of these proteins can be considered as missing proteins. After filtering for low abundance proteins, more than 2000 proteins have been quantified by NSAF with a median correlation coefficient of 0.73 among laboratories. Functional analysis showed that classical

monocytes were enriched metabolic proteins involved in hexose and nucleotide metabolism, which is related to the phagocytic function of these cells. On the contrary, non-classical monocytes were enriched in immune system response and organelle organization, which is also related with their T-cell activation and patrolling activities of this monocyte population.

Conclusion

We have performed a multicentric proteomic characterization of the classical and non-classical monocytes populations using relatively simple experimental approach. The high reproducibility of the results obtained in different laboratories demonstrated the robustness of the procedure. The obtained results constitute the first protein abundance catalogue of pure classical and non-classical monocyte populations and its functional analysis supports the established functions of these cell types. The results can be a useful proteomic tool for the study of the biology of monocytes in different pathological conditions serving as a reference set of healthy individuals.

Keywords: monocyte pure populations, label-free quantitation, immune system, inflammation

MP07-07

Quantitative Proteomics Study of Macrophage Kinases after Interaction with Candida albicans

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Introduction and Objectives

Candida albicans is an opportunistic pathogenic fungus that as commensal is harmless, but in immunosuppressed patients these fungi can outgrow and cause symptoms of disease. The key phagocytes that mediate fungal killing are macrophages and neutrophils. For that, a tight regulation of several signalling pathways is required. ATP binding proteins, among them kinases, are an important familiy of proteins that are involved in these signalling pathways of differentiation, immune response and others. Recent developments in mass spectrometric workflows allowed proteome analysis of the macrophage proteins abundance, many of them differentially expressed in processes like inflammation, cytoskeletal rearrangement, stress response and metabolism [1]. Here, we developed a quantitative proteomic approach for the study of human macrophage kinases after interaction with C. albicans cells that allowed the quantification of the proteins involved in the regulation of different signaling pathways.

Methods

For this approach, the human monocytic (THP1) cell-line was used and labelled by SILAC method with switched labelling. Monocytes were differentiatied into macrophages by adding PMA. After incubation of the macrophages with C. albicans cells (MOI 1:1) during 3 hours, the protein lysate was enriched in ATP-binding proteins using the ActivX desthiobiotin ATP probes (Thermo Scientific) kit. Then, proteins were reduced, alkylated and in-gel digested using trypsin. Then, samples were analysed by LC-MS/MS using an LTQ-Orbitrap and the fragment ions extracted for a protein database using Mascot.

Results and Discussion

Overall, 548 proteins were quantified, among them 46 kinases, mainly protein kinases. Regarding the distribution of the proteins ratio, a cut-off of 2% was used to consider proteins more or less abundant in the interaction. This resulted in 24 differentially abundant proteins that are involved in energy production, nucleic acid metabolism, cell morphology and cell death and survival, being three of them kinases (MAP2K2, NME1 and SPRK1). Moreover, we are optimizing a method for studying a specific subset of kinases, by a targeted approach, for a tempo-spatial proteomic study of the macrophage response to Candida cells.

Conclusion

These approaches are crucial for unravelling new proteins involved in the immune response to C. albicans that in the future can be applied in antifungal therapies.This work is supported by FP7-PEOPLE-2013-ITN, IMRESFUN Project. [1] Reales-Calderón, J, et al., (2013), JOP, 91, 106

Keywords: Quantitative Proteomics, Macrophages, Candida albicans , Kinases

MP07-08

Proteomic Study of Monocyte Chemoattractant Protein-1 Induced polarisation

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Introduction and Objectives

The balance of M1 and M2 macorphages are important in vascular disease. Chemokine receptor 2 (CCR2) and chemokines (Monocyte Chemoattractant Proteins, MCPs) are well-established to mediate monocyte recruitment, however, we have recently observed that MCP-1 strongly induced mRNA expression of several M1 polarisation markers with minimal impact on the classical M2 markers in a THP-1 cells. To further characterise the observed polarisation, we performed a global proteomics comparison between THP-1 macrophages treated with MCP-1 versus classical M1 and M2 inducers and a vehicle control.

Methods

A data-dependent acquisition mass spectrometry based label-free quantification was used to compare the global protein profile in THP-1 macrophages treated with M1, M2, MCP-1 and untreated cells. Further, the significantly regulated proteins from the four treatments were validated with targeted mass spectrometry (PRM) and Western blot. Data-independent acquisition mass spectrometry focusing on the significantly regulated proteins was used to determine the EC50 of MCP-1 for our future inhibition experiment. Western blot was used to verify the result. Q Exactive mass spectrometer was used for data collection and MaxQuant, Perseus, Sklyine for data analysis.

Results and Discussion

From the global LFQ proteomics, we identified 7,051 proteins and quantified 5,448 proteins. Statistical analysis (ANOVA, FDR=0.05) identified 202 proteins that show significantly different expression levels. Hierarchical clustering showed MCP-1 samples are more closely clustered with the M1 phenotype samples than the M2 phenotypes samples or the untreated control samples. Among 202 proteins with significant differences between conditions, the protein expression levels in MCP-1-treated cells correlated strongly (r^2=0.91) with those in M1 controls but there is no correlation with M2 or control. Eleven of the M1 markers were validated with PRM and 4 by Western blot. The PRM and Western results are consistent with DDA result. A concentration response was applied to determine the EC50 of MCP-1. From the DIA experiment, statistical analysis showed the pEC50 of MCP-1 is 7.6.

Conclusion

From these results, we conclude that treatment of THP-1 macrophages with MCP-1

induces polarisation towards an M1-like phenotype. Based on our new finding, we postulate that selective inhibition of MCP-1 could potentially stabilise atherosclerotic plaques and prevent rupture by: i) reducing monocyte trafficking per se; or ii) limiting the degree of M1 polarisation of any newly arrived macrophages. From a therapeutic perspective, it is anticipated that selective inhibition of individual chemokines may have less impact than CCR2 receptor blockade on immune function.

Keywords: MCP-1, macrohpage polarisation, proteomics

MP07-09

Proteomic analysis of murine norovirus-infected Raw264.7 cells reveals the induced expression of host immune response proteins

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Introduction and Objectives

Norovirus is a major epidemic cause of acute food-borne gastroenteritis worldwide. Although the public health significance of norovirus pathogenesis has been considered for a long time, little is known about the host response in the basis of proteomic alterations by norovirus infection. This ignorance has been postulated due to the absence of human norovirus culture system. Thus, we attempted to analyze the norovirus-infected host proteome using the murine norovirus and macrophage Raw264.7 cells as a human norovirus surrogate system.

Methods

After the in vitro infection of murine norovirus into Raw264.7 cells, the host cell lysates were taken at the time-point of dpi 0 hour (early phase), 16 hours (mid-infection phase), and 24 hours (late-infection phase) and subjected by the label-free quantitative proteomics using tandem MS analysis (Q Exactive, Thermo Fisher Scientific). Progress of murine norovirus infection was observed by the confocal imaging analysis using immunofluorescence-conjugated norovirus capsid protein.

Results and Discussion

Proteomic profiling and network analysis revealed the up-regulation of STAT1 and STAT2 involved in the infectious disease and identified the interacting proteins such as dedicator of cytokinesis 8 and interferon-induced proteins (IFI35, IFIT1B, IFIT2, and IFIT3).

Conclusion

This preliminary information gives the snapshot insight of understanding norovirus-host interaction and host-immune response system. [This study was performed by the KBSI fund, C36945 to Joseph Kwon and the UST-KBSI cooperation fund, PBF080 to Jong-Soon Choi.]

Keywords: Label-free; Murine norovirus; Quantitative proteomics; Raw264.7

MP07-10

Proteomic analysis of human saliva samples obtained from caries-free and caries-susceptible people

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Introduction and Objectives

Most people worldwide suffer from dental caries. Only a minor part of the population in the age of 30 is caries-resistant and reason of this resistance in unknown. Our study was aimed to proteomics of saliva and to find the differences in the abundances of the responsible proteins in these tissues between caries-resistant and caries-susceptible people. Only few studies compared the protein saliva composition of people with carious teeth and people with no caries (Vitorino, 2006; Preza, 2009). Proteomic analysis of human saliva revealed differences in protein expression according to gender and age (Fleissig, 2009).

Methods

Unstimulated whole saliva samples were collected from healthy females and males volunteers aged between 23 and 38 years (4 caries-resistant and 4 caries-susceptible (more than five dental caries in their oral cavity)). The samples were centrifuged and were divided on two parts of samples: supernatant and pellet and then the proteins of oral fluids were separated by two-dimensional electrophoresis and the resulting protein maps were quantitatively evaluated. Spots exhibiting statistically significant changes were excised and analyzed by nano-liquid chromatography coupled to Q-TOF mass spectrometer (MaXis, Bruker).

Results and Discussion

We detected the significant differences in the protein composition between the samples of saliva separated from caries-susceptible and caries-resistant females and males. We detected for example zinc-alpha-2-glycoprotein with significantly higher expression in the caries susceptible females supernatant and cystatin-SN with significantly higher expression in the caries-free females supernatant, significant differences between the groups.

Conclusion

Our result demonstrates that the observed differences in the protein levels might have influence on the anti-caries resistance. This result should be further verified on a larger group of respondents.

Keywords: human saliva, dental caries, two-dimensional electrophoresis, comparison

MP07-11

Quantitative Proteomic Analysis of Different Clinical Manifestation of Severe Falciparum Malaria Patients

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Introduction and Objectives

Malaria is a life-threatening infectious disease caused by Plasmodium falciparum species. It results in high rate of morbidity and mortality worldwide. The aim of this study was to identify changes in the human serum proteome as an outcome of different clinical manifestations severe falciparum malaria patients and to understand malaria pathobiology.

Methods

In discovery phase of this study, a comprehensive quantitative proteomics analysis was performed on ten biological replicates of healthy control (HC; n = 10), non-severe falciparum malaria (NSFM; n = 10), severe falciparum (SFM; n = 10), severe anemia malaria (SA; n = 3), cerebral malaria (CB; n = 3) and hyperbilirubinemia malaria (HB; n = 3) using gel-free (iTRAQ) technique on Q-TOF mass spectrometry. Potential protein targets have been selected from this analysis, and Multiple Reaction Monitoring (MRM) and ELISA assays were performed to validate the potential target proteins like alpha-1 acid glycoprotein 1, alpha-1-antichymotrypsin, haptoglobin and ceruloplasmin. The FASTA sequences of proteins were used to generate in silico trypsin digested peptides using Skyline. After MRM screening, chromatographic, MS and source temperature conditions were optimized for selected best MRM transitions. In-solution digested samples were run on LCMS-8050 using Shim-pack XR-ODS II column with mobile phase.

Results and Discussion

The alternation in proteome of severe falciparum malaria patient shows the modulation in blood coagulation and complement cascades pathways. Few proteins which inhibit the platelet aggregation like alpha-1-acid glycoprotein 1 ($p<0.05$) were found to be up-regulated in severe anemia and cerebral malaria patients as compared to healthy control. Proteins related to cell adhesion such as alpha-2 macroglobulin, alpha-2HS glycoprotein were found to be down-regulated in cerebral malaria as compared to severe anemia and hyperbilirubinemia. Differentially expressed proteins, including plasma protease C1 inhibitor, haptoglobin, alpha 1-antichymotrypsin, zinc alpha-2 glycoprotein, alpha-2 macroglobulin and alpha 1 acid glycoprotein 1 in different severity levels of malaria could be further validated and investigated for their potential as a prognostic marker for disease severity and to understand falciparum malaria pathogenesis.

Conclusion

This is the first comprehensive quantitative proteomic analysis of severe falciparum malaria patients with different clinical syndromes as compared to non-severe falciparum malaria patients. This study has been performed on ten individual biological replicates

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and potential targets were validated by MRM assay. Analysis of individual patients based on severe complications such as severe anemia malaria, cerebral malaria and hyperbilirubinemia is the first comprehensive analysis to identify prognostic biomarkers, which can monitor the severity of falciparum malaria and understand disease pathobiology.

Keywords: Malaria pathobiology, Severe anemia malaria, Cerebral malaria, Hyperbilirubinemia

MP07-12

Quantitative Proteomic Analysis from Acute to Chronic Stages Hepatitis C Virus Infection by iTRAQ Technology

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Introduction and Objectives

Hepatocellular carcinoma (HCC) is one of the most prevalent and mortal cancer in the world, and 20% to 30% of patients with chronically hepatitis C virus (HCV) lead to liver cirrhosis and liver cancer. Most infected acute HCV people develop chronic HCV easily. Due to genetic variability of HCV, the development of antiviral drugs and vaccines becomes a real challenge. In this study, we aim to investigate the protein profiles of HCV infections by differential proteomics strategy.

Methods

Naïve Huh7.5-SEAP cells were established and infected by hepatitis C virus, then isobaric tags for relative and absolute quantitation (iTRAQ) strategy was applied to investigate the protein profiles in acute and chronic infections. The resulting iTRAQ labeled peptides were fractionated by solution isoelectric focusing (sIEF), hydrophilic interaction liquid chromatography (HILIC) and basic reverse phase chromatography (bRP) column, followed by nano-LC tandem mass spectrometry analysis.

Results and Discussion

A total of 2615 proteins were identified, and 1816 of them were also quantified in both acute and chronic stages. Two-dimensional liquid chromatography technique that employed on iTRAQ labeled peptides provided results with excellent complementarity and orthogonality. Moreover, 78 and 140 differentially expressed proteins were selected in acute and chronic infection phases for GeneGo analysis. As a results, these proteins were found to be associated with cytoskeleton remodeling and cell adhesion.

Conclusion

In this initial differential proteomics analysis of HCV infections, Sec61B and SNX3 which related to secretory lipoprotein secretion, were subsequently confirmed and validated by Western blot. They could serve as novel diagnostic biomarkers for hepatitis C virus infection. We believe this study opened new directions for elucidating the molecular mechanisms of HCV infections.

Keywords: hepatitis C virus(HCV), HuH7.5-SEAP, iTRAQ, HILIC, isoelectric focusing, basic reverse phase chromatography, LC-MS/MS

MP07-13

Serum Proteome and Cytokine Analysis in a longitudinal cohort of Dengue Fever Patients for Identification of Predictive Markers

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Introduction and Objectives

Dengue Virus infection is the arboviral infection, which has been raised as an international public health problem with the largest incidence in tropical and subtropical regions of the world. Clinical manifestation of dengue virus infection ranges from an asymptomatic to a flue like state dengue fever (DF) and to a life-threatening complication dengue hemorrhagic fever (DHF). Our study directed towards the identification of altered human serum proteome as a consequence of dengue fever (in a longitudinal cohort) for studying the viral pathogenesis and host immune responses.

Methods

Initial phase of this study involved quantitative proteomic analysis of dengue fever patients (n=6) at two different time points; after 3-4 days of infection and after 7 days of treatment to healthy controls (n=8) using gel-based (2D-DIGE) and gel-free (iTRAQ) technique using high resolution mass spectrometry instrument. In solution digested samples were fractionated on offgel fractionators and run on 6550 i-Funnel QTOF LCMS/MS (Agilent Technologies, US) with mobile phase containing 0.1% formic acid in water and 90:10% acetonitrile: water in 0.1% formic acid. Data was analysed using Spectrum mill software with SPI% as 60 and 1% false discovery rate for the identification of differentially expressed proteins.

Results and Discussion

Out of several identified proteins, few potential protein targets like Haptoglobin, Apolipoprotein B-100, alpha-1-antitrypsin, Serotransferrin, Vitamin-D-binding protein and fibronectin were selected for MRM assay. The data was also searched for viral proteins against Dengue virus database and few proteins like Non-structural protein 2, Genome Polyprotein, Polyprotein were found in most of the samples. Further, pathway analysis was performed using DAVID software tool and KEGG pathways, and several proteins like fibronectin, Apolipoprotein A-II, Angiotensinogen, Complement C3 were found to be involved in coagulation and complement cascades, ECM- Receptor interaction, Vitamin digestion and absorption. For the very first time we have successfully identified some of the viral proteins like nonstructural protein 2 (NS2) and polyproteins from the host serum samples.

Conclusion

This study described a comprehensive and systematic molecular analysis of serum proteome and their striking association with the functional/physiological pathways from a longitudinal cohort of dengue fever patients. We have extended this study to include the analysis of cytokines in dengue patient sera with healthy controls. The same study needs to be validated using MRM assays for a large biomarker panel evaluation and also to increase the efficiency of biomarker development.

Keywords: Dengue, Longitudinal, Proteomics

MP07-14

Phosphoproteomics and proteomics profiling of macrophage cells infected with dengue virus

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Introduction and Objectives

Dengue virus (DENV) is the most prevalent mosquito-borne human pathogen worldwide. It is responsible for 390 million infections annually, mainly in tropical and subtropical regions, where the vectors, Ae. aegypti and Ae. albopictus, live. Human infection with DENV may be asymptomatic or causes a spectrum of clinical manifestations ranging from self-limiting dengue fever to severe hemorrhagic fever, which can be fatal. To date, there is no licensed protective prophylactic vaccine and no specific antiviral drugs available.DENV host-cell interaction includes many cellular players. The proteins as well as their modifications and the dynamics of the biological processes hijacked during virus replication are only a piece of the puzzle in understanding DENV pathogenesis. In this work, we analyzed the proteins and phosphoproteins change in a human macrophage cell line (THP-1) infected with dengue virus at 24 and 48 hours post-infection (hpi).

Methods

Differentiated THP-1 cells infected and mock-infected with DENV were lysed and digested with trypsin. To identify and quantify the peptides, isobaric Tags for Relative and Absolute Quantitation (iTRAQ) methodology were used, then, 95% of the total peptides were enriched using titanium dioxide chromatography. Subsequently, peptides and phosphopeptides were analyzed by LC-MS/MS.

Results and Discussion

Here, we describe changes in site-specific phosphorylation dynamics in response to dengue virus infection. We were able to identify 206 and 208 phosphoproteins that change in phosphopeptides level at 24 and 48 hpi, respectively, and 143 phosphoproteins that were altered in both conditions. The proteome differences between DENV infected and mock infected THP-1 cells showed few proteins that change in expression, 10 proteins at 24 hpi and 12 proteins at 48 hpi. Gene ontology enrichment analysis showed that the majority of the identified proteins are phosphoproteins involved in Fc gamma R-mediated phagocytosis, endocytosis, cGMP-PKG signaling, cytoskeleton, splicing, MAPK signaling, among others.

Conclusion

Our results help to increase understanding of the macrophage response to DENV infection, also highlight the importance of MAPK signaling in antiviral cell condition.

Keywords: dengue, infectious diseases, proteomics, phosphoproteomics

MP07-15

Multipronged Quantitative Proteomics Analysis Reveal Oxidative Stress and Cytoskeletal Proteins as Possible Markers for Severe Vivax Malaria

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Introduction and Objectives

Even though, vivax malaria is often considered as a benign infection, in recent years several severe case series of Plasmodium vivax infection have been reported from different endemic countries. The exact molecular mechanisms associated to vivax malaria severity; particularly the factors triggering the progression of disease severity is largely a “black-box”. Investigation on the plasmodium induced alterations in human proteome can provide valuable information regarding malaria pathogenesis and host-parasite interactions. In the present study, we have compared clinical parameters and serum proteome profiles of the patients diagnosed with non-severe (n = 166) and severe (n = 34) vivax malaria with healthy community controls (n = 146) from there different endemic regions of India with an intention to identify surrogate biomarker for severity and for a better understanding of the pathobiology of severe vivax malaria.

Methods

In our study, 2D-DIGE and iTRAQ-based quantitative proteomics in combination with ESI-Q-TOF and Q-Exactive mass spectrometry platforms were used in the discovery phase; while differential serum abundance for a few selected proteins were validated further by MRM-based mass spectrometric analysis, immunoassay-based approaches (ELISA), and label-free quantification methods (SPR and Octet).

Results and Discussion

Our findings suggest that Superoxide dismutase, Nebulin, Profilin and Carbonic anhydrase along with 23 other proteins were modulated only in severe vivax malaria; while and a few differentially abundant proteins such as SAA, Haptoglobin, Apolipoprotein A-I and Apolipoprotein E were altered progressively with the increase in disease severity. Several pathways including cytokine signaling, acute phase response, lipid metabolism, oxidative stress and anti-oxidative pathways, cytoskeletal regulation and complement cascades were observed to be altered in the vivax malaria patients. We have demonstrated that an increased abundance of free-radical scavenging and anti-oxidative enzymes can provide a glimpse regarding the higher echelon of oxidative stress in severe malaria patients. Moreover, elevated serum levels of various muscle proteins in circulation indicate the possibilities of muscle damage and microvasculature lesions in severe malaria.

Conclusion

Comprehensive clinicopathological and proteomics analyses revealed activation of the oxidative stress and counteractive pathways, as well as elevated serum levels of cytoskeletal proteins as the possible cues contributing towards severe vivax malaria. Our results suggest that SOD, Vitronectin, Titin, Apo E, SAA, and Haptoglobin are potential

predictive markers for malaria severity. Taken together, this is the foremost comprehensive proteomics study which identified some possible pathogenesis for severe P. vivax infection.

Keywords: Host response; Malaria pathogenesis; Plasmodium vivax; Quantitative Proteomics; Serum marker; Severe malaria

MP07-16

Characterization of specific antibody-peptide sequences in glaucoma sera using discovery proteomic strategies.

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Introduction and Objectives

Glaucoma is a neurodegenerative ocular disease and one of the primary causes of blindness. Recently, our group demonstrated that there exist significant glaucoma-related level changes in naturally occurring autoantibody profiles in human sera and those are correlated with the continuous loss of retinal ganglion cells. For that reason, the major objective of this study was to investigate glaucoma-specific peptides from the variable region of human sera-derived immunoglobulins G (IgG) employing mass spectrometry-based proteomic strategies.

Methods

Individual sera samples were taken from 13 patients diagnosed with primary open-angle glaucoma (POAG, N=13, mean age=60±7) and 15 healthy controls (CTRL, N=15, mean age=56±5). IgG were isolated by anti-Fc (crystallisable fragment) resin beads and subsequently digested into Fab (antigen-binding fragment) and Fc by papain. Fab was further purified from the digested mixture by anti-Fc resin beads followed by In-solution trypsin digestion. Afterwards the purified samples were measured on a liquid chromatography-Orbitrap-mass-spectrometry system (LC-Orbitrap-MS). Peptide identification and label-free quantification (LFQ) were done employing state-of-the-art bioinformatic tool MaxQuant v. 1.5. Identified peptides were aligned to IgG germline sequences using IMGT database (ImMunoGeneTics Information System) and IgBLAST algorithm to annotate complementarity determining regions (CDR) and framework regions (FR) of the variable IgG domain.

Results and Discussion

In total 1699 heavy and 1607 light chain variable peptides were sequenced in CTRL and POAG group with a false discovery rate of < 1 %. Statistical analysis identified 40 peptides of the variable IgG domain to be significantly differently expressed between POAG and CTRL group (p=0.05). Thirteen of these peptide sequences were annotated as complementarity determining regions (CDR1 and CDR3) whereas the other peptides were mainly located within the framework regions (FR1, FR2 and FR3) of the variable IgG domain. Moreover, glaucoma-related changes in the distribution of variable heavy chain families (VH) were observed, indicating that specific (auto-) antigenic pressures influence the clonal selection and expansion process during B cell maturation: VH2 was decreased in POAG compared to CTRL group (p=0.01), whereas VH3 was overrepresented in POAG (p=0.04).

Conclusion

The present study shows for the first time that peptides from the variable region of

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antibodies are associated to glaucoma. These outcomes provide further evidence that mass spectrometry is a powerful tool to sequence Fab of immunoglobulins without the need of previously known antigen panels. However, it is still unknown how exactly these quantitative changes in the antibody-peptide composition influence the pathomechanism of glaucoma. Further studies are of great importance to investigate the participation of autoimmunity and autoinflammation in neurodegenerative diseases.

Keywords: Glaucoma, Autoimmunity, Antibodies, LC-Orbitrap-MS

MP07-17

Delineation of the human Ramos B cell proteome and the substrate network of the protein tyrosine phosphatase 1B by quantitative proteomics

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Introduction and Objectives

A functional interplay of kinases and phosphatases is crucial for every signal transduction process in cells. Dysfunctions of protein tyrosine phosphatases (PTPs) can lead to hyperactivity and autoreactivity of B cells. The tyrosine phosphatase PTP1B is a negative regulator of the insulin receptor and other receptors. Although PTP1B is expressed in hematopoietic cells, little is known about its role in B cell signaling. To study the function of PTP1B, we chose the human Ramos B cell line as model system and established an absolute quantitative map of its proteome. The aim of our study is to delineate the substrate network of PTP1B by quantitative proteomics to better understand PTP1B's function in human B cells. We hypothesize, that the newly identified substrates can be linked to B cell auto-reactivity and autoimmunity. This is based on the observation, that a B cell-specific PTP1B deficiency causes systemic autoimmunity in aged mice.

Methods

We characterized the Ramos B cell proteome by large-scale MS-based proteomic analysis and used histones as a proteomic ruler for absolute quantification. Next, knockout PTP1B Ramos cells were generated by CRISPR/Cas9. We used substrate trapping mutants combined with affinity purification, SILAC, and quantitative MS to identify B-cell specific PTP1B substrates. Novel substrates were validated by proximity ligation assays (PLAs) and co-IPs. To determine the specific site of substrate dephosphorylation, a MS-based phosphatase assay has been developed.

Results and Discussion

We analyzed the human Ramos B cell proteome (about 8,300 proteins) and determined the copy numbers of individual PTPs, including PTP1B, and their counterpart kinases. We further compared our quantitative proteome data with RNAseq data to reveal regulatory events at a posttranscriptional level. Remarkably, the mRNA of PTP1B is very low expressed in Ramos B cells, whereas PTP1B is more abundant at protein level, even exceeding the level of the well-described PTP SHP-1. To study PTP1B's function, we created CRISPR/Cas9-based knockouts. We identified over 15 novel candidate substrates of PTP1B under anti-lambda or CD40 receptor stimulation, potentially contributing to a break in B cell tolerance and leading to autoimmunity. Among our top candidates are proteins involved in BCR signal transduction, surface receptors and

endosomal proteins. We are validating selected candidates in Ramos and primary B cells. We have already identified p38 MAPK as a new PTP1B substrate by co-IP and PLA with Tyr-182 as the specific dephosphorylation site. Our newly developed MS-based phosphatase assay allows to accurately determine the specific dephosphorylation site of new PTP1B substrates.

Conclusion

With our project we significantly contribute to the elucidation of PTP1B's function in human B cells. The newly identified PTP1B substrates provide novel insight into the role of this important phosphatase in autoimmunity.

Keywords: Absolute Quantification Proteomic rulerSILACPhosphatasesPTP1BAutoimmunityB cellsCRISPR/Cas9

MP07-18

Selective modulation of host cellular and mitochondrial proteome during time resolved phagocytosis of MSSA and MRSA Staphylococcus aureus

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Introduction and Objectives

Methicillin-resistant Staphylococcus aureus (MRSA) represents a serious problem for human health because it is highly resistant to antibiotics and it is considered a life-threatening bacteria. In the veterinary field it is a leading cause of infections of some economically significant livestock species and, as a prominent bacterial cause of contagious bovine mastitis, a major economic burden for the dairy cattle industry. During the last decade several livestock-associated MRSA has become one of the possible determinants in livestock-to-human host jumps and should be considered a possible public health threat (Feltrin 2016). Although the majority of investigations has been directed to MRSA characterization also methicillin-susceptible Staphylococcus aureus (MSSA) has to be considered a leading cause of infections, starting from farmed animals to the hospital and clinical setting, more important, represents the global reservoir for the emergence of MRSA following the acquisition of PVL and SCCmec. (Robinson 2005, Monaco 2016). Moreover, Staphylococcus aureus , as extracellular pathogen, has proved capable to immune evasion mainly modulating the autophagic mitochondrial interplay thus facilitating the dissemination of this pathogen (O'Keeffe 2015). The aim of this project is the investigation of the host-pathogen molecular response during the phagocytosis of selected MRSA and MSSA strains using a label free approach with a particular interest to the strain-specific host mitochondrial response.

Methods

Two microarray-genotyped MSSA and MRSA strains has been used for time-resolved phagocytosis using a THP1 cell line. Protein expression changes in MSSA+THP1 and MRSA+THP1 groups have been detected using a label free approach. Protein extracts from infection samples has been analysed in quadruplicate using a SYNAPT G2-Si (Waters) applying the MSE data acquisition strategy coupled to a functional bioinformatics analysis.

Results and Discussion

After MSE data processing forty mitochondrial proteins resulted differentially expressed during time resolved phagocytosis. In particular, seventeen proteins has been detected in both experimental infections and are mainly involved in energetic metabolism and ROS detoxification in phagocyte. Five mitochondrial proteins are exclusively over expressed in MSSA and eighteen in MRSA phagocytosis. Functional bioinformatics analysis

highlighted strain-specific differences mainly in host cell death pathways. In particular, in respect to MRSA, the MSSA strain targeting different host proteins could delay the apoptotic process into the phagocyte and could promote survival and diffusion inside the host.

Conclusion

Those results will provide new data to understand the infection dynamics of different SA strains during the interaction with the host phagocyte.

Keywords: Keywords Staphylococcus aureus , MSSA, MRSA, mitochondria, phagocytosis, apoptosis, label-free MSWork supported by MIUR under grant “Futuro in ricerca 2013” project code RBFR13PIQE_001 to A.S.

MP07-19

Attenuation of Coactosin-like Protein-1 Protects Liver from Steatosis and Injury

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Introduction and Objectives

The prevalence of nonalcoholic fatty liver disease (NAFLD) has increased dramatically, and the underlying mechanisms of the syndrome remain elusive.

Methods

Two-dimensional difference gel electrophoresis (DIGE) was employed to inspect stage-specific protein expression patterns in a methionine-choline- deficient (MCD) diet-fed murine model.GOfact and DAVID online analysis tool was utilized to facilitate function categorization of identified proteins.Small Interfering RNA and Hydrodynamic Tail Vein Injection were used for RNA silencing.One cytoskeleton protein, COTL1 was validated both in MCD/FAD NAFLD models and patient with biopsy-proven hepatic simple steatosis and hepatitis.

Results and Discussion

Conclusion

The COTL1 overexpression was associated with NAFLD. Knock down of COTL1 attenuation could protects liver from steatosis and injury in MCD diet mouse. These findings may help understanding the pathogenesis of NAFLD and providing candidates for diagnosis or drug targets.

Keywords: NAFLD,COTL1; ALOX5; mechanism

MP07-20

Investigation of IgG heavy chain variable region peptides for differential diagnosis of autoimmune pancreatitis and pancreatic adenocarcinoma

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Introduction and Objectives

Autoantibodies (immunoglobulin) have been reported as potential diagnostic or prognostic biomarkers for different kinds of diseases, such as autoimmune diseases, infectious diseases, cancer, and Alzheimer's disease, etc. Specific peptides from immunoglobulin G variable region have been reported as disease related and are potential as biomarkers. In the current study, label free proteomics workflow was applied to the investigation of IgG variable region peptides from patients with autoimmune pancreatitis (AIP) and pancreatic adenocarcinoma (PDAC). Due to the similar imaging features and laboratory parameters, the differential diagnosis of AIP and pancreatic cancer is still challenging. Therefore, we aim to develop a panel of IgG variable region peptides as biomarkers for accurate diagnosis.

Methods

IgG purification was performed with protein G magnetic beads, and parameters including types of beads, beads volume, and incubation time were optimized to achieve high extraction efficiency. Purified IgG samples were introduced to SDS-PAGE and the heavy chain subunits were collected for in-gel digestion. Tryptic peptides from IgG heavy chain were analysed by nano LC-FT-ICR-MS, and the peptides with statistical significance were identified by using Mascot.

Results and Discussion

From the results of data analysis, several variable region peptides were found to be higher for patients with AIP. Summation of five peptide ratios was used to test the potential of using IgG variable region peptides for differential diagnosis of AIP and PDAC in this study. The accuracy in distinguishing AIP and PDAC by using variable region peptide ratios is 83.3 % (n= 30).

Conclusion

The results showed that IgG variable region peptides have potential as biomarkers for specific disease. Our future work is to investigate the peptide profiles and identify the functional autoantibodies for both AIP and PDAC.

Keywords: proteomics, IgG variable region, autoimmune pancreatitis, pancreatic adenocarcinoma

MP07-21

HLA class I peptidedome analysis to identified allergic peptides for drug hypersensitivity

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Introduction and Objectives

Drug allergy has reported to have strong genetic association with human leukocyte antigens (HLA) and the characterization of HLA-bound peptides affected by culprit drugs is of fundamental importance to the pathomechanism of how culprit drug can activate drug-specific T cells and induced immunological reactions. Previously, immunological studies with T cell derived from patients revealed that HLA played the key role to presented allergic drugs to cytotoxic T cells through different pathways. However, the understanding of the pathological immunopeptidome of drug hypersensitivity is not clear. Here, we used mass spectrometric analysis to identify the drug-affecting HLA-bound peptides.

Methods

The cell lysates were collected and the HLA complexes were purified though affinity purification by pan-HLA class I antibody (W6/32). Peptides were analyzed by LS-MS/MS using Orbitrap mass spectrometer and analyed by using Proteome Discoverer software. The post-translational modifications (PTM) of variable modifications including all common PTMs as well as drug modifications were analyzed by PEAKS Q software.

Results and Discussion

HLA-B*57:01 is associated with susceptibility to drug-induced liver and skin injury induced by flucoxacillin and abacavir, respectively. Comparing the peptide repertoire under different drug treatment, we observed the peptide repertoire alteration when treating with abacavir and flucoxacillin; whereas, no alteration was observed when treating with vehicle and controlled drug, penicillin. The P9 anchoring peptide preference was only changed under abacvir treatment (Phe and Trp alter to Lue), and P2 anchoring peptide preference remained under all conditions. Intriguingly, although previous literature reported that flucloxaciilin covalently bound to the methyl esters of N-acetyle-Lys on human serum albumin, we didn't find any flucloxacillin-modified HLA-B*57:01-bound peptides. Instead, we found that flucoxacillin induced significant post-translational modification on HLA-B*57:01-bound peptide, and this phenomenon was not seen under the incubation with other drugs. We found a relatively high proportion of tryptophan oxidized peptides, including dihydroxylation, oxidation and oxidation to kynurenine. Taken together, abacvir and flucoxacillin utilized different mechanisms to affect the peptide repertoire of HLA-B*57:01 to induce drug allergy.

Conclusion

Recent studies reveal that drug-responding HLA present culprit drug to specific T cell and further induced tissue damage, but few studies focus on the role of drug-affecting peptides. Here we used immunocapture and mass spectrometric method to analyze the immunopeptidomics of HLA-eluted peptide from drug treated and non-treated cells. The

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notorious culprit drugs, abacavir and flucoxacillin, affected the HLA-B*57:01 peptide repertoire, which may trigger inflammatory reactions. Further immunological studies were warrant to certify the pathological role of the drug-affecting peptides.

Keywords: HLA, immunopeptidome, drug hypersensitivity

MP07-22

Quantitative shifts in the influenza immunopeptidome reveal the relative contributions of direct and cross-presentation to T cell mediated immunity

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Introduction and Objectives

CD8+ T cells control infection through recognition of viral peptides in complex with major histocompatibility complex class I molecules (MHCI) that are presented on the surface of infected cells and cross-presented on the surface of professional antigen presenting cells. T cell immunity directed against conserved epitopes from internal influenza proteins provides an alternative strategy to current antibody-based vaccines. However, the development of suitable peptide-based vaccines is currently limited by our limited understanding of the factors that control the immunogenicity of T cell epitopes.

Methods

We have used a comprehensive mass spectrometry approach to identify and quantify naturally presented influenza epitopes following direct infection and cross-presentation of viral antigen and correlated this to the CD8+ T cell response in C57/BL6 mice. The identification of viral peptides is based on two methods: 1) information dependent data acquisition (IDA) mass spectrometry on a SCIEX TripleTOF® 5600+ system and 2) Multiple Reaction Monitoring (MRM) on a SCIEX QTRAP® 5500 system which is a targeted analysis for each peptide of interest.

Results and Discussion

In total, 22 viral peptides were identified including the two immunodominant epitopes NP3 66-374 and PA224-233, 11 previously known subdominant epitopes and 8 novel peptides. These peptides were of the expected length for MHCI (8-11mers) and with sequences corresponding to the canonical binding motifs of H-2K^b/D^b except for one novel peptide, HA41-49, found to bind to both alleles.Quantitation of epitope abundance by targeted mass spectrometry (MRM) after direct infection of two different murine cell lines revealed that NP366-374 abundance was the highest (presenting over 4000copies/cell), whereas PA224-233 abundance was the lowest (~10copies/cell). The difference in surface abundance between NP366-374 and PA224-233 is over 400 fold, in stark contrast to the co-dominant T cell response observed towards these two epitopes during primary infection in mice. However, cross presentation data showed a 5-fold increase in PA224-23 3 abundance, whilst the majority of other epitopes were presented in lower abundance compared to direction presentation.

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Conclusion

Our results provide novel insights into the relationship between peptide abundance and virus-specific $CD8^+$ T cell immunodominance hierarchies, with data suggesting that cross presentation may differentially affect the immunogenicity of influenza epitopes. This study holds promise to optimise future antiviral vaccination strategies.

Keywords: Influenza virus, epitope, direct and cross presentation, immunodominance, T cells, mass spectrometry, MRM, IDA,

MP07-23

Interactome analysis of the NS1 protein encoded by influenza A virus reveals a positive regulatory role of host protein PRP19 in viral replication

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Introduction and Objectives

Influenza A virus, which can cause severe respiratory illnesses in infected individuals, is responsible for worldwide human pandemics. The NS1 protein encoded by this virus plays a crucial role in regulating the host antiviral response through various mechanisms. In addition, it has been reported that NS1 can modulate cellular pre-mRNA splicing events. Although NS1 has been reported to interact with numerous cellular molecules implicated in multiple host response, its functions and the mechanism underlying NS1-mediated regulation of cellular mRNA processing have not been fully described to date.

Methods

To investigate the biological processes potentially affected by the NS1 protein in host cells, we systemically explored the NS1 interactome in NS1-expressing cells using immunoprecipitation followed by SDS-PAGE coupled with liquid chromatography-tandem mass spectrometry (GeLC-MS/MS).

Results and Discussion

The cellular proteins identified were found to be involved in the cellular processes of RNA splicing/processing, cell cycle, and protein folding/targeting. Importantly, one of the identified proteins, pre-mRNA-processing factor 19 (PRP19; encoded by the PRPF19 gene), was confirmed to interact with the NS1 protein in influenza A virus-infected cells, and depletion of cellular PRP19 decreased influenza A viral replication.

Conclusion

This study comprehensively profiled the interactome of the NS1 protein in host cells, and the results demonstrate a novel regulatory role for PRP19 in replication of influenza A virus.

Keywords: influenza A virus, NS1, interactome, PRP19, RNA splicing/processing

MP07-24

Temporal regulation of Lsp1 O-GlcNAcylation and phosphorylation during apoptosis of activated B cells

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Introduction and Objectives

O-GlcNAcylation and O-phosphorylation was found to have lots of interplays in many cellular events and even disease progressing, However whether these interplay also play critical role in immune cells it still unknown. Since the signaling events of BCR crosslinking involves plenty of O-phosphorylation signaling cascade, we check whether O-GlcNAcylation and the cross-talk between these two post-translational modification control this process.

Methods

Western / Immunoprecipitation / Flow Cytometry

Results and Discussion

Crossing-linking of B cell receptor (BCR) sets off an apoptosis program, but the underlying pathways remain obscure. Here, we decipher the molecular mechanisms bridging B cell activation and apoptosis mediated by post-translational modification (PTM)We found that B cell activation and apoptosis induced by B cell receptor (BCR) cross-linking were enhanced upon O-GlcNAcase inhibition. The proteome-scale analysis identifies the phosphorylation of lymphocyte specific protein-1 (Lsp1) is sensitive to O-GlcNAcylation accumulation. More importantly, we found the temporal interplay between Lsp1 O-GlcNAcylation and phosphorylation after B cell activation determines B cell survival. O-GlcNAcylation at S209 of Lsp1 was required for PKC-β1 recruitment and subsequent S243 phosphorylation that leads to the following hyper-phosphorylation of Erk kinase and anti-apoptosis gene BCL-2 and BCL-xL down regulation.

Conclusions

Our study depicted the critical interplay between O-GlcNAcylation and phosphorylation of Lsp1 in linking apoptosis and BCR signaling.

Keywords:

B cell / Apoptosis / O-GlcNAcylation and phosphorylation

MP07-25

Inorganic nanomaterials react with tumor infiltrating immune cells to modulate tumor progression

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Introduction and Objectives

Inorganic nanomaterials are used as a delivery system to offer increased drug efficacy and as an immunostimulant adjuvant to activate or enhance immunity for cancer therapy. The tumor infiltrating immune cells, cancer-associated fibroblasts and angiogenic endothelial cells in the tumor microenvironment are associated with tumor progression, invasion or destruction of the tumor cells. The aim of this study was to characterize the effects of inorganic nanomaterials on the regulation of tumor progression.

Methods

We examined the effect of inorganic nanomaterials on tumor bearing mice. After stimulation with inorganic nanomaterials, the tumor infiltrating immune cells were isolated from tumor of tumor bearing mice. The production of reactive oxygen species (ROS) were analyzed by luminol-dependent chemiluminescence assay. The cell population reacts with inorganic nanomaterials were surface stained and analyzed by flow cytometry. The expression level of inflammatory cytokines from the supernatant of tumor infiltrating immune cells was analyzed by ELISA at different time interval of stimulation. The migration ability of tumor cells with tumor infiltrating immune cells was analyzed by transwell culture system.

Results and Discussion

The tumor infiltrating immune cells population was increased during inorganic nanomaterials treatment in tumor bearing mice. The majority of tumor infiltrating immune cells reacted with nanoparticle was CD11b positive cells. The production levels of cytokines and ROS were modified after inorganic nanomaterials incubation. The migration ability of tumor cells was changed at the inorganic nanomaterials-stimulated tumor infiltrating immune cells co-culture group. The regulation of migration was abrogated by ROS inhibitor.

Conclusion

These data provide impetus for further studies the inorganic nanomaterials affect on tumor infiltrating immune cells in many tumor therapy models.

Keywords: Inorganic nanomaterials, tumor progression, tumor infiltrating immune cells, ROS

MO05-01

Proteomics reveals individual patient responses to therapeutic treatment for dry eye

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Introduction and Objectives

A common treatment for dry eye, punctual plug occlusion works to increase the amount of tears in the eye, which should decrease the disease severity. However, this invasive treatment has not been evaluated on an individual patient basis which is the objective of this study. Proteomics due to the ability to provide objective evaluation of the health of the ocular surface with a liquid biopsy has considerable clinical potential.

Methods

In this study 23 patients with moderate dry eye had non-absorbable punctal plugs inserted bilaterally into the lower punch of the eyelid.In the more severe eye, dry eye symptoms, fluorescein corneal staining, Schirmer's I test, tear film break-up time (TBUT) and safety were assessed at baseline, week 1 and 3. Proteins in tear samples were collected using Schirmer's test strips, quantified and compared relative to preocclusion baseline levels using isobaric tagging for relative and absolute quantification (iTRAQ).

Results and Discussion

Two broad groups of proteins were found: lacrimal gland proteins (LGP), lysozyme, PIP, lacritin and lactoferrin and inflammatory proteins (IP) S100A8, S100A9, S100A4, S100A11,ENO1 and TGM2. At one week of treatment, the beneficial LGPs were up-regulated in 5 patients and the adverse IP down-regulated. Other patients showed no clear response profile at this point. At 3 weeks, only 10 patients showed a beneficial response and 13 did not gain by the treatment. Logistic regression analysis revealed that those 10 patients had significantly lower baseline Schirmer's scores (a clinical assessment score to evaluate the tear secretion) compared to another 13 non-responders (mean [SD]: 4.3 [1.3] vs 6.8 [2.6]; P=0.006).

Conclusion

The results of this study suggest that proteomics could be used to determine which patients would benefit by an invasive therapeutic treatment, allowing non-responders to be removed from treatment. Two other outcomes are suggested: patients could be followed at 1 week to evaluated which ones will benefit from the treatment, and the results also suggest that prolonging the time of the treatment may be beneficial.

Keywords: dry eye, S100 proteins, tears, inflammatory proteins, lacrimal gland proteins

MO05-02

A comprehensive inter-grade proteomic analysis of serum, CSF and tissue in glioma

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Introduction and Objectives

Gliomas are brain tumors arising from glial cells characterized by rapid proliferationand are associated with poor prognosis.They account for ~28% of all CNS tumors where 80% of them are malignant with a median survival of about 10-12 months. W.H.O classified gliomas into four grades depending on their immunohistochemical and microscopic properties. An inter-grade comparative analysis of different biospecimens could provide an insight in the molecular features responsible for progression of the disease.

Methods

Both gel-based and gel-free proteomic approaches were used for identification of significantly altered proteins in different grades of gliomas. Serum and CSF proteome was extracted using TCA-Acetone precipitation, while tissue proteins were extracted using Trizol extraction method. The extracted proteins were labeled using CyDyes or iTRAQ4-plex reagents to perform 2D-DIGE and iTRAQ experiments respectively. The differentially expressed proteins were subjected to gene set enrichment analysis and were further validated using western blots.

Results and Discussion

Inter-grade tissue proteome profiling resulted in proteins involved in cell cycle regulation and proliferation, metabolic pathways associated with neurodegenerative disorders, energy metabolism, antigen-presenting and processing pathways were found to be differentially regulated. Certain proteins like thioredoxin, thymosin beta-4-like protein 3, L-lactate dehydrogenase A chain and ferritin light chain showed positive correlation with an increase in grade of the tumor. Proteins identified by inter-grade serum and CSF profiling were associated with immune system signaling, integrin signaling pathway, cytoskeletal regulation, lipid metabolism, gluconeogenesis and glycolysis. Various proteins identified in CSF like vitronectin, zinc-alpha-2-glycoprotein, transthyretin and afamin were found to be up-regulated while proteins like secretogranin-1, chromagranin-A and brevican core protein showed a significant down-regulated in higher grades of glioma. On the contrary proteins like vitronectin, zinc-alpha-2-glycoprotein and afamin were down-regulated in the serum of high grade gliomas. Intriguingly proteins involved in complement and coagulation pathways and extra-cellular matrix remodeling were found to be up-regulated in all the three bio-specimens.

Conclusion

This comprehensive inter-grade proteome profiling of glioma using different bio-specimens will provide an in-depth and mechanistic insight into glioma progression. A panel of differentially identified proteins may act as potential diagnostic and prognostic

biomarkers. However, further validation of identified protein targets on a larger cohort of glioma patients is needed before anticipating their clinical impact.

Keywords: Glioma, Biomarker, Central Nervous System (CNS), Tissue, Serum, Cerebrospinal Fluid (CNS)

MO05-03

Building a comprehensive chick retinal proteome dataset by liquid chromatography (LC) fractionation for tandem MS and SWATH analysis

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Introduction and Objectives

The sequential window acquisition of all theoretical fragment ion spectra (SWATH) is an advanced and important strategy to efficiently extract target fragment ions mass spectra from data independent acquisition (DIA) dataset for quantification of proteomic dataset. Prior to targeted extraction process, a spectral library is always constructed by the information dependent acquisition (IDA) approach. The capacity and quality of the library is critical to the sensitivity and specificity of targeted extraction data for subsequent analysis. In this study, we propose an approach to construct an expanded ion library using the IDA data generated by sample pre-fractionation in order to build a comprehensive coverage and high quality library for future SWATH analysis.

Methods

White Leghorn Chicks were sacrificed at 14 days after hatching to extract the retinal tissues. A pooled retinal protein sample from 50 chicks was used in this study. This retinal lysate (200 µg) was subjected to off-line SCX fractionation using the Eksigent ekspert™ ultraLC 110 system (Sciex). A strong cation-exchange (SCX) (PolySULFOETHYL ATM, PolyLC INC) column was used in this study. Multidimensional separation of tryptic peptide mixtures was performed using ammonium formate (PH=3.0) and ACN at a fixed flow rate of 0.8 mL/min under linear gradients. Seventy equal fractions were collected from an 80 minutes LC run before they were pooled into 20 fractions. Individual IDA acquisition for each fraction was performed using a TripleTOF 6600 MS (SCIEX). Protein identities were searched against Uniprot gallus gallus database using ProteinPilot 5.0 software and a combined proteome library was generated. In this study, comparison of proteome coverage between two libraries (with and without fractionation) was performed.

Results and Discussion

The combined library identified a total of 4832 proteins and 38335 peptides (at 1% FDR) from all collected fractions while there were only 1417 proteins and 5907 peptides (at 1% FDR) identified from the unfractionated Library. Compared with the ion library built from the unfractionated LC-MS, there are approximately 3.41 folds and 6.49 folds increase at proteins and peptides levels respectively from the expanded ion library.

Conclusion

This study provided one of the largest proteome datasets of chick retina recorded in the literature. Compared with the unfractionated ion library, the fractionated ion library could greatly expand the extracted proteins and peptides for building a more comprehensive and higher quality library, which will be beneficial to further SWATH targeted extraction and quantification analysis.

Keywords: SWATH, LC, MS, proteome, fractionation, dataset, library

MO05-04

Do Platelet-derived extracellular vesicles contain specific biomarkers allowing for early diagnostics of Alzheimer's Disease?

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Introduction and Objectives

Alzheimer dementia is a neurodegenerative disorder which affects about 6% of the general population over 65. Aggregated proteins contained in amyloid plaques and neurofibrillary tangles are the hallmarks of AD. Besides all efforts targeting these protein aggregates over the last 20 years to develop effective AD-therapies no success could be reported so far.

Methods

Few of the AD-patients are affected because of a known gene variant in the APP-protein or the ß-or γ-secretase triggering an early onset of disease. For these cases it could be shown that the first biomarker changes can be detected about 25 years before the manifestation of the clinical symptoms of dementia.Most of the AD-patients belong to the group of the so called "sporadic" type; however, many of them carry gene allele variants like apo E4, apo J, PICALM or CR1 which are found to be risk factors for getting AD later in life. I.e. if somebody carries the apoE4 allele in the homozygote form his/her relative risk is about 15-fold higher compared with carriers of the other isoforms apoE3 or E2. Diabesity in combination with E4 substantially increases AD-risk.The AD susceptibility genes like apo E4, apo J, PICALM or CR1 are all somehow connected to lipid and glucose metabolism, innate immunity and inflammation.

Results and Discussion

In a joint effort in finding early onset biomarkers by analyzing extracellular vesicles in the blood which are produced by senescent platelets and other cells in the circulation we found that subfractions of these EVs are enriched in AD-related proteins, lipids and miRNAs.

Conclusions

These may be useful for the early diagnosis of a beginning Alzheimer disease years before the clinical symptoms are arising. This may be a starting point for the development for therapeutical interventions.

Keywords: early biomarkerAlzheimer's Diseaseextracellular vesicles

MO05-05

Generating a Proteomic Profile of Neurogenesis, through a quantitative comparison of neuroepithelial and radial glial like stem cells

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Introduction and Objectives

Neurogenesis, the development of new neurons, starts soon after the formation of the neural tube and is largely completed by birth. Development of the brain after birth is mainly reliant on the formation of new connections between surviving neurons. However, adult neurogenesis does continue in the subgranular zone of the Hippocampus from quiescent adult neural stem cells. Traditionally neural stem cells were cultured as neurospheres, a heterogenous agglomeration of neural cells at various stages of differentiation. This heterogeneity prevented accurate quantitative analysis. In 2008 Sun et al produced the first non-immortalised human foetal neural stem (NS) cell line from nine week old human foetal cortex. These cells are cultured as monolayers, have a radial glia like appearance, self renew and form all three neural cell types, neurons, astrocytes and oligodendrocytes upon differentiation. More recently human foetal neuroepithelial like (NES) stem cells have been produced from five week old human foetal hindbrain, they resemble neuroepithelial cells, with characteristic rosettes, upon differentiation they form a pure population of neurons. These homogeneous monolayer cultures enable quantitative proteomic analysis, to increase our understanding of early brain development.

Methods

Three NES and two NS cells lines were available for analysis. They proliferate with stimulation from FGF and EGF, removal of these growth factors results in spontaneous differentiation. Proliferating NES and NS cells were compared using SILAC labelling. In addition, each cell line was differentiated for 12 days, 6 timepoints were taken and compared using label free quantitation.

Results and Discussion

4916 proteins were quantitated with 665 differentially expressed, revealing fundamental differences between NES and NS cells. NES cells are less differentiated, expressing SOX2 and LIN28, have active cell cycle processes, DNA elongation, histone modification and miRNA mediated gene silencing. Whereas NS cells are more developmentally defined, express multiple membrane proteins, have activated focal adhesion, thereby increasing their binding and interaction with their environment. NS metabolism is more oxidative, utilises lipid metabolism, the pentose phosphate pathway and produce creatine phosphate. Upon differentiation the cell cycle processes are downregulated and neurogenic and gliogenic processes increased.

Conclusion

This work represent a detailed in vitro characterisation of non immortalised human foetal neural stem cells, it describes the regulatory, metabolic and structural changes occurring within neural stem cells in early brain development. The information herein points towards

MO05-05

de-differentiation as a means to produce more neurogenic neural stem cells in vitro thus aiding regenerative therapies, as well as provides a wealth of information for better understanding neurological developmental disorders.

Keywords: neural stem cells, neuroepithelial, radial glia

MP08-01

Comparison of protein extraction protocols for label free proteomic studies in retinal tissue.

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Introduction and Objectives

Soluble protein extraction is the first and one of the key challenges in proteomics studies. There is limited information on optimizing protein extraction methods in species-specific retinal proteome. An evaluation of the extraction protocol to maximize proteome coverage is of great importance to improve reproducibility and representation of total proteome for label free retinal studies. Using both a Triple TOF MS and an IonTrap MS, this pilot study evaluated an experimental workflow to extract and identify chick retinal proteins using two different lysis buffers.

Methods

Four normal chick eyes (10 days old) were included. Ocular biometrics was checked before retinal tissues were harvested. The tissues were divided into EB2 group (n=2)and T-PER group (n=2). Proteins were extracted using 200 ul customized lysis buffer or 200ul of commercially available T-PER® Tissue Protein Extraction Reagents for EB2 group and T-PER group respectively. The protein concentrations were determined by Bradford protein assay. All samples were tryptic digested in solution for protein identification using two types of nanoLC MS including a Triple TOF 6600 MS (SCIEX) and an Ion Trap amaZon speed ETD (Bruker). The MS/MS spectra were extracted and searched by ProteinPilot 5.0 for datasets generated by the Triple TOF MS while Mascot 2.5.0 were employed for analyzing the Ion Trap generated datasets. The UniProt protein database (Gallus) was used for protein identification. Functional comparison of Gene Ontology annotations of identified retinal proteins was performed using PANTHER gene classification analysis.

Results and Discussion

Similar ocular parameters were observed in all chick eyes. Nearly fifty percentage increase was found in terms of total protein concentration using EB2 buffer. In terms of protein IDs (with <1% FDR), a total of 1906 proteins (9859l peptides) in EB2 group and 1724 proteins (10931 peptides) in T-PER group got identified by Triple TOF 6600 MS. Using Ion Trap MS, we identified 1267 proteins in EB2 group and 1150 proteins in T-PER group respectively. Despite of different MS design, there was consistently about 10% more proteins that could be found in using EB2 extraction. According to analysis from gene ontology, similar protein profiles were found in aspect of molecular functions and biological processes. However, some cellular component functions were found different in different extraction protocols.

Conclusion

We provided a workable proteomic protocol for studying retinal tissues. Global protein profiling of chick retina using different protein extraction methods were evaluated using

MP08-01

different MS systems. Our data suggested that the customized protein lysis buffer may have the advantages of providing stronger extraction ability by yielding more proteins IDs as well as offering a more economic alternative to commercial kit. It represents one of the fundamental ground works towards exploring quantitative label free proteomic studies for retina.

Keywords: Extraction methods, retina, proteomics, MS, chick

MP08-02

Comparison of fixed Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra (SWATH) windows for normal chick vitreous proteome

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Introduction and Objectives

Vitreous humor (VH) is a gel-like substance which represented up to 80% of the total volume of the eyeball. With recent breakthrough in LCMS-based profiling techniques, the protein quantification in the VH has emerged as an important area for studying various ocular diseases. Using chick as an animal model, this study aims to optimise the workflow of VH profiling by comparing two fixed windows sizes (25Da and 10Da) during Data Independent Acquisition (DIA) using Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra (SWATH).

Methods

2 weeks old normal growing White Leghorn Chicks were employed for this study. A 12/12-hour dark/light cycle was used to represent the normal light exposure. Chicks were sacrificed with CO2 overdose and eyeballs were removed and hemisected immediately to harvest the VH. Frozen VH samples were homogenised with T-PER lysis buffer (Thermo) using a homogeniser (Mikro-Dismembrator U). Protein concentrations were determined by Bradford protein assay (BioRad). Equal amount of proteins in samples were then reduced with DTT and alkylated with IAA. VH samples were digested in solution with trypsin (n=3) and were then analysed by the Eksigent ekspert NanoLC 400 system coupled with a TripleTOF 6600 system (SCIEX). SWATH window size was adjusted in Analyst 1.7 (SCIEX). Data obtained were exported using ProteinPilot 5.0 (SCIEX). Functional classification was analysed using PANTHER classification system. SWATH analysis was carried out with PeakView 2.2 (SCIEX) and web based OneOmics platform (SCIEX).

Results and Discussion

A total of 975 non-redundant proteins (10315 distinct peptides) were identified from the 2 weeks old chicks VH at 1% Global FDR. In terms of major molecular function, 33% was found to be involved in binding and 23% for catalytic activity. In terms of biological process, 26% was identified to be involved for cellular process and 15% for metabolic. In addition, we also detected a number of possible post translational modifications in the dataset. With FDR cutoff threshold set at 1%: 91.5% proteins were co-detected from both windows, while 3.7% and 4.8% were found individually from 25Da and 10Da window, respectively. Peptides count at the cutoff was confirmed to have no significant differences ($p>0.05$) between the two window size. For SWATH comparison: At 30% coefficient of variance (CV) threshold, 72% and 54% of the total protein were identified from 25Da and 10Da window, respectively. Statistical analysis indicated that there were no significant differences ($p>0.05$) between the two.

MP08-02

Conclusion

We demonstrated a label free proteomics workflow from sample preparation to protein quantification for studying chick vitreous using the nanoLC with TripleTOF 6600 MS system. Using a narrower window during SWATH acquisition did not show any statistical differences on the total number of proteins and %CV of VH, indicating there were no overall improvements on the quality of the acquisition for VH.

Keywords: Vitreous, SWATH, Myopia, Proteome

Discovering protein regulations of human trabecular meshwork in response to corticosteroids using SWATH acquisition and MRMHR

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Introduction and Objectives

Trabecular Meshwork (TM) of the anterior chamber of the eyes plays an important role on the regulation of intraocular pressure (IOP). It offers resistance to the evacuation of aqueous humor from the eye. Corticosteroids can increase the intraocular pressure (IOP) of the eye and prolonged treatment with steroids can even lead to glaucomatous optic neuropathy. To better understand the underlying biological mechanisms of corticosteroid-induced ocular hypertension (CIH), expression profiles of human trabecular meshwork (hTM) cells after treated with dexamethasone (DEX) and prednisolone (PRED) were investigated by SWATHTM-MS previously. In current study, we performed MRM-HR for further verification.

Methods

Primary human TM cell cultures from 3 healthy donors were incubated with and without either corticosteroid (DEX or PRED) for 7 days. The differentially expressed proteins in response to these treatments were quantified by a high resolution QTOF MS using SWATH approach. 20 commonly expressed protein sets measured in the hTM cells of the same donors were further verified using a novel MRM-HR approach.

Results and Discussion

In the present study, we have used a combination of DDA-based ion library and SWATHTM-MS in order to perform high-throughput relative quantitative analysis in hTM cells. The data demonstrated good reproducible quantitation by both MRM-HR and SWATH-MS. The correlation plot showed a good linear correlation between the quantification results (slope >0.9, R2> 0.9) and benchmarks of the quantification accuracy obtained by SWATHTM-MS targeted analysis to the level of quality delivered by MRM-HR data acquisition. Besides, the deviation between the two methods was typically within 5% in terms of fold changes.

Conclusion

The combinatorial use of SWATHTM-MS and MRM-HR provides a powerful workflow for biomarker discovery and verification on a single MS platform.

Keywords: Trabecular meshwork; Glaucoma; Corticosteroids; MRMHR; SWATH

MO10-01

Changes in Protein Expression Patterns in Islets of Langerhans: Implications for Treatment of Children with Obesity and Type 2 Diabetes

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Introduction and Objectives

The number of individuals with obesity and type 2 diabetes mellitus (T2DM) is increasing dramatically world-wide. An alarming aspect is the decline in age of onset. The European consortium "Beta-Cell Function in Juvenile Type 2 Diabetes and Obesity" (Beta-JUDO) was formed to work on the hypothesis that insulin hypersecretion is an early etiological factor promoting lipid deposition, insulin resistance and eventually loss of functional beta-cells. The consortium aims to identify novel strategies reducing insulin hypersecretion.

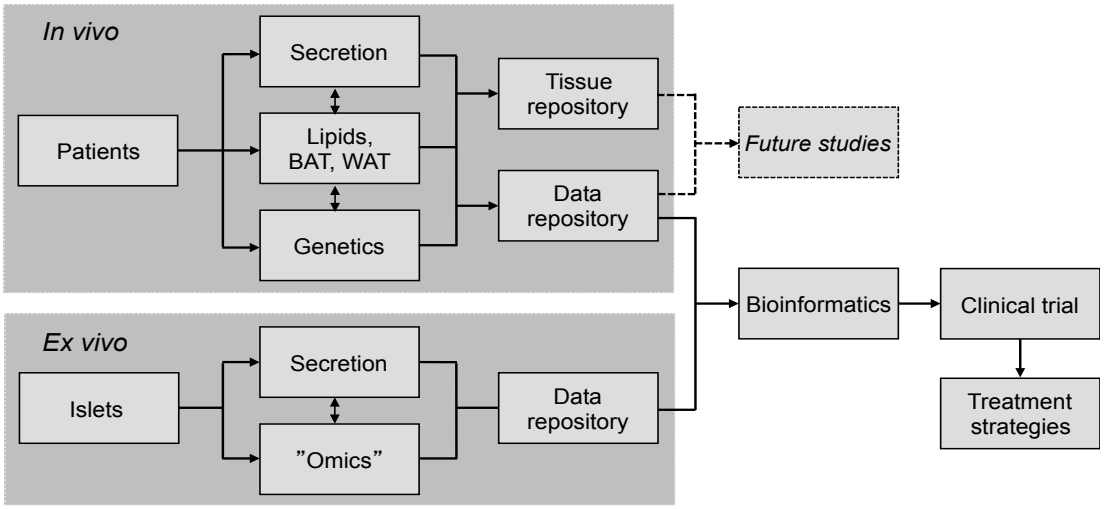
Methods

A translational approach is applied. *In vivo* obese children coming from a new European pediatric obesity cohort are characterized with regard to insulin secretory response during oral glucose tolerance test (OGTT), levels of circulating lipids, free fatty acids and incretins, body composition measuring brown (BAT) and white (WAT) adipose tissue, and genetics. *In vitro* isolated human islets of Langerhans are cultured in the presence of free fatty acid palmitate. In addition, analogues of the incretin GLP-1 or metformin, drugs used in patients with T2DM, are included or not during culture. After culture islets are functionally characterized by measuring glucose-stimulated insulin secretion. Islet protein expression patterns are subsequently generated by LC-MS/MS with isobaric mass tagging and bioinformatically analyzed utilizing GO, KEGG and IPA. An interventional trial is conducted, where pediatric patients with morbid obesity showing insulin

hypersecretion are subjected to intervention with GLP-1 analogue.

Results and Discussion

Obese children with high circulating palmitate levels had higher insulin levels both at fasting and during OGTT than obese children with low palmitate levels. Obese adolescents with high palmitate levels showed impaired insulin secretory patterns and developed impaired glucose tolerance and T2DM to a greater extent than obese adolescents with low palmitate levels. Isolated islets exposed to palmitate mimicked this development by showing hypersecretion of insulin after two days culture but impaired insulin secretory response after seven days culture. When either GLP-1 analogue exendine or metformin was included during culture the secretory pattern from the palmitate-exposed islets was essentially normalized. Protein expression sets of islets exposed to palmitate in the presence or absence of the drugs were generated. The analysis of the protein sets indicated that the pharmacological compounds changed activation of specific pathways and pointed towards specific upstream regulators involved.



Schematic overview of the project components.

Conclusion

Novel therapeutic strategies aiming at normalizing insulin secretion are expected to emerge from the proteomic and bioinfomatic analysis of the human islets. Such results are of the utmost importance to halt development of overweight and T2DM in children.

Keywords:

Childhood, obesity, type 2 diabetes, islets, palmitate, GLP-1, metformin, LC-MS/MS, IPA

MO10-02

Proteomic Phenotyping of Human Arterial Samples Identifies Novel Markers of Early Atherosclerosis

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Introduction and Objectives

The inability to reliable detect premature atherosclerosis significantly hinders our ability to implement personalized therapy to prevent coronary heart disease. A complete understanding of the protein network topology in arterial tissues and the ways this topology changes in the early stages of atherosclerosis could identify new biomarkers for disease detection and new therapeutic targets.

Methods

To study this we performed data dependent LC MS/MS (Orbitrap) analysis of proteins from the left anterior descending (LAD) coronary artery and the abdominal aorta of 99 human autopsy cases of men and women <50 years who died of trauma. 1890 and 1495 unambiguous proteins were identified in the LAD and AA samples respectively. The proteins represented a wide range of cellular functions, processes, components and canonical pathways and exhibited a scale-free network topology typical of molecular systems in a wide variety of living organisms. Unsupervised convex analysis of mixtures (CAM) was used to de-convolve the protein data into distinct signatures that were subsequently shown to correlate with pathologist ratings of extent of fibrous plaque, fatty streak and normal tissue in the individual samples.

Results and Discussion

A series of 100 elastic net models predicting extensive disease identified a subset of 20 highly informative proteins yielding an average AUC of 0.88 (S.E: +/- 0.003) and misclassification rate of 13% (S.E: +/- 0.0010). The LAD proteins most strongly associated with fibrous plaque provided clear indication of up-regulation of TNF-α and insulin receptor signaling and down-regulation of PPAR-α and PPAR-γ signaling. Moreover, differential dependent network analysis reveal specific features of these signaling networks that are re-wired in the setting of atherosclerosis.

Conclusions

Importantly, many of the sentinel proteins for early disease are secreted and likely detectable in plasma. If verified in additional samples and model systems, these data may provide new biomarkers for early disease detection and novel targets for treatment of insipient atherosclerosis.

Keywords: Atherosclerosis, Novel Markers, Human Arterial Samples

MO10-03

Proteomic Analysis of Membranes in Mouse And Human Cardiovascular Tissues

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Introduction and Objectives

Analyzing cardiac membrane proteins is crucial to a better understanding of heart function and development. Our objectives were to examine the major cell populations in the mouse and human heart by shotgun proteomics.

Methods

We employed cationic silica-bead coating coupled with shotgun proteomics to enrich for and identify cell-surface associated proteins from primary mouse neonatal and human fetal ventricular cardiomyocytes, fibroblast, endothelial cells, and muscle cells. Human coronary artery smooth muscle cells, human coronary artery endothelial cells, and human cardiac muscle derived cardiomyocytes or fibroblasts, and mouse neonatal ventricular derived cardiomyocytes were cultured for 2-3 weeks. Freshly dissociated human fetal ventricular myocytes were acutely dissociated and analyzed. Membrane proteins were cross-linked to cationic silica beads to isolate cytosolic proteins and a membrane fraction attached to the beads. Samples were analyzed by LC-MS MuDPIT strategies on a Thermo LTQ Orbitrap or Q-Exactive.

Results and Discussion

Shotgun proteomics identified >3,000 mouse and >2,500 human proteins. Enrichment of membrane protein and depletion of cytosolic proteins was confirmed by immunoblotting. Mapping of orthologous proteins between mouse and human resulted in 1717 proteins. Focussing on the cardiomyocytes, QSpec statistical analysis calculated differential spectral counts between proteins found in the membrane enriched and membrane depleted fraction and provided a dataset of 555 cardiomyocytes proteins including many known membrane proteins. Bioinformatic integration with transmembrane helix predictions, Phenotype Ontology (PO), and publically available microarray data sets, identified a rank ordered set of cardiac-enriched surface proteins; Tmem65 was the highest ranked candidate and explored in detail. We also examined subcellular fractions from in vitro human cardiac fibroblasts, cardiomyocytes, coronary endothelial, and coronary smooth muscle cells by shotgun proteomics. We identified a total of 2853 proteins across all cell types. Statistical analysis identified 367 fibroblast-enriched, 97 cardiomyocyte-enriched, 340 endothelial-enriched and 52 smooth muscle enriched proteins. We further ranked these proteins by statistical enrichment and by staining in the Human Protein Atlas database to identify top ranked, single cell-type enriched candidates.

MO10-03

Comparison of these proteins with heart failure demonstrated that many single cell type enriched proteins were also differentially expressed in disease. Fibroblast candidates GRB2, AKR1B1, and PRDX1 were further examined in a myocardial infarction mouse model, and demonstrated increased immunofluorescent staining in the fibroblast enriched, infarct- and adjacent border-zones.

Conclusion

Global protein expression of the major cell types in the mouse and human heart identified significant protein enrichment of single cell-type enriched proteins.

Keywords:

MO10-04

Glycoproteomics of the Aortic Extracellular Matrix: An Approach for Studying Diabetes and Cardiovascular Risk

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Introduction and Objectives

Cardiovascular disease (CVD) is the main cause of death and disability in patients with type II diabetes mellitus (T2DM). Previous proteomics studies into the diabetic vasculature have explored the extracellular matrix (ECM) proteome but have not investigated glycosylation, a key post-translational modification of ECM proteins. The aim of this study is to use the latest glycoproteomics workflows available on the Orbitrap Fusion™ Tribrid™ MS (Thermo Scientific) for comparing the aortic ECM from patients with and without T2DM.

Methods

Thoracic aortic samples from patients with and without T2DM were enriched for ECM proteins and subjected to indirect and direct glycoproteomics approaches. For indirect glycopeptide analysis, deglycosylation with PNGase F was performed in the presence of $H_2{}^{18}O$ before analysis by untargeted proteomics. For direct glycopeptide analysis, a glycopeptide enriched sub-proteome was analysed by two different HCD-ETD workflows: HCD-data dependent decision tree-ETD and HCD-product dependent-ETD.

Results and Discussion

The three glycoproteomic methods identified 4720 glycopeptides from 65 ECM proteins and 229 glycosylation sites, of which 22 were novel. Subsequently, 125 ECM proteins were quantified by MRM for analysis at the proteome level. Comparison of proteomic and glycoproteomic data revealed some notable differences; proteins like tubulointerstitial nephritis antigen-like and laminin subunit alpha-5 showed the same trend between the two sets of data, indicating a change in protein abundance rather than glycosylation. However, other proteins like microfibril-associated glycoprotein 4 and fibromodulin displayed distinct patterns of glycosylation but showed no change in overall protein levels.

Conclusion

This study is the first comprehensive glycoproteomic characterisation of the ECM in the human aorta demonstrating that T2DM does not just alter the expression but also the glycosylation status of vascular ECM proteins.

Keywords: Glycoproteomics, Diabetes, Extracellular matrix, Orbitrap Fusion, HCD-product dependent-ETD

MO10-05

Phosphoproteomics Identifies CK2 as a Negative Regulator of Beige Adipocyte Thermogenesis and Energy Expenditure

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Introduction and Objectives

Since the prevalence of brown adipose tissue (BAT) and its contribution to energy homeostasis have been widely appreciated in adult humans, it is considered that increasing BAT-mediated thermogenesis via uncoupling protein 1 (UCP1) serves as an alternative approach to modulate energy balance. Recent studies suggest that rodents and humans possess at least two populations of UCP1-positive thermogenic adipocytes: classical brown adipocytes and beige adipocytes. Beige adipocytes reside sporadically within white adipose tissue (WAT) where they emerge in response to certain external stimuli, such as chronic cold exposure, exercise, and long-term treatment with PPARγ agonists. Molecular analyses indicate that adult human BAT contains beige-like adipocytes. For instance, RNA-sequencing analyses of clonal adult human brown adipocytes indicate that their gene signatures resemble murine beige adipocytes (Shinoda et al., 2015). These results further emphasize the potential importance of beige adipocytes in human obesity and metabolic diseases. Here, we employed phosphoproteomics to map global and temporal protein phosphorylation profiles in beige, brown, and white adipocytes in response to norepinephrine treatment.

Methods

To identify the downstream signaling pathways of norepinephrine that are unique to brown, beige, and white adipocytes, norepinephrine was added into differentiated immortalized brown adipocytes, white adipocytes (F442A cells), and a model of beige adipocytes in which PRDM16 is ectopically expressed in F442A adipocytes. The differentiated adipocytes were harvested before the treatment (time point 0) and at 5, 10, and 20 min after norepinephrine treatment. Phosphoproteins, isolated from the collected adipocytes, were enriched by TiO2-based hydroxy acid-modified metal oxide chromatography (HAMMOC) (Sugiyama et al., 2007). Phosphopeptides were desalted by StageTips (Rappsilber et al., 2007) and suspended for subsequent nanoLC-MS/MS analyses.

Results and Discussion

By applying PhosphoMotif finder database to the phosphoproteome, we unexpectedly found that Casein Kinase 2 (CK2), an evolutionarily-conserved serine/threonine kinase, is activated by norephinephrine stimulation preferentially in white adipocytes.

MO10-05

Blockade of CK2 by genetic or pharmacological approaches promotes the cAMP-induced thermogenesis in white adipocytes. Furthermore, inhibition of CK2 by small compound or antisense drugs promotes beige adipocyte biogenesis *in* vivo and protects mice from diet-induced obesity and insulin resistance.

Conclusions

The phosphoproteome analysis of the three types of adipocytes provides insights on the physiological role of CK2 in the regulation of brown/beige adipocyte-selective thermogenesis and also illuminate the therapeutic potential of CK2 inhibitors in combating obesity and obesity-related diseases.

Keywords:

Obesity, Diabetes, Adipocyte Development, Stem CellReferencesShinoda, K., et al. (2015). *Nature Medicine* 21, 389–394. Shinoda, K., et al. (2015). *Cell Metabolism* 22, 997–1008.

MO10-06

Protective Effects of GLP-1 Analogues against Cellular Stress: An in Vitro Proteomic Study

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Introduction and Objectives

Increase in glucagon-like peptide-1 (GLP-1) activity has recently emerged as a useful therapeutic tool for the treatment of type 2 diabetes (T2D) by enhancing the glycemic control and also helping in maintaining or even decreasing body weight of most patients. The action of GLP-1 and its mimetics on pancreatic β-cells as well as on nervous and digestive systems are relatively well established. The effect of this peptide and its analogues in other tissues including adipose, muscles and liver, however, are still poorly defined. We therefore investigated the potential beneficial effects of GLP-1 mimetics on those peripheral tissues using established cell lines.

Methods

Using cell lines from liver (HEPG2), muscle (L6) and adipose tissue (3T3-L1), we analysed the effect of GLP1 mimetics (Liraglutide and Exendin-4) on cellular stress markers including Heat Shock Response (HSR) and Endoplasmic Reticulum (ER) in the presence of stressing amounts of glucose and palmitic acid (PA). Differential protein expression pattern was investigated using LC-MS/MS Orbitrap-Velos system and label-free quantification with a focus on the common markers of HSR (HSP-60, HSC-70, HSP-72 and HSP-90) and ER (GRP-78, ATF-6, IRE-1α, PERK, and eIF-2α). Results were validated using Western blotting and RT-PCR approaches.

Results and Discussion

In all used cell lines, PA (400uM) was found to clearly induce ER stress as reflected by increased levels of the ER stress proteins and GLP-1 analogues attenuated their expression levels. This effect was limited on HSR where only HSP60 levels were significantly decreased by PA and increased by GLP-1 analogues. Glucose (30mM) has shown however a mild effect on those cell lines. More interestingly, a set of proteins related to lipid homeostasis were also modulated by GLP-1 analogues under lipotoxic concentrations of PA. Proteomic data were further validated by Western blot and qRT-PCR and GLP-1 analogues showed indeed a similar effect in attenuating HSR and ER stress responses. Finally, we showed that JNK and ERK MAP Kinases were among the proteins that were highly modulated by GLP-1 analogues both at the expression and phosphorylation levels.

Conclusion

Our results suggest that GLP-1 mimetics alleviate the lipotoxicity-related cellular

stress in peripheral cells and thus recovering their normal homeostasis.

Keywords: GLP-1, GLP-1 analogues, Proteomic profiling, ER stress

MO10-07

Poorly controlled diabetes mellitus is associated with decreased aspirin-mediated acetylation of platelet cyclooxygenase 1 (COX-1) at serine 529

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Introduction and Objectives

Aspirin is a cornerstone treatment in the prevention of ischemic vascular events through the alteration of platelet function. Its main antithrombotic action is driven by the acetylation of cyclooxygenase 1 (COX-1) at serine 529. However, diabetic patients do not benefit from its preventive property to the same extent than non diabetic patients, particularly in primary prevention. Several studies have suggested that non-enzymatic protein glycation might potentially be an important factor in platelet function changes through its competition with acetylation modification.

Methods

The goal of this study was to evaluate the molecular and biological impact of in vivo hyperglycemia on ex-vivo aspirin-mediated acetylation (500 µM) of platelet-derived COX-1. Two mass spectrometry strategies were used (data dependent acquisition – DDA and parallel reaction monitoring- PRM) to detect and quantify blood platelet protein acetylation and COX-1 acetylated sites from control (HbA1c < 6%) and diabetic (HbA1c ≥ 8%) subjects matched for age and sex. COX-1 peroxidase activity was also measured.

Results and Discussion

More than 1500 acetylated platelet proteins were identified and quantified including COX-1 by DDA. Using PRM, from the 5 COX-1 modified amino acid sites, the four lysine residues that were found acetylated (Lys 168, 221, 252 and 572) after aspirin incubation in both groups of patients were not altered by hyperglycemia. In contrast, a significant decrease of acetylation was observed for serine 529 in hyperglycemic patients ($p < 0.01$). A partial inhibition of platelet COX-1 peroxidase activity was also observed with increasing glucose levels (p for trend < 0.01).

Conclusions

In conclusion, we demonstrated that hyperglycemia is associated with an impaired aspirin effectiveness through a reduced non-enzymatic acetylation modification of COX-1 at serine 529, thus providing a potential mechanism to the platelet hyper-reactivity observed in aspirin-treated diabetic patients.

Keywords: Diabetes, cardiovascular, platelet, COX-1, aspirin, acetylation, glycation

MP09-01

Silac-Based Proteomics of Human Kidney Cells Reveals a Novel Link between Male Sex Hormones and Impaired Energy Metabolism in Diabetic Kidney Disease

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Introduction and Objectives

Male sex predisposes to many kidney diseases. Considering that androgens exert deleterious effects in a variety of cell types within kidney, we hypothesized that dihydrotestosterone (DHT) would alter the biology of the renal tubular cell by inducing changes in the proteome. We aimed to perform an in-depth analysis of the sex hormone-regulated proteome in proximal tubular cells.

Methods

We employed stable isotope labeling with amino acids (SILAC) in an indirect spike-in fashion to accurately quantify the proteome in DHT- and 17β-estradiol (EST)-treated human proximal tubular epithelial cells (PTEC). Tryptic peptides were subjected to SCX fractionation on an HPLC system. Proteome analysis of DHT- and EST-stimulated PTEC was performed using two-dimensional LC-MS/MS in a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer. The MS files were processed with the MaxQuant software and searched with Andromeda search engine against the human UniProt database. Perseus software was used for calculation of significance A of protein ratios. BiNGO and Enrichment Map plugins in Cytoscape were used for bioinformatic analyses. Top candidate proteins were verified in vitro and validated in vivo by Western Blot. Renal oxidative stress was assessed by nitrotyrosine immunostaining.

Results and Discussion

Of the 5043 quantified proteins, 104 were differentially regulated. Biological processes related to energy metabolism were significantly enriched among DHT-regulated proteins. SILAC ratios of 3 candidates representing glycolysis, N-acetylglucosamine metabolism and fatty acid β-oxidation, namely glucose-6-phosphate isomerase (GPI), glucosamine-6-phosphate-N-acetyltransferase 1 (GNPNAT1) and mitochondrial trifunctional protein subunit alpha (HADHA), were verified in vitro. In vivo, renal GPI and HADHA protein expression was significantly increased in males. Furthermore, male sex was associated with significantly higher GPI, GNPNAT1 and HADHA kidney

protein expression in two different murine models of diabetes. Enrichment analysis revealed a link between our DHT-regulated proteins and oxidative stress within the diabetic kidney. This finding was validated in vivo, as we observed increased oxidative stress levels in control and diabetic male kidneys, compared to females.

Conclusions

This is the most in depth quantitative proteomic study of human primary PTEC response to sex hormone treatment. In this study, we suggest for the first time that male sex hormone stimulation results in perturbed energy metabolism in kidney cells, and that this perturbation results in increased oxidative stress in the renal cortex. These alterations associated with androgens may play a crucial role in the development of structural and functional changes in the diseased kidney. With our findings, we propose a novel link that may help to understand the more rapid kidney disease progression ascribed to male sex, especially in the context of diabetes.

Keywords: SILAC, Kidney, Sex hormones, Metabolism, Diabetes.

MP09-02

Quantitative simultaneous multiple PTMomics characterization of arteries from patients with atherosclerosis and type 2 diabetes

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Introduction and Objectives

Atherosclerosis is the primary cause of cardiovascular diseases, and an increase risk of atherosclerosis is associated with type 2 diabetes. Metabolic stress induced oxidative stress is essentially involved in the initiation and progression of atherosclerosis and T2D, as it can seriously affect the protein structure and signaling pathways of artery endothelial wall by posttranslational modifications (PTM) and other mechanisms. The ROS mediated cysteine oxidation and other essential PTMs such as phosphorylation and N-linked glycosylation involved in atherosclerosis are largely unknown. Characterization of PTMs and protein expression difference in human artery related to atherosclerosis and T2D can be helpful to reveal molecular mechanisms and etiology of atherosclerosis.

Methods

We analyzed four types of human artery samples from male patients in ages around 62 to 68 with and without atherosclerosis and T2D using our newly developed simultaneous multiple PTMomics strategy. We identified and quantified the difference of protein expression and multiple PTMs including Cys oxidation, phosphorylation and glycosylation among these four types of artery samples, revealed and characterized the regulated proteins and related signaling pathways in atherosclerosis and T2D.

Results and Discussion

In total, 9419 peptides with PTM sites were identified from 1866 proteins, 509 of them contained 2 or more different PTMs. 5988 PTM sites (3182 phosphosites, 1999 modified Cys sites and 807 N-glycosylation sites) were identified and mapped. We found 723 proteins at expression level, 544 unique phosphopeptides (206 proteins), 680 unique modified Cys peptides (329 proteins), and 259 N-glycosylated peptides (106 proteins) were significantly (P?0.05) regulated among four types of artery samples. Significant difference was mainly observed between normal and atherosclerotic samples, minor difference in atherosclerotic arteries between T2D patients and nonT2D patients, but not in normal artery. Functional analysis revealed that proteins involved in blood coagulation and inflammation were highly expressed in the atherosclerotic artery, but the proteins related to integrin signaling pathway showed the reverse case. Proteins with role in vascular smooth muscle contraction activity were highly phosphorylated. Proteins related to redox activity, complement and coagulation cascades and response to vascular injury showed high level of Cys oxidation. Meanwhile, proteins for fibrils formation, ECM receptor interaction and coagulation showed high N-linked glycosylation level.

Conclusion

These results suggested that differential protein expression and diverse changes of multiple PTMs were synergically involved in the regulation of artery and formation of

MP09-02

artery plaque in atherosclerosis, and multiple signaling pathways, such as coagulation and complement cascades, inflammation, vascular muscle contraction, fibrils formation and redox were affected in the process.

Keywords: Atherosclerosis, Diabetes, Metabolic stress, Quantitative PTMomics

MP09-03

A quantitative proteomic analysis of liver samples from liraglutide vs. placebo treated GIPR^{dn} pigs

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Introduction and Objectives

Methods

In this study the effect of liraglutide treatment in the transgenic pig model has been examined at the proteome level. To do this, liver samples of transgenic pigs GIPR^{dn} treated with placebo (PT, n = 5) or liraglutide (LT, n = 5) have been analysed using label free quantification. Peptides were separated on a 50 cm nano-LC column without prefractionation and analysed on a 5600+ triple-TOF instrument.

Results and Discussion

In total we were able to identify 1800 proteins, from which 40 proteins were found to show significant differences in abundance (P > 0.05) between the two sample groups.The data were further bioinformatically analysed by gene-set enrichment analysis (GSEA) in order to find enriched gene ontology categories. 1226 proteins were matched against protein sets based on gene ontology from Molecular Signatures Database using GSEA. 20 protein sets were observed to be significantly enriched for differentially expressed proteins (FDR > 0.05). Of these protein sets; 12 protein sets contained proteins that were more abundant and 8 protein sets contained proteins that were less abundant in the LT group.The 12 protein sets that included proteins that were more abundant in the LT group were enriched for proteins associated with cellular processes such as: regulation of cell proliferation, RNA processing, and tRNA biosynthesis. This is in accordance with previous findings which have shown that stimulation of incretin receptors promote cell proliferation. The 8 protein sets of the proteins less abundant in the LT group were enriched for proteins associated with cellular processes such as: amino acid metabolism, nitrogen metabolism and lipid binding.

Conclusion

We could demonstrate that treatment of the transgenic pig model alters the abundance of various liver proteins being involved in different biological processes. The corresponding proteins represent interesting targets for further investigations with the aim to improve the knowledge of physiological and pharmacological effects of liraglutide treatment on the liver.

Keywords: Diabetes, liraglutide, liver, large animal models

MP09-04

Differential Membrane Proteomics of Diet-induced Insulin Resistance Mice featured by Disorganized Actin and Myocardial Dysfunction

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Introduction and Objectives

Obesity accompanied by insulin resistance is associated with higher risk of preserved ejection fraction heart failure (HFpEF), though the underlying mechanism remains to be elucidated. Here, we aimed to explore the molecular changes in membrane proteome of cardiomyocyte and associated cardiac cytoskeleton alterations in elderly, high fat diet (HFD) fed mice model featured by phenotypic obesity and insulin resistance.

Methods

Male C57BL/6J mice aged 44 weeks were fed 12 weeks of a standard chow (SC) or HFD. Real-time Quantitative Reverse-Transcriptase Polymerase Chain Reaction (RT-qPCR), immunoblotting were performed for evident marker of myocardial damage and fibrosis with myocardial functional assessed by echocardiography. Immunofluorescence staining for isolated cardiomyocytes cytoskeleton Actin was examined and coupled to a variety of membrane-specific proteomic profile changes. We applied an iTRAQ-based quantitative membrane proteomics analysis for isolated cardiomyocytes from the control and HFD mice.

Results and Discussion

After 12-weeks HFD feeding, we successfully replicate early stage type 2 diabetes mice model featured by overt weight gain and insulin resistance. Compared with SC group, aging HFD group showed unfavorable cardiac remodeling and systolic functional decline, and markedly myocardial tissue fibrosis and up-regulated HF markers, including ProBNP or Galectin-3 etc. (all $p<0.05$). The differential membrane proteome profile revealed an impaired mitochondria dysfunction that was consistent with previous studies. In addition, the HFD group displayed increased expressions of membrane receptors, transporters, and channels involved in myofilament contraction as well as aberrant cytoskeleton organization with decreased levels of a number of actins, cytoskeletal keratins, troponins and myosins. The higher degree disorganized network of thick/parallel F-actin stress fibers also caused drastic decrease of F-to-G-actin ratio ($p<0.05$).

Conclusion

HFD fed elderly mice model displayed a certain phenotype that mimics early stage diabetes, which was accompanied by altered membrane proteomic profiling, interfered cytoskeletal rearrangement and evidence of myocardial dysfunction. Such findings may shed light on pathogenetic mechanisms involved in HFpEF.

Keywords: High fat diet, HFpEF, early stage type 2 diabetes, actin disorganization

MP09-05

The changes of proteomic profiling in the human four chambers from heart failure and additional insights in their biological function

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Introduction and Objectives

Heart failure (HF) is a worldwide cause of mortality and morbidity. It is not a uniform disease entity, but a syndrome with various causes, of which dilated cardiomyopathy (DCM) disease is one of the major causes of HF. However, although many mouse studies have provided essential insights into the role of myocardium, the relative protein expression patterns are difficult to apply to human hearts. Meanwhile, previous different studies mostly focused on the role of individual proteins and this promising variant molecular evidence indicates a clear need for a large-scale approach. The global heart proteomics could be particularly significant to provide an integrated view and better understanding of DCM-associated HF. It is well accepted that the LV (left ventricle) remodeling were of particular importance to attenuate initial cardiac event. In spite of its importance, the three other chambers have not been studied with the same degree and remains relatively poorly understood. Thus, a comprehensive knowledge of four-chambered protein expression alteration in heart failure is essential for the greater understanding of chamber-specific function and putative novel drug targets.

Methods

In the study, iTRAQ-8 plex label method was used in the MS-based analysis of three pairs of human heart tissues including four healthy and four failing chambers, and we also validated some molecules using western blotting.

Results and Discussion

Hundreds of chamber-enriched protein groups in the adult healthy heart were harvested. GO analysis of the chamber-enriched proteins revealed that atria might be more suitable for various genetic studies, but not directly related to muscle work that ventricle focused on. A total of 1039 significant changed proteins were identified and might be regarded as potential therapeutic targets for HF diagnose. These altered proteins were different in the four chambers, which revealed that every chamber was responsible to different function and have different remodeling process in HF. However, they also have 11 proteins changed uniformly in the four chamber.In addition, the cell death process were intensified in atriums, especially in LA. Actually, we also validated that Atf6, chop and caspase-3 were expressed higher in atrium.

Conclusion

In our study, proteome of four chambers in adult healthy hearts and heart failure were analyzed. We have identified potential chamber-characteristic biomarkers of recovered heart function from transplant patients. The energy production process of LV were prominent in healthy state, while a remarkable alteration of energy metabolism was detected more remarkable in LA in failing state. In addition, the cell death process were intensified in atriums, especially in LA, while ventricles didn't focus on it. Thus, this study

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provides key insights that enhance our understanding of the cellular mechanisms related to the pathophysiology of HF and could lead to the development of chamber-specific heart failure therapies.

Keywords: DCM, four chambers, energy, cell death, LA

MP09-06

Glycoproteomics of Aortas from Patients with Marfan Syndrome

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Introduction and Objectives

Marfan syndrome (MFS) is a genetic disorder caused by mutations in fibrillin-1. Patients with MFS develop aortic dilatation with a high risk of aortic rupture resulting in high mortality rates at a young age. The aim of this study is to characterize the glycoproteome of the aortic extracellular matrix (ECM) from MFS patients.

Methods

Ascending aortic tissue from MFS and control patients were sequentially extracted using our previously published extraction method (Didangelos et al, Mol Cell Prot 2010). The ECM extract was enriched for glycopeptides and analysed by LC-MS/MS using a combination of HCD-ETD fragmentation on an Orbitrap Elite mass spectrometer (ThermoFisher).

Results and Discussion

Glycopeptide analysis detected 179 different glycoforms from 35 glycoproteins in the human aneurysmal aorta. Notably, microfibril-associated glycoprotein 4 (MFAP4) showed a marked increase in the diversity of glycan structures in patients with MFS. Further proteome profiling of the salt and guanidine fractions identified 99 and 86 ECM proteins, respectively. The vast majority of ECM proteins showed decreased levels in aortic specimen from patients with MFS, including regulators of TGF-beta signalling. For most protein changes, there was no corresponding change in gene expression. MFAP4, however, was up-regulated at the mRNA level. MFAP4 plays an important role in the organisation of elastin fibre-related proteins, which together constitute the mature elastin fibre. Immunohistochemistry staining localized MFAP4 throughout the vessel wall with prominent staining near the internal elastic lamina and in the media. Silencing MFAP4 expression in human aortic smooth muscle cells resulted in decreased transcription of fibrillin-1, a known interaction partner of MFAP4, but increased expression of elastin, a key component for the mechanical properties of the aortic wall.

Conclusion

Our glycoproteomics analysis identified alterations in the glycosylation of MFAP4 in MFS patients. This study highlights an important role of MFAP4 in regulating the distensibility of the human aorta following fibrillin-1 mutations.

Keywords: Marfan syndrome, aortic aneurysms, extracellular matrix, microfibril-associated glycoprotein 4, elastic fibre.

MP10-01

Proteome Identification of Human Beta-Defensins in Male Reproductive System, and Induced Expression by Epigenetic Regulation

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Introduction and Objectives

Human beta-defensins (DEFBs) comprise one of the largest groups of host defence peptides. They are small cysteine-rich cationic proteins against bacteria, fungi and many viruses. DEFBs are also closely related to male reproductive system function. However, DEFBs are almost all missed in proteome identification. Aim to find why DEFBs are missed in regular tissue samples in MS data, and how DEFBs work in spermatogenesis and sperms fertilization in female reproductive system, we detect the protein expression in male reproductive system used immunohistochemistry (IHC). Next we used target proteomics technology to verify DEFBs highly positive expression tissues samples. When clarified the correlations between DEFBs expression in male reproductive system tissues, in the next step we can use epigenetic regulation tools to challenge to active the DEFBs missing block in related chromosomes regions, which is may be help to clarify the DEFBs functions in male sterility in the future.

Methods

We detected the expression level of DEFBs by IHC in the 6 types of human male reproductive tissues, including testis, fetal testis, epididymis, fetal epididymis, seminal vesicle and vas deferens. Then verified DEFBs in human testis and epididymis by the MRM assay. Targeting on 34 DEFBs by 132 synthesized unique peptides (each protein has 3 to 5 unique peptides). Lastly, we activated β-defensins expression by transcription regulatory mechanisms. The expression of DEFBs was deeply influenced by multiple transcription regulatory mechanisms including DHS and histone modifications. We conducted three epigenetics related agents to activate the DEFBs gene expression. The another strategy we conducted is RNA-guided activation of human DEFBs genes by dCas9-VP64.

Results and Discussion

The IHC in male reproductive system related tissue samples results showed DEFB103 is highly expressed in testis, fetal testis and epididymis. DEFB106 is highly expressed in epididymis. And DEFB136 is highly expressed in seminal vesicle and vas deferens. These positive areas are all related to spermatogenesis, sperms maturation, storage and transportation. The tissue microarray also included prostate samples as negative control. The same trend of DEFBs expression also occurs in the chromosome 6 and 20.Through MRM assay, we verified DEFB104, DEFB105, DEFB106, DEFB119, DEFB123, DEFB126 and DEFB129 in human testis and

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epididymis tissues. Through epigenetic regulation, we can see DEFB1, DEFB103, DEFB116 and DEFB130 can be activated several times. Through dCas9-VP64 strategy, we designed to target DEFB104, DEFB110, DEFB119 and DEFB126 in the 3 different cell lines. As the results showed, the four genes are activated many times in the three cell lines.

Conclusions

We verify the DEFBs expression in male reproductive system related tissue. We proposed that DEFBs may function as preventing the immune recognition. Furthermore, we activate the genes expressions by epigenetic regulation strategies.

Keywords: DEFBs, IHC, MRM, epigenetic regulation

MP10-02

Looking for missing proteins in the proteome of human spermatozoa: an update

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Introduction and Objectives

The Chromosome-Centric Human Proteome Project aims to identify proteins classed as « missing » in the neXtProt knowledgebase. The present study originated with the Franco-Swiss contribution to the C-HPP initiative to map chromosomes 14 (France) and 2 (Switzerland) by identifying additional missing proteins. Here, we combine the search for proteins that are currently classed as “missing” with an extensive examination of the sperm proteome.

Methods

A pool of spermatozoa from 5 healthy donors was treated using different fractionation/separation protocols along with different protein extraction procedures. The most recent version of the HPP guidelines for the identification of missing proteins was followed. Additional MS-based strategies (spectral comparison and parallel reaction monitoring assays) were applied to validate some of these missing proteins. Data mining was also applied to determine which proteins would be selected for validation by immunohistochemistry on human testes sections.

Results and Discussion

Using a range of protein extraction procedures and through LC-MS/MS analysis, 4974 protein groups were identified that passed the 1% PSM-, peptide- and protein-level FDR thresholds. Mapping of unique peptides against the most recent neXtProt release (2016-01-11) revealed 238 proteins (202 PE2, 22 PE3, 14 PE4) and eight proteins annotated with a PE5 (uncertain) status in neXtProt for which no evidence of protein expression was previously available. Combination of LC-MS/MS and LC-PRM analysis, data mining and immunohistochemistry (IHC), allowed confirming the expression of 215 missing proteins in line with HPP guidelines (version 2.0). The expression pattern for some of these proteins was specific to the testis and in particular to the germ cell lineage at late steps of spermiogenesis (i.e. from early spermatocytes to late spermatids). Precisely, IHC studies not only allowed us to confirm the existence of the proteins in sperm, but also to hypothesize a biological role for some of them (i.e., CXorf58, C20orf85, CFAP46, FAM187B and AXDND1).

Conclusion

Our results show how the use of a range of sample preparation techniques combined with

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MS-based analysis, expert knowledge and complementary antibody-based techniques can produce data of interest to the community. The information gleaned will help to extend our knowledge on the potential roles of these proteins in sperm function and/or maturation; some of which could potentially be valuable markers with applications in the assessment of human fertility. In addition to contributing to the Chromosome-Centric Human Proteome Project, we hope the availability of our data will stimulate the continued exploration of the sperm proteome.

Keywords: human proteome project, spermatozoon, missing proteins, mass spectrometry proteomics, immunohistochemistry, bioinformatics, data mining, cilia

MP10-03

State of the Chromosome 18-centric HPP in 2016: Transcriptome and Proteome Profiling of Liver Tissue and HepG2 Cells

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Introduction and Objectives

Gene-centric approach was applied for a large-scale study of expression products of a single chromosome. The main effort of the Russian Consortium in 2012-2016 was intended to investigate a master proteome of the chromosome 18 (Chr 18) in three types of biomaterial, included liver samples, HepG2 cell line and human plasma. Generally, proteome investigations are focused on the master proteins which resembling at least one of the many possible proteoforms, coded by the gene and containing the at least one MS-detectable proteotypic peptide.At the pilot stage of the current Chr 18 project, relevant proteins were profiled using SRM with non-labeled standards. This method of proteome inventory corresponded to the exploratory phase of study designated as Tier 3 assay in guideline paper by Carr et al., 2014. At the current phase of the project, we switched SRM assays to the use of stable isotope labeled peptide designated before as Tier 2. Increase in the degree of analytical validation expectedly modified the result of proteome inventory.

Methods

Transcriptome profiling for liver tissue and HepG2 cell line was independently performed using two RNA-Seq platforms (SOLiD and Illumina), as well as by Droplet Digital PCR (ddPCR) and quantitative RT-PCR. Proteome profiling was performed using the shotgun LC-MS/MS as well as by selected reaction monitoring with stable isotope-labeled standards (SRM/SIS) for liver tissue and HepG2 cells.

Results and Discussion

Based on SRM/SIS measurements, protein copy numbers were estimated for Chromosome 18 (Chr 18) - encoded proteins in the selected types of biological material. These values were compared with expression levels of corresponding mRNA. As a result of this pipeline, we obtained information about 158 and 142 transcripts for HepG2 cell line and liver tissue, respectively. SRM/SIS measurements and shotgun LC-MS/MS allow us to detect in total 91 Chr 18-encoded proteins, while an intersection between HepG2 cell line and liver tissue proteomes was about 66%. In total, there are 16 proteins specifically observed in HepG2 cell line, while 15 proteins were found in liver tissue. Comparison of proteome to transcriptome revealed quite a poor correlation (R2~0.1) between corresponding mRNA and protein.

Conclusion

Rise up to the higher level in proteome profiling (Tier 2) showed that only 30% of proteins (81 proteins) encoded on the Chr 18 can be detected in the study of two types of biomaterial – liver tissue cells (73 proteins) and the HepG2 cell line (66 proteins). The comparison of the results of transcriptome and proteome profiling showed that only for 30-40% of the genes, the expression of which was confirmed at transcriptome level, it is

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possible to detect the corresponding protein in the same sample using SRM/SIS (Tier 2).

Keywords: Human Proteome Project, transcriptome, proteome, human chromosome 18, RNA-Seq, SRM

MP10-04

Mining Missing Membrane Proteins by High-pH Reverse Phase StageTip Fractionation and Multiple Reaction Monitoring Mass Spectrometry

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Introduction and Objectives

Despite significant efforts in the past decade towards complete mapping of the human proteome, 3537 proteins (neXtProt, 01-2016) are still “missing proteins”. Membrane annotation shows that 1285 (36%) of these missing proteins are membrane proteins. Among the missing membrane proteins, topology analysis shows that 1198 and 760 proteins belong to integral membrane and plasma membrane proteins, respectively. Using non-small cell lung cancer (NSCLC) as a model study, we aim to apply our developed membrane proteomic approach to mine missing proteins from disease-associated membrane proteome, which may be still under-represented.

Methods

We used a high-pH reverse phase stage tip pre-fractionation of membrane enriched samples of 11 NSCLC cell lines bearing different EGFR mutation status and varying sensitivity to tyrosine kinase inhibitors, to enhance identification coverage. We also included membrane proteome of 20 pairs of tumor and adjacent normal lung tissue sample from NSCLC patients. Besides, multiple reaction monitoring (MRM) mass spectrometry was used for validation of selected missing proteins using synthetic peptides. In an effort for complete identification of missing membrane proteins, we assessed in silico digestion capability of multiple proteases to yield unique detectable peptides of missing membrane proteins and utilize for membrane proteome analysis from NSCLC cell lines.

Results and Discussion

Based on the 6820 proteins identified from the NSCLC cell lines and 4406 proteins identified from the tissue membrane, we were able to identify a total of 7702 unique proteins (66% membrane proteins) with PSM-, peptide-, and protein-level FDR of 1% from 64277 nonredundant peptides. Mapping further to neXtProt database (2014-09) 178 were found to be missing proteins (74 missing membrane proteins) of which 77% (139) already

have transcript level evidence. For further validation of these missing proteins, we also provided additional evidences of eight missing proteins including seven with transmembrane helix domains (TMH) using MRM mass spectrometry. To further enhance the peptide detectability from the relatively more hydrophobic membrane proteins, we have adapted multiple enzymes digestion approach. Computational analysis of 18 sole enzymes and 59 two enzymes combinations to yield unique detectable peptides (7-25 amino acid residue) for the missing membrane proteins, distinguished Pepsin, Chymotrypsin and Glu-CDE as suitable alternative enzymes. Preliminary result of the combined digestion by chymotrypsin and trypsin followed by Hp-RP pre-fractionation enabled us to detect additional 9 missing proteins, 6 of which are annotated as membrane proteins among 2052 proteins (67% membrane proteins)) from membrane samples of PC9 NSCLC cell line.

Conclusion

This study demonstrates that cancer membrane sub-proteome may be a rich resource to mine missing proteins and greatly contribute to mapping the whole human proteome.

Keywords: Missing protein, membrane protein, lung cancer, Hp-RP StageTip, MRM

MP10-05

The Spanish HPP: Detection of chromosome 16 missing proteins by targeted proteomics

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Introduction and Objectives

In the last years the global proteomics community has engaged in the generation of conclusive experimental evidence for human proteins that have not been previously detected at the protein level. We present here a workflow based on the generation of recombinant proteins to develop targeted proteomics methods for the identification of the so-called missing proteins of the human chromosome 16. Our work is currently integrated in the C-HPP IVTT consortium that harmonizes the efforts of chromosome 5, 10, 15, 16 and 19 teams.

Methods

Using an IVTT cell-free expression system 48 missing proteins from chromosome 16 were expressed. Recombinant proteins were digested with trypsin and analysed by shotgun proteomics to identify the high-responding peptides. Therefore 2-4 peptides per protein were selected based on intensity and sequence properties and they were synthesized as isotopically labelled peptides to generate the corresponding reference spectral libraries (Orbitrap Velos Pro, HCD). Next, we selected the biological matrices with the highest probability of detecting these proteins according to the information found in public repositories and on previous transcriptomics experiments. Samples were collected by the different ProteoRed nodes, and they were fractionated either by SDS-PAGE or reverse-phase liquid chromatography, and subsequently analyzed by scheduled SRM using spiked-in heavy synthetic peptides as reference standard.

Results and Discussion

Using the workflow described in the methods section we were able to detect several peak groups that potentially could correspond to the targeted missing proteins based on their co-elution with the standard peptides, and on the heavy-light transition intensity correlation. Further confirmation experiments using dilution curves are being pursued to generate conclusive experimental evidence of the detection of the selected proteins.

Conclusion

We show a workflow consisting of the combination of expression of recombinant proteins with scheduled targeted SRM proteomics methods that enhances the probabilities of detection of the so-called human missing proteins.

Keywords: human proteome, missing proteins, chromosome 16, CHPP

MP10-06

Detection of missing proteins using the PRIDE database as a source of mass-spectrometry evidence

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Introduction and Objectives

The current catalogue of the human proteome is not yet complete as elusive experimental proteomics evidence is available at present for a group of proteins known as the missing proteins. The Human Proteome Project (HPP) has been successfully using technology and bioinformatic resources to improve the characterization of such challenging proteins. In fact, different methodological approaches applied to characterize missing proteins confirmed that the selection of the tissue or cell type is critical to the success of these experiments.

Methods

We propose a pipeline starting with the mining of the PRIDE database to select a group of datasets enriched in missing proteins that are subsequently analysed for protein identification with a method based on the statistical analysis of proteotypic peptides. After the selection of our target samples (HEK293 cell line, spermatozoa, seminal plasma, placenta, blood plasma, retina, aorta thoracica and frontal cortex), we compared two different methods of shotgun dataset analysis for the identification of missing proteins at proteome level.

Results and Discussion

Spermatozoa and HEK293 cell line, a new promising source of missing proteins, deserve special attention for further studies. The functional analysis of the missing proteins detected confirmed their tissue specificity and the validation of a selected set of peptides using targeted proteomics (SRM/MRM assays) further support the presence of missing proteins in the analysed samples. In particular, DNAH3 and TEPP in spermatozoa, and UNCX and ATAD3C in HEK293 cells were some of the more robust and remarkable identifications in this study. We provide evidences indicating the relevance to carefully analyse the ever-increasing MS/MS data available from PRIDE and other repositories as sources for missing proteins detection in specific biological matrices as revealed for HEK293 cells.

Conclusion

Resources as the PRIDE database contain a large amount of information about peptides and proteins detected using MS in many different sample types. The analysis of these spectrometry evidences can be used to gain more accurate estimates about the biological

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sources in which the missing proteins are enriched. On the basis of this consideration, we proposed an approach to localize the missing proteins based on the analysis of all the human PSMs stored in the PRIDE database.

Keywords: C-HPP, missing proteins, MS/MS proteomics, PRIDE database

MP10-07

Proteomic Analysis of Human Placenta Stem Cell in Search of Missing Proteins

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Introduction and Objectives

To better understand the function of genes and proteins involved in human disease and biological process, the International Consortium of the Chromosome-Centric Human Proteome Project (C-HPP) has been thriving great efforts to identify and characterize missing proteins (MPs) which have weak or no mass spectral evidence. We were interested in identifying MPs from human placenta stem cells which are known to contain a large number of genes and perform many biological functions. Despite stem cells have special features to differentiate into various organizations, studies on MPs have not been well explored.

Methods

Placenta stem cells driven from the placental cells of both normal and pre-eclampsia [PE] patients were analyzed by the 1DE gel fractionation and Orbitrap MS analysis. Using the label-free quantitative analysis and bioinformatics quantitative programs (Sieve), search of MPs were sought by Trans-Proteome Pipeline plus X!tandem search engine and cross-checked with HPP Data Guidelines (ver 2.0.1) for verification.

Results and Discussion

In our pilot experiment, we were able to detect more than 1540 proteins present in placental stem cells with high confidence. Among these proteins, 85 proteins were found to be differentially expressed in PE (up-regulated 72, down-regulated 13). In addition, 5 proteins were turned out to be newly identified MPs (KRTAP2-1, OR10J4, MAGEB17, C1orf101 and ZSCAN5B) among which two proteins were at PE2 (transcript evidence) and three were at PE3 level (homology evidence), respectively.

Conclusion

We show that proteomic profiling of placenta-driven stems gave rise to identification of 85 differentially expressed proteins in PE, which also led to detection of 5 MPs that meet the criteria of MP claim set by HPP Guidelines (ver 2.0.1). Currently, a scale-up fractionation and proteomics analysis are underway in order to search for more MPs and PE-specific marker candidates from the placenta stem cells.

Keywords: C-HPP, Missing Proteins, Placenta, Stem Cell, Mass spectrometry

MP10-08

IVTT C-HPP Consortium. Targeting Missing Proteins In Biological Matrices

Horvatovich PL, Fuentes M, Domont G, Nilsson CL, Park J, Rezeli M, Segura V, Clemente LF Nogueira FCS, Ferolla C, Hernáez ML, González-González M, Marko-Varga G, LaBaer J, Gil C, Corrales FJ

Introduction and Objectives

According to neXtProt database, about 15% of human proteins still have no reliable experimental evidence, and are considered to be the "missing proteins". One of the goals of the C-HPP is to identify as many of these missing proteins as possible in human samples by mass spectrometry. A consortium of C-HPP teams (Chr5, Chr10, Chr15, Chr16 and Chr19) has recently joined forces to tackle this challenge by means of developing SRM methods based on recombinant proteins to be used for screening biological matrices with high probabilities of expression of missing proteins based on transcript evidence.

Methods

SRM methods have been developed using Skyline and optimized with the recombinant proteins produced using an *in vitro* transcription/translation cell free system (IVTT). Peptides were chosen according to the following characteristics: no trypsin miscleavages, 9-25 amino acids in length, no Met, Trp or Cys. All peptides selected for SRM were unambiguous according to the information of the neXProt database.

Results and Discussion

25 PE2 proteins were selected, expressed and analysed by targeted MS/MS, leading to the optimization of SRM procedures based on the detection of at least 3 unambiguous peptides and 3 co-eluting transitions in each case. More than 75% of the detected peptides are coincident with those proposed in the SRM Atlas for the selected proteins. Based on our previous studies suggesting the cell type or tissue where the probability of finding missing proteins is most likely, we have started the screening in selected samples including COV318 and KLE cells.

Conclusions

We believe that the IVTT system, when coupled with downstream mass spectrometric detection and specifically selected biological samples, is a valuable resource to identify proteins that have eluded more traditional methods of detection.

MP11-01

Functional characterization of a novel oncogene C20orf24 in colorectal carcinoma

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Introduction and Objectives

To characterize the expression pattern, biological functions and clinical significance of C20orf24 in colorectal cancer progression.

Methods

Gene expression profiles of several cohorts of patients from Gene Expression Omnibus (GEO) database were analyzed to determine the expression level and clinical significance of C20orf24 in colorectal cancer (CRC). The binding proteins of C20orf24 were identified by immunoprecipitation coupled with mass spectrometric measurement, and were systemically analyzed by Ingenuity Pathway Analysis (IPA). HCT116 CRC cells with C20orf24 overexpression or knockdown were compared with the respective control cells for migration and invasion abilities by using transwell assay, as well as for expressions of Epithelial-mesenchymal transition (EMT) markers including Vimentin, E-cadherin, β-catenin, and expressions of phosphorylated-AKT, AKT, phosphorylated-Erk, Erk by using Western blot.

Results and Discussion

The data from GEO indicated that C20orf24 was significantly upregulated in CRC, and more importantly, C20orf24 overexpression was associated with poor survival of patients. Our functional studies revealed that ectopic expression of C20orf24 not only significantly enhanced the migration and invasion abilities of CRC cells, but also resulted in the increased expressions of vimentin and β-catenin and the decreased expression of E-cadherin. Knockdown of C20orf24 exerted the opposite results. In addition, colony-formation assay showed that C20orf24 promoted cell growth. Mechanistically, IPA analysis of C20orf24 interactome suggested that AKT and Erk signaling pathways were markedly involved in the functional mechanisms of C20orf24 in CRC progression, which was confirmed by western blot data showing that C20orf24 overexpression increased, whereas C20orf24 silencing decreased the expression levels of p-AKT and p-Erk in CRC cells.

Conclusions

C20orf24 activates PI3K/AKT and Erk signaling, and promotes CRC invasion. Our data suggest the potential of C20orf24 as a biomarker and therapeutic target in colorectal cancer.

Keywords: colorectal cancer / C20orf24 / invasion and migration / EMT/ biomarker

MP12-01

FROM SNP TO GLYCOSYLATION: A COMPREHENSIVE GENO-GLYCOMIC APPROACH TO DISCOVER NEW LUNG DISEASE GLYCOBIOMARKERS

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Introduction and Objectives

One of the recent major focuses of biomedical sciences is glycobiomarker targets of interest in biological systems. Examples include cell surface membrane receptors, critical proteins in signaling pathways and key enzymes just to mention a few. The structure of most of these targeted glycoproteins possesses a variety of post-translational modifications (PTMs) including glycosylation, which is critical to their function. However, as glycosylation patterns are strongly related to the activity and expression level of glycosyltransferases and glyosidases, genetic variations in the genes coding these enzymes might play an important role. Understanding the effect of the genetic variants at the SNP level of these relevant enzymes may shed light on the genetic factors of glycosylation changes in serious lung diseases. In this presentation, a revolutionary and comprehensive geno-glycomic approach will be introduced to identify genetic risk factors that play a role in the glycosynthetic pathways and affect the glycosylation of several important serum glycoproteins at this important posttranslational modification in COPD and lung cancer.

Methods

SNP analysis and genomwide association study was accomplished by a multicapillary DNA analysis system. Immunoprecipitation of the relevant glycoproteins was done by a multiimmunoaffinity cartridge. The glycans from the purified glycoproteins were released by PNGase F, APTS labeled and analyzed by capillary electrophoresis with laser induced fluorescence (CE/LIF) and mass spectrometry detection (CESI-MS).

Results and Discussion

SNPs were identified in a group of enzymes playing crucial roles in glycosylation pattern generation and investigated by high-throughput genotyping techniques in a case–control study setting. We also introduced a novel multidetection separation platform to analyze the N-glycosylation modifications on the immunoprecipitated serum glycoproteins with cancer glycobiomarker potential at the low fmole level. The sugar moiety from these glycoproteins were released by N-glycanase treatment, followed by high yield derivatization with a charged fluorophore (APTS) and analyzed by capillary electrophoresis with high sensitivity laser induced fluorescent and MS detection. The glycoforms of the investigated glycoproteins were compared between normal individuals as well as and lung cancer and COPD patients also with co-morbidity. All major glycoforms >1% have been characterized.

Conclusion

The work to be presented will describe new opportunities to find sensitive and specific glycobiomarkers, even at early stages of lung serious diseases of cancer and COPD. The

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platform solution particularly addresses issues related to 1) local and global genetic studies, 2) sample preparation, 3) high sensitivity glycoanalytics using capillary electrophoresis with LIF and MS detection, 4) data processing/analysis, and 5) interpretation of the biological relevance of the results.

Keywords: Genetics, glycomics, capillary electrophoresis, mass spectrometry

MP12-02

Establishment of an omics database to study AOM/DSS mouse model of colorectal cancer

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Introduction and Objectives

Mouse model of colorectal cancer (CRC) induced by azoxymethane (AOM) and dextran sodium sulfate (DSS) is widely accepted in the field of CRC study, especially for studying on the carcinogenesis mechanism and chemopreventive intervention for CRC. Cancer genomics in human CRC has achieved a great progress, whereas the genomic variation of AOM/DSS model has yet to be explored. Herein, based upon a matured animal model we try to comprehensively investigate the CRC cancer genome landscape by establishing an omics database including genome, transcriptome and proteome analysis.

Methods

For genome analysis, we introduced exome sequencing on 14 neoplastic tissues in tumor-normal paired strategy, including 4 aberrant crypt foci which were regarded as precancerous lesion of CRC, 5 adenomas and 5 adeno-carcinomas. Meanwhile, the paired samples were collected from another five mics, and were taken for preparation of RNA and proteins simultaneously. For transcriptome study, mRNA samples were further isolated and deeply sequenced with HiSeq 4000 platform. For proteome study, two analysis methods were adopted, 1) a label-free proteomics using OrbiTrap Fusion MS to achieve higher coverage of identified peptides per proteins, and 2) a label proteomics with TMT10plex reagent to quantitative compare of the differential proteins between tumor and its adjacent tissues. In addition, the peptides were enriched by affinity resins for quantification of the phosphorylated and N-linked sialylated glycosylated proteins.

Results and Discussion

Coverage statistics of genome dataset showed a high enrichment efficiency of exons, with over 98% coverage and over 180X sequencing depth on consensus coding sequence (CCDS). Further analysis identified 6932 somatic mutations in these 14 neoplastic tissues. On the basis of RNAseq data, the transcriptome dataset in each sample was covered over 70% of CCDS. A total of 88162 single nucleotide variants were identified, and were established to a self-built mutation database for searching mutated peptides. On account of LC MS/MS data for label-free proteomics, averagely over 7000 unique proteins were identified in each sample, while for TMT-labeling proteomics, a total of 9191 unique proteins were identified in the five pair samples, including 2815 phosphorylated proteins and 711 sialylated N-linked glycoproteins. The integration analysis of these three datasets, including the identification of both sequentially mutated genes and differentially expressed genes, is being carried out.

MP12-02

Conclusion

We have established a complete databank for AOM/DSS mouse model, which includes the datasets of the exome sequencing, RNAseq, and proteome acquired from the individual mice. The databank sets a solid base to employ proteogenomic strategy for systematically evaluating how the mutation messages are transferred from genetic to functional molecules.

Keywords: Omics research, AOM/DSS mouse model, CRC

MO01-01

Verification of colorectal cancer biomarker candidates in plasma/serum extracellular vesicles by targeted proteomics

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Introduction and Objectives

Many biomarker candidate proteins for colorectal cancer (CRC) have been reported in the earlier literature including our report (Kume et al, Mol Cell Proteomics 2014), however, none of them has been applied for clinical test or validated as useful biomarker. Recent advances in targeted proteomics enabled to validate hundreds of biomarker candidates. In this study, we aimed to verify previously reported CRC biomarker candidate proteins in human CRC tissues and serum extracellular vesicles (EVs) of CRC patients by selected reaction monitoring (SRM).

Methods

We performed PubMed literature search for CRC biomarker candidates from 2003 to 2014. A total of 687 proteins were found to be functionally correlated with CRC and were potential CRC biomarker candidate proteins. Expression level of the proteins were verified in human CRC tissues and serum EVs of CRC patients by SRM.

Results and Discussion

First, we examined how many proteins of previously reported 687 CRC biomarker candidates can be verified in CRC tissues. We performed quantitative shotgun proteomic analysis in pooled extracts of non-cancer, adenoma and cancer tissues. A total of 319 CRC biomarker candidates were identified and about half of them were differentially expressed between each group. Next, we examined how many proteins of the CRC biomarker candidates can be verified in serum EVs of CRC patients. Among 687 biomarker candidates, about 400 proteins were able to be detected in EVs of CRC patient serum or in EVs derived from CRC tissues or cell lines using SRM. Quantitation of the biomarker candidates in serum or plasma EVs of healthy control and CRC patients are in progress.

Conclusion

Targeted proteomics is a significantly effective method for verification of biomarker candidates in various clinical samples. It will enable to develop a novel clinical test by SRM-based multiple biomarker quantitation.

Keywords: colorectal cancer, targeted proteomics, SRM, biomarker, extracellular vesicle

MO01-02

Proteomic Multimarker Panel Complements CA19-9 Insufficiency in the Diagnosis of Pancreatic Ductal Adenocarcinoma

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Introduction and Objectives

Pancreatic cancer (PC) is one of the most lethal gastrointestinal malignancies. The absence of symptoms in its initial stages and insufficient early detection lead to poor prognoses. In this study, we discovered biomarkers and developed a multimarker panel for the diagnosis of pancreatic ductal adenocarcinoma (PDAC).

Methods

We validated biomarker candidates in a large clinical cohort (n=1008) from five centers. All candidates were measured by multiple reaction monitoring-mass spectrometry (MRM-MS). Differentially abundant proteins, as determined by multivariate analysis, were assembled into a multimarker panel from a training set (n=684) and validated in independent set (n=318). The level of panel proteins were also measured by immunoassays.

Results and Discussion

The panel comprised leucine-rich alpha-2 glycoprotein (LRG1), transthyretin (TTR), and CA19-9, and had a sensitivity of 82.5% and a specificity of 92.1% in an independent validation set. These panel proteins also demonstrated the corresponding results by immunoassays. The triple-marker panel exceeded the diagnostic performance of CA19-9 by more than 10% using Delong's test (AUCCA19-9 = 0.826, AUCpanel = 0.931, $P < 0.01$) in all PDAC samples and by more than 30% (AUCCA19-9 = 0.520, AUCpanel = 0.830, $P < 0.001$) in patients with normal range of CA19-9 (< 0.01) and other cancers (AUCCA19-9 = 0.796, AUCpanel = 0.899, $P < 0.001$).

Conclusion

This study is the first and the largest-scale validation of pancreatic cancer markers that is based on MRM-MS to our knowledge and should have clinical applicability to early detection of PC.

Keywords:

Pancreatic cancer; proteomics; biomarker; multiple reaction monitoring-mass spectrometry; CA 19-9

MO01-03

Pathology-driven comprehensive proteomic profiling of the prostate cancer tumor microenvironment

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Introduction and Objectives

Gleason grading is an important predictor of Prostate Cancer (PCa) outcomes and is influential in determining patient treatment options. Clinical decisions based on a Gleason grade of 7, are difficult, as the clinical outcomes vary between G3+4 and G4+3 tumors. Laser capture micro-dissection (LCM) is a highly precise method to isolate specific cell populations or discrete micro regions from tissues. As part of a detailed molecular characterisation of the tumor microenvironment in PCa we undertook proteomic analysis of epithelial and stromal regions from tumor foci of different Gleason grades. Regions of interest were identified using telepathology to leverage specialized pathology expertise, isolated by laser capture micro-dissection and subjected to label-free LC MS/MS for unbiased proteomic analysis. Potenially significant differences between G3 and G4 tumour foci and between epithelial and stromal cells were identified.

Methods

Pathology-guided LCM was performed on patient tumor tissue to isolate G3 stromal and epithelial (n=4) and G4 stromal and epithelial (n=4) tumor regions. Cells captured from each region of interest (ROI) were extracted, pooled and subject to short range SDS-PAGE. Concentrated protein bands were excised and digested with trypsin (Promega) prior to LC-MS/MS analysis on a Q-Exactive mass spectrometer (Thermo). Raw data was processed through PEAKS (version 6) for protein identification. Statistical analysis of protein changes between G3 and G4 tumor regions was performed using Perseus (version 1.5.0.15) software. Expression of selected proteins that were significantly different in expression was evaluated in a separate cohort of 96 patients by immunohistochemistry using tissue microarrays.

Results and Discussion

Regions of G3 and G4 stromal and epithelial tissue were isolated through pathology-guided LCM and subject to label-free LC-MS/MS analysis. Over 2,000 proteins were identified in all ROIs following LC-MS/MS analysis. Technical reproducibility was confirmed by analysis (n=3) of a crude PCa tissue sample with CVs of <15% observed for all proteins identified. ANOVA analysis was undertaken to identify significantly changing proteins within the G3 microenvironment and the G4 microenvironment and between G3 and G4 tumor tissue. Pathway analysis of significantly changing proteins using Ingenuity Pathway Analysis (IPA) software revealed a significant increase or decrease in proteins

associated with cell cycle, carbohydrate metabolism, cell proliferation, RNA post-translational modification and immune response between stromal and epithelial tissue within and between G3 and G4 tumor. The expression of a selection of proteins that are up or down regulated between G3 and G4 epithelia was validated in a separate cohort of 96 patients by immunohistochemistry.

Conclusion

This study represents an integrated clinical and laboratory¬based investigation of the molecular diversity of G3+4 and G4+3 tumors. These data highlight a number of proteins with potential biomarker and/or therapeutic utility for management of prostate cancer.

Keywords: Prostate Cancer, Laser Capture Microdissection, Telepathology, Tumor Microenvironment

MO01-04

Tissue derived neo-antigens for T cell-based cancer immunotherapy

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Introduction and Objectives

Cancer immunotherapy reprograms the inherent capacity of immune cells to recognize molecular entities expressed specifically on tumors. Tumor antigens that are presented as human leukocyte antigens (HLA) binding peptides on the surface of cells, namely the immunopeptidome, serve as the leading targets and they may be derived from tumor-associated (over-)expressed self-proteins or mutated proteins. Recent data show that activation of the immune system by immune checkpoint blocking therapies leads to tumor rejection and that recognition of mutated antigens, known as 'neo-epitopes' plays a key role. So far, discovery of neo-epitopes relies mainly on prediction-based interrogation of the 'mutanome'. Tumor associated antigens have been regularly discovered in the last two decades by mass spectrometry (MS) based immunopeptidomics. However, it has been questioned whether MS would be sensitive enough to detect neo-epitopes as a discovery approach.

Methods

With the aim to identify the in-vivo presented neo-epitopes from human melanoma tumors, we applied our recently developed in-depth and streamlined MS-based immunopeptidomics approach. We isolated HLA class I and HLA class II peptidomes from 25 melanoma primary tissues. We separated the peptides by a nanoflow HPLC and sprayed them directly into a Q Exactive HF mass spectrometer. We developed a new module in the MaxQuant computational environment that integrates next generation sequencing data and generates customized patients' specific references that contain nonsynonymous somatic mutations for the direct identification of neo-epitopes.

Results and Discussion

We accurately identified the most comprehensive immunopeptidome from human melanoma tissues, comprising 95,662 unique peptide ligands with a false discovery rate of 1%, among them more than 300 known and novel peptide ligands derived from known melanocyte-associated differentiation and cancer testis antigens. Although we did not

specifically enrich for them, we detected 365 phospho HLA-I and 26 phospho HLA-II peptides, with 261 of these corresponding to known phosphorylation sites. We generated customized reference databases using exome sequencing data for five patients, and for the first time, using our discovery approach, we identifed 11 neo-antigens from three patients. We confirmed their identification with corresponding synthetic peptides. Four of eleven mutated ligands proved to be immunogenic by antigen-specific T-cell responses, hence are confirmed neo-epitopes. Moreover, tumor-reactive T cells with specificity for two of the neo-epitopes identified by MS were detected in the patient`s peripheral blood.

Conclusion

We established a comprehensive resource of the melanoma in-vivo immunopeptidome with a high number of attractive novel targets. We showed the feasibility of identifying the in-vivo and immunogenic neo-epitopes, directly from tumor tissues. This is a promising discovery approach for the development of immunotherapies.

Keywords: Immunopeptidomics, antigen discovery, immunotherapy

MO01-05

LinkedOmics: discovering associations between genomic, proteomic and clinical attributes in human cancer

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Introduction and Objectives

Traditionally, human tumors are characterized by a small number of clinical attributes. Technology advancements now enable comprehensive molecular characterization of tumors. For example, through the combined effort from The Cancer Genome Atlas (TCGA) and the Clinical Proteomic Tumor Analysis Consortium (CPTAC), the TCGA tumors have been characterized by genomic, epigenomic, transcriptomic, and proteomic attributes, increasing the dimensionality of the attribute space by several orders of magnitude. Many algorithms and tools have been developed for analyzing data generated by these studies. Standardized data sharing mechanisms, such as the TCGA and CPTAC data portals, have also been established. However, it remains a major challenge for biologists and clinicians to make direct use of these data.

Methods

We developed a web application LinkedOmics (http://bioinfo.vanderbilt.edu/linkedomics) that helps reveal novel biological and clinical insights by allowing users to fully explore the relationships between all molecular and clinical attributes using the well-understood association analysis. LinkedOmics relies on an R package OmicsAssociations to perform association analysis based on various statistical tests depending on the types of data being analyzed. The web application was developed based on PHP, Javascript, Perl, R and MySQL database.

Results and Discussion

For each of the 35 TCGA cancer types, the LinkedOmics database has data for >500,000 attributes including clinical attributes, mutations at site and gene levels, copy number alterations at region and gene levels, methylations at site and gene levels, mRNA expression, and miRNA expression. For the breast, colorectal, and ovarian tumors analyzed by CPTAC, the database also includes protein expression data and protein phosphorylation data at site and protein levels. LinkedOmics has three analytical modules: LinkFinder, LinkInterpreter and LinkCompare. LinkFinder allows users to search for attributes that are associated with a query attribute, thus enabling gene and protein signature analysis, biomarker identification, and regulatory relationship prediction, etc. The analysis results can be visualized by scatter plot, box plot or Kaplan-Meier plot. To facilitate a systems level understanding of the association results, the LinkInterpreter module performs enrichment analysis based on the gene ontology, pathway, network module, phenotype, disease and drug and chromosomal location data. The LinkCompare module uses visualization functions (clickable venn diagram, interactive scatter plot and sortable heat map) and meta-analysis to compare and integrate association results generated by the LinkFinder module. These features facilitate multi-omics and pan-cancer analyses. We demonstrate the usefulness of LinkedOmics using a few use case scenarios.

MO01-05

Conclusion

LinkedOmics provides a unique platform for cancer biologists and clinicians to access, analyze and use pan-cancer multi-omics data to gain novel insights.

Keywords: cancer, TCGA, CPTAC, multi-omics

TO05-04

Proteome analysis of microdissected tumor cells reveals Annexin A10 as biomarker candidate for differentiation of ICC and liver metastases of PDAC

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Introduction and Objectives

Intrahepatic cholangiocarcinoma (ICC) and pancreatic ductal adenocarcinoma (PDAC) are highly aggressive cancer types that arise from epithelial cells of the pancreatobiliary system. Owing to their histological similarity, differential diagnosis between ICC and metastases of PDAC located in the liver (mPDAC) frequently proves an unsolvable issue for pathologists. Yet, differential diagnosis of ICC and mPDAC is highly relevant as it leads the patient either to possibly curative resection (for ICC) or to palliation (for mPDAC). Therefore, novel biomarkers capable of improving this task are urgently needed.

Methods

We performed laser-capture micro-dissection combined with a label-free proteomics approach and analyzed isolated tumor cells from nine ICC and eleven primary PDAC (pPDAC) patients. The resulting biomarker candidates were evaluated in a large independent cohort of 87 ICC and 88 pPDAC tissue samples using immunohistochemistry. Moreover, we also tested biomarker candidates published in the literature (MUCIN 1, Agrin, S100P, MUC5 AC, Laminin, VHL, CK 17, N-Cadherin, ELAC2, PODXL and HSPG2) and applied the biomarker candidates with the most promising results to an independent sample set including biopsies of 27 ICC and 36 mPDAC.

Results and Discussion

In the proteome analysis, we found 180 proteins with a significantly differential expression between ICC and PDAC cells (p value < 0.05, absolute fold change > 2) of which nine candidate proteins were chosen for the immunohistochemical verification. Annexin A1 and Annexin A10, together with three previously published biomarker candidates (MUC5 AC, CK17 and N-Cadherin), showed the highest AUC values for the discrimination of ICC and pPDAC (between 0.72 and 0.84). These five biomarker candidates were applied to the independent set including biopsies of ICC and mPDAC, emulating the challenging situation in clinical practice. Here, Annexin A10 showed the highest diagnostic potential with 75.0% correctly classified mPDAC (sensitivity) and 85.2% correctly classified ICC (specificity), respectively.

Conclusion

We performed a quantitative proteome analysis to identify new biomarker candidates for the challenging differential diagnosis of ICC and mPDAC. We assessed newly discovered

biomarker candidates and evaluated previously published ones. We propose Annexin A10 as a biomarker meeting the challenging task of differentiating ICC from mPDAC. Our results highlight the great potential of proteomics techniques in biomarker discovery when a specific diagnostic problem is addressed and illustrate possible solutions to overcome technical challenges like sample heterogeneity.

Keywords: Intrahepatic cholangiocarcinoma, pancreatic ductal adenocarcinoma, laser-capture micro-dissection, label-free proteomics, tissue micro array

WO07-03

Quantitation and Evaluation of Candidate Biomarkers of Pancreatic Cancer in Plasma Using Multiple Reaction of Monitoring Method

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Introduction and Objectives

Pancreatic carcinoma (PC) is one of the deadliest epithelial malignancies with a low five-year survival rate of 5%, which has changed minimally for decades. Without active treatment, patients with metastatic pancreatic cancer has a median survival of 3–5 months and 6–10 months for those with locally advanced disease. The study aims to develop a pipeline for a novel clinical biomarkers discovery of pancreatic carcinoma.

Methods

Serum samples from healthy people(N), patients with benign disease of pancreas(PB) and patients with PC were utilized for high abundant protein depletion followed by Isobaric Tags for Relative and Absolute quantitation (iTRAQ) and Tandem Mass Tag(TMT) coupled with two-dimensional liquid chromatography－tandem mass spectrometry analysis. Pseudo MRM Quantitation(MRMHR) were applied to validate and relatively quantify the interesting proteins in different conhorts of sample groups without depletion of high abundant proteins .Based on the iTRAQ and MRMHR results, selected proteins were further verified and absolute quantified using Multiple Reaction Monitoring (MRM) method.

Results and Discussion

A total of 1381 proteins were quantitated, in which 94 proteins were differentially expressed according to our defined criteria. Based on the iTRAQ and MRMHR results, 54 selected proteins were further verified and relative quantification using Multiple Reaction Monitoring (MRM) method. 5 proteins in serum differentially expressed were used for absolute quantification. Finally, APOA1, APOL1 and ITIH3 were chosen as novel candidate biomarkers for pancreatic carcinoma. The sensitivity and specificty of these proteins were 85.0%, 85.3%; 67.5%, 85.3% and 75.0%, 73.3.3%.

Conclusion

In conclusion , we established a high efficient pipeline for biomarker discovery and verification, APOA1, APOL1 and ITIH3 could be an accurate diagnostic model for pancreatic carcinoma.

Keywords: pancreatic carcinoma/ serum /iTRAQ/MRMHR/MRM/biomarker

TP01-01

Clinically-Actionable Proteomic Biosignatures of Colorectal Cancer

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Introduction and Objectives

Detection of colorectal cancer (CRC) at the early stages (i.e., stage A/B) combined with surgical resection provides the greater chance of patient survival. Despite this, only 9% or 24% of CRC patients are diagnosed at stage A or B respectively, with the majority diagnosed at later stages (i.e., C (23%) or D (44%)) where 5-year survival rates fall dramatically especially when evidence of metastasis is found.

Methods

Detection of blood biomarkers expressed in early stage CRC would be enormously efficacious but discovery of low abundance biomarkers is extremely challenging due to presence of high abundance proteins coupled with the lack of technologies to detect markers at pg-ng/mL in blood by current mass spectrometry methods. We employed a SWATH-MS data independent acquisition (DIA) method to collect the complete record of all fragment ions of detectable peptide precursors from pooled ultradepleted plasmas (n=20 per stage depleted using MARS-14 in combination with an in-house chicken IgY bead-based method) from the same Dukes' stage A-D CRC patients with age-, sex- and other criteria matched controls. These plasma biomarker studies appeared, for the first time, to have achieved detection of early stage CRC 'signatures' with differences between normal and all Dukes' stages studied for at least three (3) proteins.

Results and Discussion

Conclusion

Keywords: colorectal cancer, plasma biosignatures, uPAR, interin avb6, SWATH-MS

TP01-02

Integrative Analysis for the Discovery of Non-small Cell Lung Cancer Serological Markers and Validation by MRM-MS and ELISA

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Introduction and Objectives

Non-small cell lung cancer, consists of two subtypes: adenocarcinoma and squamous cell carcinoma, constitutes ~80% of diagnosed lung cancers. Enormous efforts have been made to discover tissue or plasma/sera biomarkers about NSCLC patients, but unfortunately, clinical use of those markers might be limited. To overcome this problem, we applied the high-throughput omics-based technologies in which tumor-based transcriptomic, cell-based secretomics and plasma/sera proteomics were combined to find the serological biomarkers and validated these candidates by using SID-MRM and ELISA.

Methods

10 GEO datasets included 20,297 genes (271N vs 547T) which performed t-test (P <0. 001), FDR (Q <0.001), and fold-changes > ± 15%. The six lung cancer cell lines were cultured to identify the secretome by LTQ XL-Orbitrap mass spectrometer (Thermo Scientific). The acquired MS/MS spectra from the cell secretome were searched using SEQUEST against the UniProtKB (released in March 2012). Authorization to use the clinical plasma/sera for research purposes was obtained from the IRB. The Eksigent chip LC system was coupled to a 5500 Qtrap for LC-MRM assays. The serum levels of GPX3 and BCHE were determined by the commercialized ELISA kits (AdipoGen; R&D systems).

Results and Discussion

Conclusion

In this study, we hypothesized that if some proteins show quantitative changes in cancer tissues compared to normal tissues and are present in the cell secretome, those proteins would stand a better chance of being detected in plasma/serum. We challenged this hypothesis with NSCLC integrative tissue transcriptomes and cell secretomes and the resulting candidates were verified using MRM-MS and ELISA to measure the concentrations of candidate proteins in sera. Finally, two potential biomarkers (BCHE and

TP01-02

GPX3; AUC = 0.918 and 0.728, respectively) and one two-marker panel (BCHE/GPX3; AUC = 0.943) were verified by MRM-MS. In an ELISA validation study, the GPX3 and BCHE also were lower in NSCLC group with statistical significance. (BCHE and GPX3; AUC = 0.676 and 0.732, BCHE/GPX3 panel: AUC=0.768)

Keywords: lung cancer, transcriptome, secretome, MRM, ELISA, serological marker

TP01-03

Paradigm shift in accurate assessment of HER-2 in breast cancer patients by expression proteomics: Precision medicine and cost saving implications

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Introduction and Objectives

There are widespread disparities in the accuracy of HER-2/neu status among breast cancer patients especially among the borderline or equivocal scores. This lapse predisposes reasonable percentage of patients to either be denied of therapy or inappropriately treated. Of additional, research interest towards precision treatment of breast cancer, is the prospect of positive/negative- conversions of patient's status following treatment with HER2 targeted agents. Therefore, discovery of disease specific surrogate protein markers for HER2+/- subtypes could unravel the inherent heterogeneity within the sub population of triple negative breast cancer and assist clinicians in screening, choice of therapy and post-treatment surveillance towards achieving the goal of personalized medicine.

Methods

We have analyzed peripheral blood plasma (PBP), from 20 HER2+ and HER2- breast cancer patients using quantitative label-free liquid chromatography tandem mass spectrometry (LC/MS/MS). Differentially expressed protein patterns would be validated using immuno-based method in large archival materials to test their clinical usefulness.

Results and Discussion

We identified over 320 and 1100 proteins from PBP and peripheral blood mononuclear cells (PBMC) respectfully. Significantly differentially expressed protein fingerprints were able to discriminate HER2 + from HER2- samples using principal component analysis (PCA). Among the identified proteins are members of the calpain family of proteins of which one of them has been implicated in HER2 expression status. Other dysregulated proteins among the Her2 negative that might be of potential clinical interests include ANKRD18A, NEB, AZFYVE19, PTGS1, RRAB11A, TTECPR1, and TBCEL AND ERC1. Some of these identified proteins functions as extracellular matrix protein, protease, transporter, transferase, oxidoreductase, signaling molecule, enzyme modulator, and transfer/carrier proteins others are membrane traffic protein, chaperone, kinase, isomerase and receptor.

Conclusion

We anticipate that this study would be able to identify sub population of women that are otherwise labeled as HER2 – but expresses HER2 + intracellular domain phenotype, (the so called "negative inside the positive"). We advocate that development of protein expression profile would lead to unbiased stratification of breast cancer patients in the context of HER2 phenotypes and improved precision therapy for triple negative breast cancer patients. These results are preliminary but very exciting and would require

analysis of larger sample cohort to validate their clinical utility.

Keywords: Her-2-Status, Expression-Proteomics, Prognostication, Breast-Cancer, Intracellular-Domain, Precision-Medicine

TP01-04

Identification of new biomarkers for castration resistant prostate cancer by secretome analysis

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Introduction and Objectives

A prostate-specific antigen (PSA) is widely used as a diagnostic marker for prostate cancer (PCa) because of its high specificity. However, PSA level has no reliable correlation with pathological malignancy of PCa, and is not a predictor for castration resistant prostate cancer (CRPC). This study sought to discover a new biomarker that would allow more reliable prediction of CRPC via cancer secretome-based approach.

Methods

Using mass spectrometry, we compared proteins in conditioned media from 6 PCa cell lines and 8 other cancer cell lines originated from renal cell cancer and urinary cancer. The biomarker candidate proteins were selected through bioinformatics analysis and gene expressions analysis in proteins common to the PCa cell lines. Using mouse monoclonal antibodies produced in-house, these biomarker concentrations in plasma samples from 33 patients with benign prostatic hyperplasia (BPH), 31 with untreated PCa and 35 with CRPC were measured by a sandwich-type, one-step immunofluorometric assay on an automated immunoassay analyzer (AIA) system.

Results and Discussion

Around 1,500 proteins were identified in conditioned media from each cell line. Among them, we selected 12 proteins satisfying the following parameters as new biomarker candidates for PCa; a) which were identified commonly in PCa cell lines, but not detected from other cancer cell lines, b) which were categorized into “extracellular protein” or “membrane protein”, and c) which were neither abundantly nor widely expressed in various tissues. Furthermore, we investigated the gene expression levels of the biomarker candidates in various cell lines and tumor tissues by real-time RT-PCR. By these analyses, we found that growth and differentiation factor 15 (GDF15) was highly expressed in tumor tissues from patients with PCa and CRPC, and not only the mature region of the protein but several fragments of the propeptide region (named GDDP) were released from PCa cell lines. The AIA analysis revealed that the plasma level of GDDP-1, one of the processing forms of GDDP, was significantly higher in CRPC than those in BPH and untreated PCa ($P<0.01$). Receiver operating characteristic analysis also showed that GDDP-1 (AUC = 0.86) was superior to mature GDF15 (AUC = 0.76) in discriminating CRPC from PCa. There was a significant difference of overall survival (OS) in CRPC patients between those with more than 4.0 ng/mL compared to less than 4.0 ng/mL of GDDP-1, but no significant difference of OS by PSA in CRPC patients

Conclusion

GDDP-1 is thought to be a potential plasma biomarker for CRPC.

Keywords: cancer secretome, biomarker, prostate cancer, GDF15

TP01-05

Muscadine Grape Skin Extract Induces an Unfolded Protein Response Mediated Autophagy in Prostate Cancer Cells

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Introduction and Objectives

Muscadine grape skin extract (MSKE), derived from muscadine grape (Vitis rotundifolia), is a common red grape used to produce red wine. It has been reported to induce apoptosis in prostate cancer cells via phosphoinositide 3-kinase (PI3K) and mitogen activated protein kinase (MAPK) signaling. Endoplasmic reticulum (ER) stress activates the unfolded protein response (UPR) that serves as a survival mechanism to relieve ER stress. However, when persistent, ER stress can alter the cytoprotective functions of the UPR to promote autophagy and cell death. Although MSKE has been documented to induce apoptosis, it has not been linked to ER stress/UPR/autophagy. We hypothesized that MSKE may induce a severe ER stress response-mediated autophagy leading to apoptosis.

Methods

As a model, we treated C4-2 prostate cancer cells with MSKE and performed a quantitative Tandem Mass Tag Isobaric Labeling proteomic analysis. ER stress//UPR and pro-apototic marker upregulation, autophagy and apoptosis were analyzed respectively by western blot, acridine orange and TUNEL staining.

Results and Discussion

Our results indicated that MSKE significantly up regulated component of the ER stress/UPR response (GRP78, PDIA4, PDIA6, EIF2, EIF4 and Ire-1 alpha) and autophagy (ACIN1, PI4KA, PGK2 and MTDH). Pro-apoptotic markers PARP and Casp12 were also significantly up-regulated in the presence of MSKE. Moreover, increased acridine orange staining was detected in MSKE-treated C4-2 cells suggesting an autophagic response.

Conclusion

Our results suggest that MSKE induces apoptosis through signaling pathways that modulate a UPR/ER stress-driven autophagic response leading to apoptosis in prostate cancer cells, opening the door to novel therapeutic and clinical exploitations.

Keywords: Muscadine grape skin extract, unfolded protein response, Endoplasmic Reticulum, autophagy, prostate cancer

TP01-06

Biomarkers of Colorectal Carcinoma Liver metastasis in Urine

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Introduction and Objectives

Colorectal carcinoma (CRC) is one of the most common gastrointestinal carcinomas and more than 1/3 of CRC patients accompanied by liver metastasis. The routine diagnostic methods of CRC liver metastasis are ultrasonography and CT scan. But these methods are high-cost and need good medical conditions. Urine can be obtained non-invasively and continually, can serve as good resource of biomarkers.

Methods

We used a new filter device to gather the urine proteins onto the NC membrane, then extract the urine proteins and the proteins can be used for Western Blotting experiments. The arbitrary absorbance units of the Western blot bands were normalized to the urinary creatinine excretion.

Results and Discussion

We have collected urine samples from CRC liver metastasis patients. The preliminary results showed a high quality of urine proteins. Then we tried to detect Cathepsin D and the result shows that there is a significant difference between CRC liver metastasis patient and healthy urine samples.

Conclusion

The enrichment approach of urine proteins provides a new way of biomarker discovery and urinary Cathepsin D maybe a potential marker of CRC liver metastasis.

Keywords: Urine protein/CRC/Biomarker

TP01-07

Targeted proteomics based clinical investigation of serum early detection biomarkers for malignant pleural mesothelioma cancer

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Introduction and Objectives

Malignant Pleural Mesothelioma is an aggressive cancer of the pleural layers caused by asbestos exposure. The disease is mainly identified at advanced stages limiting efficacy of treatments. Here we apply Selected Reaction Monitoring (SRM) based targeted proteomics for the verification of multiplexed early disease detection serum biomarkers for mesothelioma cancer. We investigate the biomarkers in clinical cohorts of asbestos exposed donors and mesothelioma patients.

Methods

We investigate serum samples from more than 400 mesothelioma patients and asbestos exposed individuals. Samples are collected from the USA, Europe and Australia. Samples are enriched for N-linked glycoproteins using a multiplexed procedure on 96-well plates. Peptide analysis is performed on a UPLC-MS/MS triple quadrupole.

Results and Discussion

SRM traces are visualized using the software Skyline. For the quantititative large scale analysis across samples we use the MSstats software package. Spiked-in reference proteins and stable isotope labeled peptides are used for quantification across samples. We investigate robustness of the workflow and discrimination power of the biomarkers in distinguishing between asbestos exposed individuals and cancer subjects.

Conclusion

SRM based targeted proteomics allows for the efficient clinical investigation of early detection serum biomarker candidates in mesothelioma cancer.

Keywords: mesothelioma, cancer, biomarkers, early detection, targeted proteomic

TP01-08

Development of a Multiplexed LC-MRM MS Method of Salivary Proteins for Evaluation of Oral Cancer Biomarkers

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Introduction and Objectives

Multiple-reaction-monitoring (MRM) of peptides is a growing technology for target protein quantification because of the robustness, precision, accuracy, high throughput, and mulplexicity than antibody-based techniques. The technique has been optimized or performed for large-scale quantification of multiple target proteins in clinical applications using multiple types of fluids. However, less focus was put on the sample preparation workflow and analytical performance of precise protein quantification in saliva, a non-invasive body fluid, by LC-MRM-MS.

Methods

In this study, we applied MRM- based targeted proteomics in a simple and robust fashion to evaluate the analytical performances for 56 salivary protein quantification. The optimized platform was used to evaluate a subgroup of proteins that were previously associated with human cancer as putative salivary biomarkers.

Results and Discussion

Among the 56 proteins, 49 (87.5%) and 55 (98.2%) proteins were quantified with CV less than 10% and 20%, respectively. Only one protein showed the CV more than 20%. Using a clinically well-characterized cohort of two independent clinical sample sets (total n=119), our results quantitatively characterized the behavior of these protein biomarker candidates in saliva. 45 out of 56 (80%) proteins were quantifiable with average concentrations higher than their LOQ values in more than 50% of OSCC patients which indicates the potentials for routine analysis in clinics. The results clearly evidenced the statistically significant elevation of most targeted proteins in the saliva of the oral cancer group compared to non-diseased controls.

Conclusion

In the standpoint of technology development, we provided novel knowledge to optimize the workflow for salivary protein digestion and assay development, quantification performance, robustness and technical limitations in analyzing clinical sample samples. Overall, this platform demonstrates the most highly multiplexed panel of salivary protein biomarkers and highlights the clinical utility of MRM-MS in oral cancer biomarker studies.

Keywords: oral cancer, targeted proteomics, MRM, SRM, biomarker, saliva

TP01-09

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Introduction and Objectives

Methods

Results and Discussion

Conclusion

In conclusion, we revealed that the Mt of A549 cells were re-activated in the form of Mt biogenesis, hyperfused networks and increased energy metabolism. The Mt reactivation during EMT emphasizes the unique role of Mt in cancer cell retro-development to switch the metabolism pattern toward fitting the normoxia environment upon metastasis.

Keywords: EMT; Mitochondria; Metabolic reprogram; SILAC; Cancer

TP01-10

Quantitative profiling of plasma proteome in Kawasaki disease

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Introduction and Objectives

Kawasaki disease (KD) is an acute febrile vasculitic syndrome that commonly occurs in children less than 5 years old. The pathogenesis and etiology of KD are still unclear. Currently, KD is diagnosed mainly based on long lasting fever for >= 5 days, yet there is no specific molecular test to assist diagnosis for KD. In this study, we aim to develop a sensitive analytical workflow for quantitative profile of the plasma proteome between KD and febrile control (FC) groups to identify protein candidates for KD diagnosis.

Methods

Results and Discussion

In order to detect low abundant proteins in plasma, we adopted the immunoaffinity depletion methods to remove high abundant proteins. By using ProteoPrep20, all of the samples showed obvious decreases in high abundant proteins, such as albumin and IgG. The BCA assay showed that 80.0% and 61.4% of protein amount was removed by using ProteoPrep20 and Aurum Kits, suggesting a higher depletion efficiency of ProteoPrep20 Kit.We further integrated the ProteoPrep20 kit with quantitative analysis of plasma proteome of KD and FC groups. In the result, we identified 25 differentially expressed proteins in KD group. A majority of up-regulated proteins were involved in complement pathway, e.g. IGHA1, C1S, C3, C4A, and C8G, with a range of 2.0 to 10.6-fold higher expressions compared to FC group. The higher expression of these proteins suggested activation of classical complement pathway via antibody which mediates inflammation and response for phagocyte recruitment. The up-regulated GSN also promotes phagocytosis. Moreover, CLU, the complement inhibitor to C5b-9, showed up-regulated (8.3-fold) and C8B was down-regulated (2.1-fold), revealing a lack of response for membrane attack complex.

Conclusion

Our findings supported that the classical complement pathway serves an important role in KD with the underlying antigen binding to IGHA1, activates C1, triggers C3 to be cleaved into C3a and C3b, mediates inflammation and recruits phagocytes for phagocytosis. These results implied an acute activation of inflammation induced from bacterial infection in KD. Yet we need to improve the depth of quantitative proteome analysis in plasma sample to have better understanding for the pathogenesis of KD.

Keywords: Kawasaki disease; plasma proteome; complement pathway

TP01-11

Evaluation of biofluid biopsies by targeted proteome analysis for oral cancer biomarker discovery and verification

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Introduction and Objectives

Disease markers in body fluids provide a good chance to diagnose the disease in early status with less invasive. For oral cavity cancer (Oc), saliva is the fluid with much closer to the lesions than blood. However, blood is the much popular samples collected in clinic. In this study, we systematically determine the abundance of the potential disease protein biomarkers in saliva and plasma samples from the same subjects to evaluate their performance as biomarkers for oral cancer diagnosis.

Methods

Thirty paired saliva and plasma samples from the same subjects in Oc and healthy control (Hc) groups were enrolled. The stable Isotope labeled tryptic peptide fragments of target proteins were designed and synthesized as internal standard to evaluate the endogenous abundance of their correspondent proteins by using targeted proteomics analysis. The targeted multiple reaction monitoring mass spectrometry (MRM-MS) analyses were conducted on triple quadruple mass spectrometer (QTRAP 5500, AB Sciex).

Results and Discussion

The targeted multiple reaction monitoring mass spectrometry (MRM-MS) analyses were conducted to measure the abundance of 32 candidates in the paired saliva and plasma samples from 30 health donors and 30 oral cancer patients. The abundances of candidates are calculated according to the spike-in SIS peptide standards. Using MRM measurement, 87.5 % (28) and 37.5 % (12) of 32 protein candidates could be detected in one microgram of saliva and plasma proteins, respectively. To improve the sensitivity, an immuno-MRM assay by combining peptide immunoaffinity enrichment and MRM measurement was implemented to enrich peptide targets from higher amounts of saliva (25ug) and plasma samples (100ug). Consequently, 90.6% (29) and 53.1 % (17) of 32 protein candidates are detected in saliva and plasma samples with values higher than lower limit of quantification (LLOQ). Consequently, 7 and 1 candidates are shown significantly increased in saliva and plasma samples, respectively, of oral cancer patients as compared with the healthy controls.

Conclusion

This proof-of-concept study revealed that the proximal fluid of lesions provides a better opportunity to detect the disease-related protein candidates to reflect the disease status. More than 85% candidates are detectable (with values higher than LLOQ values) in saliva. However, less than 60 % of candidates were confidently measured in plasma samples. Furthermore, more candidates showed increase in saliva samples but not in

plasma obtained from oral cancer patients. Interestingly, three protein candidates, OC23, OC32 and OC36, with Oc/Hc protein ratios higher than 10 will be further verified in large cohort to confirm their role and application in oral cancer detection and diagnostics.

Keywords: saliva, plasma, oral cancer, MRM-MS, immumo-MRM-MS

TP01-12

Identification of Salivary Biomarkers for Detecting Oral Cavity Squamous Cell Carcinoma by Quantitative Proteomics

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Introduction and Objectives

Oral cavity cancer is the most common cancer of the head and neck cancer; oral cavity squamous cell carcinoma (OSCC) accounts for up to 90 percent of oral cancers. In Taiwan, oral cavity cancer is one of the top 10 cancers and the fourth highest mortality of malignancy in males. The main reason for high mortality of OSCC is that about 50 percent of patients with this disease were first diagnosed in the advanced stage. Therefore, early detection and prevention of OSCC are urgently needed to reduce the cancer burden. Recently, analyzing the body fluid is one of the most promising approaches to identify cancer-related molecules and biomarker candidates.

Methods

To identify biomarker candidates for OSCC, the proteome of saliva, a non-invasion and easy-to-obtain body fluid, was analyzed with isobaric tags for relative and absolute quantitation (iTRAQ)-based mass spectrometry (MS). The saliva samples were collected from 10 OSCC, 9 oral potentially malignant disorders (OPMD) patients, and 10 healthy volunteers. After pooling of the samples from the same groups, the samples were digested by trypsin, labeled with iTRAQ reagent, and analyzed by 2D-LC-MS/MS for triplicate experiments. To confirm the different level of targets from discovery phase, we used two-phase quantitative procedure, including multiple reaction monitoring MS (MRM-MS) and sandwich ELISA.

Results and Discussion

In discovery phase, among 1,852 proteins identified, the salivary levels of 106 proteins were higher in OSCC patients than that in healthy controls. Moreover, the salivary levels of biomarker candidates were estimated in OSCC, OPMD patients, and healthy controls by using targeted MS quantitative assay and immunoassays. In phase 1 verification, 28 biomarker candidates were estimated by using MRM-MS and 25 of them were confirmed to be elevated in OSCC patients compared to healthy controls. With an extended sample numbers in 2nd verification phase, the salivary levels of 4 proteins were significantly higher in OSCC group than in HC group and even in OPMD group by using ELISA.

Conclusion

We established a MS analysis pipeline including quantitative MS approach in both discovery phase and verification phase for saliva proteome research and biomarker identification. These results collectively demonstrated that our strategy is practical to

identify biomarker candidates for OSCC in clinical application.

Keywords: OSCC, saliva, biomarker, iTRAQ, proteomics

TP01-13

Variabilities of potential protein biomarkers level in saliva samples from healthy donor

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Introduction and Objectives

Saliva is an easy accessible bio-fluid and considered as an attractive source for bio-molecular testing in many diseases, especially oral cancer. Unlike serum/plasma and urine, saliva is not a regular diagnostic sample in clinical use. Few studies using low-molecular-weight and SELDI fractions with relative quantification technologies to address the composition of saliva and variation of partial salivary proteins many years ago, but the comprehensive identification and absolutely quantification of specific salivary protein/biomarkers for variability issue are not particularity characterized. To evaluate whether saliva is a good diagnostic sample for biomarker testing, we not only globally understand the relative abundance in whole salivary proteins, but also determine the absolute abundance in specific protein targets for diurnal variation.

Methods

Ten healthy donors were included in this study. Saliva samples were collected at three time-points (before breakfast on Day 1, before dinner on Day 1, and before breakfast on Day 2) for each individual. These samples were labeled with iTRAQ reagents and analyzed by using 2D LC-Orbitrap MS/MS to understand the global stabilities and relative abundance in whole salivary proteins. Besides, absolute abundance and diurnal variation in specific protein targets were analyzed by using multiple reaction monitoring (MRM) with stable isotope-labeled standard peptides for quantification. In the meanwhile, 15N-labeled recombinant standard protein was spiked in each samples to monitor technical stabilities.

Results and Discussion

Conclusion

We provide a method to evaluate whether saliva is a good diagnostic sample for selected proteins, and trying to establish a standard protocol for application in clinical salivary samples.

Keywords: Saliva, biomarker variability, MRM, clinical application.

TP01-14

Discovery and verification of blood biomarker for a diagnosis of acute graft-versus-host disease based on mass spectrometry and proteomics technology

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Introduction and Objectives

Graft-versus-host disease (GVHD) is major complications of allogeneic hematopoietic stem cell transplantation (allo-HSCT) which is curative treatment for hematological malignancies. No blood biomarkers are associated with the GVHD after allo-HSCT. The aim of this study is to discover and verificate blood-based protein biomarker for a diagnosis, drug treatment response monitoring of acute graft-versus-host disease based on mass spectrometry and proteomics technology.

Methods

Blood samples from patients who received allo-HSCT were taken at the time of clinical diagnosis of GVHD and equivalent times after allo-HSCT in patients with no GHVD. 5 allo-HSCT patients presenting with GVHD and 5 allo-HSCT patients presenting with no GVHD were pooled respectively. Unbiased, label-free quantitative mass spectrometry based approach was applied for discovery of candidate proteins. We removed high abundant proteins from blood using MARS-14 column and fractionated proteins by SDS-PAGE gel. Each gel slices were in-gel digested and digested peptides was separated and quantitatively analyzed with 2D nanoLC (high/low PH) & Q-TOF mass spectrometry. Candidate proteins verification is done by multiple reaction monitoring (MRM) methods.

Results and Discussion

In total, 200 unique proteins were identified and quantified. Some important proteins which is related to GVHD was discovered. Then, We finally selected 36 peptides corresponding to 12 proteins for MRM analysis and will verify them in large patients cohort (n = 102) We are currently developing MRM method which includes heavy peptide selection, optimization of LC-MS parameter and response curve establishment of each target protein for candidate biomarker verification. Each candidate protein concentration of biomarker candidate from individual blood samples will be presented and these values will be integrated with demographic and other clinical variables using a multivariable logistic regression analysis, which might give us clear classification of patients with GVHD and patients with no GVHD. Sometimes multiple biomarker panel give us more clear results than with single biomarker, so combination of each biomarker candidate and data analysis will be done.

Conclusion

TP01-14

ConclusionsBlood concentrations of these proteins biomarkers of patients at the early onset of GVHD can be used as a tool for patient-centered care, thus improve quality of life for patients received allo-HSCT. Verified protein biomarker for diagnosis of GVHD can be validated further for serial sample obtained at regular time based blood collection, and might be used for prognostic testing and therapeutic response monitoring. Our mass spectrometry based biomarker discovery and verification platform can be applied to other bio-fluid and biomarker study of specific disease.

Keywords: Graft-versus-host diseaseAllogeneic hematopoietic stem cell transplantationProteomics Mass spectrometry

TP01-15

In-Depth Proteomic Analysis of Pancreatic Cyst Fluid in Intraductal Papillary Mucinous Neoplasm Dysplasia of the Pancreas

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Introduction and Objectives

Intraductal Papillary Mucinous Neoplasm (IPMN) is a benign tumor that grows within the pancreatic ducts characterized by the production of thick mucinous fluid by the tumor cells. IPMN is the most important precursor lesion for pancreatic cancer that is the fourth most common cause of cancer deaths. Pancreatic cyst fluid is commonly utilized in the medical field to diagnose the severity of IPMN. However, proteomic research of this has been limited so far, because of the complexity of samples, the multifocality of IPMN, and immaturity of proteomic techniques. Also, there have been no reliable markers of dysplasia in patients with discovered intraductal papillary mucinous neoplasm (IPMN) of the pancreas.

Methods

We investigated differentially expressed proteins among pancreatic cyst fluids consisted of low-grade dysplasia (LGD), high-grade dysplasia (HGD), invasive IPMN, and mucinous cystic neoplasm (MCN) patients by using our novel proteomic strategy. Our experimental workflow included removal of contaminants, filter-aided sample preparation, High-pH peptide fractionation, and single-shot analysis using high-resolution Orbitrap LC-MS/MS. Especially, we increased the depth of individual samples by using proteome library of pooled pancreatic cyst fluid samples.

Results and Discussion

In this study, approximately thousands of proteins were identified in soluble part of pancreatic cyst fluid. Our proteome dataset is the largest among the worldwide pancreatic cyst fluid proteomic researches. Interestingly, our dataset contained several pancreatic tumor markers. In addition, there were numerous differentially expressed proteins according to histological grade of IPMN, especially, it was discovered that gradually decreased proteins or increased proteins as the pancreatic dysplasia progresses. Furthermore, it was demonstrated that the proteomic differences between IPMN and MCN used as a control by our novel strategy.

Conclusion

Despite the difficulty of handling pancreatic cyst fluid, we identified a considerable amount of unique proteins in small volume of samples. To date, our study has the highest depth in comparison with previous pancreatic cyst fluid proteomic studies. Moreover, we

TP01-15

discovered the specific peptide peak patterns of 3 histological groups of pancreatic dysplasia and mucinous cystic neoplasm. In accordance with this MS analysis result, we could identify the distinct protein expression among LGD, HGD, Invasive IPMN and MCN. Overall, the MS-based comprehensive proteomics approach used in this study can suggest a novel candidate biomarkers to distinguish dysplasia in patients with IPMN.

Keywords: Intraductal papillary mucinous neoplasm, Mucinous cystic neoplasm, FASP, High-pH Fractionation, Q-Exactive

TP01-16

Identification of protein and PTMs changes in gastric cancer patients from before to after surgery

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Introduction and Objectives

A biomarker is a molecule which can be measured and used to diagnose or classify a disease and also to measure the progress and therapeutic response of disease Blood is the preferred sample for identification of biomarkers, as it can be sampled using a relative non-invasive method. Moreover, blood passes through the body tissues thereby collecting biomolecules that could indicate a disease status. The ideal biomarker is a biomolecule that is present only in diseased patients and absent in healthy controls, or vice versa. Gastric cancer is one of the most common human cancer types. It is the second leading cause of global cancer death. In recent studies, surgical resection along with chemo-radiation showed significant improvement over surgery alone. However, most gastric cancer patients have advanced diseases at diagnosis. While surgical resection is an effective therapeutic procedure for curing gastric cancer patients, the 5-year survival rate is only about 20% for patients with late stage cancer. Therefore, early diagnosis is beneficial and critical for successful surgical removal of gastric cancers since metastases often occur in the late stages of gastric cancer and greatly reduce the effectiveness of surgery intervention.

Methods

In this study, we used a comprehensive strategy for enrichment of multiple post-translational modifications combined with hydrophilic interaction chromatography or high pH reversed phase fractionation and nanoLC-MS/MS to identify biomarker candidates in serum samples. We furthermore combined this strategy with TMT 10plex labeling in order to compare serum obtained from 72 patients with gastric cancer before and after surgery.

Results and Discussion

The comprehensive strategy resulted in identification of a total number of 989 proteins, 153 unique phosphorylated peptides, 2179 unique sialylated N-linked glycopeptides, 2964 unique Cysteine-containing peptides and 7437 unique non-modified peptides. Several proteins and modified peptides were identified as significantly regulated in all gastric cancer sera from before to after surgery, and among them proteins classified as being, cell adhesion molecules, cytoskeletal, ECM proteins as well as proteins involved in immunity and defense system.

Conclusion

The proteins and PTMs changes from before to after surgery in gastric cancer patients might be involved in the regulation of cell motility, architecture and morphogenesis through cytoskeletal proteins as well as in cell migration and invasion through the ECM and cell adhesion molecules.

TP01-16

Keywords: Serum, Biomarkers, Cancer, PTMs, Mass Spectrometry.

TP01-17

Immuno-MALDI for quantifying PI3K/AKT/mTOR signaling pathway activity in breast and colorectal cancer tumors

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Introduction and Objectives

Breast cancer and colorectal cancer are responsible for 0.52 and 0.69 million deaths worldwide, respectively. Aberrations in PI3K/AKT/mTOR pathway signaling play a critical role in the development and progression of these cancers and are targeted by novel therapeutic agents. However, these targeted treatments only work in a minority of patients. Additionally, predictive protein biomarkers are lacking or only partially successful, and currently there are no reliable methods to quantify signaling pathway activity. We therefore set out to develop immuno-matrix assisted laser desorption/ionization (iMALDI) assays based on our phosphatase-based phosphopeptide quantitation (PPQ) method to determine expression levels of AKT1 (P31749) and AKT2 (P31751), as well as to measure the pathway activity by accurately and precisely quantitating stoichiometry of phosphorylation sites critical for protein function (S473 and S474, respectively) in cancer cells and tumors.

Methods

As a first step, lysates of cancer cell lines or tumor tissues undergo tryptic digestion, followed by addition of stable isotope-labelled standard (SIS) peptides corresponding to the tryptic C-terminal AKT1 and AKT2 peptides ($_{466}$RPHFPQFSYSASGTA$_{480}$ and $_{468}$THFPQ FSYSASIRE$_{481}$, respectively). The solution is split into two aliquots, of which one is treated with alkaline phosphatase. The non-phosphorylated target peptides of both aliquots are then captured by anti-target peptide antibodies coupled to magnetic Protein G Dynabeads®. The beads are washed and spotted directly onto a MALDI plate. Addition of the acidic HCCA-MALDI matrix elutes the captured peptides from the beads. A Bruker Microflex LRF MALDI-TOF instrument is used for absolute quantitation of non-phosphorylated target peptide in both aliquots, which allows determination of the degree of phosphorylation (stoichiometry) of the target phosphopeptides in the sample.

Results and Discussion

We quantified the target peptides from colon-cancer (SW480, HCT116) and breast-cancer (MDA-231) cell lines, as well as flash-frozen and formalin-fixed paraffin-embedded (FFPE) breast-cancer and HCT116 mouse xenograft colon-cancer tissues. Per sample, ~50 µg protein per sample are required (10 µg protein per capture). Target peptide levels ranged from ~ 0.1-1 fmol/µg protein lysate with phosphorylation levels of 0-50%. The lower limit of detection was 100 amol of peptide, with a linear range of 0.5-10 fmol of

peptide on the MALDI plate. CVs for iMALDI assays were consistently <10%. All liquid handling steps have been automated on an Agilent Bravo liquid handling robot.

Conclusion

We have developed iMALDI AKT1 and AKT2 assays for quantitation of non-phosphorylated AKT1 and AKT2 in fresh frozen and FFPE tumor samples which will be validated in a currently on-going study.

Keywords: iMALDI, breast cancer, colorectal cancer, AKT, PI3K/AKT/mTOR pathway

TP01-18

Proteomic analysis identified heat shock protein 72 as a possible target of combination therapy with histone deacetylase inhibitor

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Introduction and Objectives

Histone deacetylase inhibitors (HDACi) are emerging as effective therapies in the treatment of multiple malignancies including cutaneous T cell lymphoma (CTCL). Although the effective treatments by HDACi have been reported in the patients with CTCL, the sensitivities to the HDACi are variable among the patients, and the molecular backgrounds of the different sensitivities have long been explored to optimize the therapeutic strategies.

Methods

We investigated proteomic features of the 33 lymphoid cell lines, which exhibited the different sensitivity to an HDACi, valproic acid (VPA). The proteomic profiles of the cells were created by two-dimensional difference gel electrophoresis (2D-DIGE) using the IPG DryStrip gel (24 cm length, pI4-7) and Ettan DALT twelve (GE) with home-made gradient gel, and protein spots were identified by mass spectrometry. The cell proliferation status in the presence of VPA was monitored using the Cell Proliferation Kit XTT Colorimetric assay.

Results and Discussion

The 50% inhibitory concentrations (IC50) value for VPA was varied between 0.2 and 6.0 mM (average 1.8 mM) in these 33 lymphoid cell lines. We found that the intensity of 20 protein spots was positively correlated with the IC50 value on 2D map. Among these protein species, we focused on heat shock protein 72 (HSP72), because it showed the most dominant up-regulation in the resistant cell line group. To reveal the functional role of HSP72 in chemoresistance of HDACi, we established HSP72 stably knockdown cell line from Hut78, an HSP72 overexpressed CTCL cell line, and confirmed that HSP72 knockdown enhanced not only VPA induced apoptosis but vorinostat (SAHA), other HDACi, induced apoptosis. Next, we studied the role of quercetin, an inhibitor of HSP70 family, on the antineoplastic effect of SAHA on Hut78. Quercetin reduced HSP72 expression in Hut78 cells, and enhanced SAHA-induced suppression of cell proliferation of Hut78, and its effect was synergistic (the combination index was less than 1). Single use of low dose quercetin induced G2 arrest and only a slight increase of subG1 cell fraction, while quercetin significantly enhanced SAHA-induced apoptosis detected by PI staining and PARP cleavage. Quercetin also enhanced SAHA-induced caspase-3, 8 and 9 activation, and loss of mitochondrial membrane potential was further enhanced.

Conclusion

HSP72 is a possible target of combination therapy with histone deacetylase inhibitor and quercetin is a candidate for combination therapy with SAHA in patients with CTCL.

Keywords: 2D-DIGE

TP01-19

Multi-dimensional Proteomics Reveals a Role of UHRF2 in the Regulation of Epithelial-Mesenchymal Transition (EMT)

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Introduction and Objectives

UHRF1 is best known for its positive role in the maintenance of DNMT1-mediated DNA methylation and is implicated in a variety of tumor processes. Here we provided evidence to demonstrate a role of UHRF2 in cell motility and invasion through the regulation of the Epithelial-Mesenchymal Transition (EMT) process by acting as a transcriptional co-regulator of the EMT-transcription factors (TFs).

Methods

We first expressed UHRF2 in four gastric cancer cell lines SGC7901, MKN74, N87 and MKN45 with Lentivirus Infection, and performed LC-MS/MS Analysis with both WCE and CatTFRE DNA Pull-Down for proteome profiling and transcription factor screening. We also performed UHRF2 knockout with CRISPR/cas9 in two gastric cancer cell lines SGC7901 and BGC823 and did transwell assay for migration and invasion testing. To figure out if UHRF2 functions through chromatin-mediated gene regulation, chromatin immunoprecipitation (ChIP) experiment was used with an UHRF2 antibody using IgG as a control.

Results and Discussion

MS profiling of these four UHRF2 overexpression cell lines revealed downregulation of a number of epithelial markers including CDH1, JUP, TJP1, DSG2, INADL, SPINT2, CXADR, SPINT1 and TJP2. The catTFRE-MS analysis also demonstrated upregulation of multiple key transcription factors involved in EMT, including TWIST2, FOXC2, and TCF family of transcription factors. On the other hand, after UHRF2 knockout, migration and invasion inhibition showed up in SGC7901 cells, as well as spheroid formation was significantly induced. Top ranked UHRF2-enriched binding motif matches one of ZEB1-binding sequences with a significance score of 0.77. This matching sequence contains a specific subclass of E-boxes (-CACCTG-). In addition to ZEB1, others TFs such as ZEB2, SNAI1/2 and TCF3 were also reported to bind E-box, which is located in the promoter region of CDH1 to repress the expression of E-cadherin, and the binding of UHRF2 to CDH1 promoter was validated by ChIP-qPCR. Moreover, the interactome analysis with IP-MS uncovered the interaction of UHRF2 with TFs including TCF7L2 and several protein complexes that regulate chromatin remodeling and histone modifications, suggesting that UHRF2 is a transcription co-regulator together with TFs such as TCF7L2 to regulate the EMT process.

Conclusion

Taken together, our study identified a role of UHRF2 in EMT and tumor metastasis and

demonstrated an effective approach to obtain clues of UHRF2 function without prior knowledge through combining evidence from multi-dimensional proteomics analyses.

Keywords: UHRF2, LC-MS/MS, profiling, TFRE, migration, EMT, IP, ChIP

TP01-20

A Set of Mass Spectrometry-derived Prognostic Biomarkers for Prostate Cancer Survival Prediction

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Introduction and Objectives

Research in proteomic biomarker discovery has led to the identification of a vast amount of potential candidates for cancer diagnosis and prognosis. However, very few of these potential biomarkers survive clinical validation and are then routinely used in the clinic. Main challenges are (i) the selection of an adequately sized patient cohort (ii) the reliable quantitation of the appropriate set of biomarker candidates and last but not least (iii) their statistical validation.

Methods

In our research project we work towards the development of a risk stratification test (RST) capable of differentiating aggressive from non-aggressive prostate cancer in patients diagnosed with localized prostate cancer. We monitored potential prognostic biomarkers in the context of a unique set of clinically defined samples from the proCOC cohort, which provides serum and long term survival data of 304 prostate cancer patients which underwent prostatectomy. For independent validation, clinical samples from a Hamburg prostate cancer prostatectomy cohort were taken. Targeted Proteomics (PRM) was employed on a pre-selected set of 48 glycoproteins known to be involved in the development of prostate cancer from a genetic screen. A Random Forest classification model was trained on our samples.

Results and Discussion

In total, we analyzed 243 serum samples from individual patients in four batches. Over all four batches 27 biomarker candidates could be identified consistently. Of these, 22 proteins with prognostic potential and no more missing values than ? throughout the cohort were used to train the random forest classifier. Our results indicate that by using a classifier based on six prognostic proteins, regression free survival of patients with localized prostate cancer could be predicted more reliably and with a lower error rate than using the biopsy-based Gleason-Score that is currently standard in the clinics. To further validate our prognostic biomarker panel we are currently developing a set of antibodies in order to transfer our assays onto a Luminex platform that will be used for high-throughput screening of independent cohorts.

Conclusion

In our approach we could show that the combination of an hypothesis-driven targeted proteomics approach in the context of an adequately sized patient cohort and powerful statistics enables one to identify predictive signatures of diseases. This is key to future

TP01-20

success in the biomarker field and in our case will provide doctors and patients with localized prostate cancer with a tool supporting them in their decision making in-between prostatectomy and active surveillance.

Keywords: Prostate Cancer, Prognostic Biomarker, Glycocapture, targeted Proteomics, Random Forest Classification Modeling

TP01-21

Novel Risk Models for early detection and screening of Ovarian Cancer.

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Introduction and Objectives

Ovarian cancer (OC) is the most lethal gynaecological cancer. Early detection is required to improve patient outcomes. Risk estimation models were constructed for Type I (Model I) and Type II (Model II) OC from analysis of candidate biomarkers identified following mass spectrometry investigations in conjunction with CA125 levels in prospectively collected samples from the United Kingdom Collaborative Trial for Ovarian Cancer Screening (UKCTOCS).

Methods

This nested case control study included 418 individual serum samples serially collected from 49 OC cases and 31 controls up to six years pre-diagnosis. Discriminatory logit models were built combining the ELISA results for candidate proteins with CA125 levels.

Results and Discussion

Model I identifies cancers earlier than CA125 alone, with a potential lead time of 3-4 years. Model II detects a number of high grade serous cancers at an earlier stage (Stage I/II) than CA125 alone, with a potential lead time of 2-3 years and assigns high risk to patients that the ROCA Algorithm classified as normal.

Conclusion

These models have encouraging sensitivities for detecting pre-clinical ovarian cancer, demonstrating improved sensitivity compared to CA125 alone. In addition we demonstrate how the models improve on ROCA for some cases and outline their potential future use as clinical tools.

Keywords: ovarian cancer, UKCTOCS, risk estimation, ELISA, Proteomics

TP01-22

Systems-wide analysis of protein expression in formalin-fixed paraffin-embedded Secretory Breast Carcinoma Tissues

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Introduction and Objectives

The mass spectrometry based quantitative proteomics in clinical samples have been shown to be powerful strategies for the discovery of novel disease biomarkers that can provide diagnostic, prognostic, and therapeutic targets. While discovery proteomics relying data-dependent acquisition is routinely used in analysis of clinical samples such as frozen tissues or body fluids, pre-clinical and clinical applications of proteomic strategies in formalin-fixed paraffin-embedded (FFPE) tissues were limited so far. Secretory carcinoma (SC) of the breast is one of the rarest types of breast cancer accounting for less than 0.2% of all breast cancers. Although recent research show that secretory carcinoma belongs to the phenotypic spectrum of basal-like breast carcinomas, difference from conventional basal-like breast carcinomas was unclear. Here, we present a study of SC FFPE tissue specimens using novel in-depth quantitative proteomic strategy.

Methods

In this study, we analyzed three SC FFPE tissues compared with Triple-negative breast cancer (TNBC) FFPE tissues from 3 patients, respectively. To perform quantitation in one FFPE slide per each samples, we performed well-defined proteomic strategies including FFPE protein extraction, filter-aided sample preparation, TMT 6-plex labeling, and high-resolution quadruple Orbitrap LC-MS/MS. TMT report ion quantification and data analysis were performed using Proteome Discoverer 2.1.

Results and Discussion

In total, 6900 protein groups were identified using one FFPE slide per sample, of which 1300 proteins were significantly regulated in between 3 SC FFPE tissues and 3 TNBC FFPE tissues. Some important proteins and novel proteins that related to SC pathology were discovered using bioinformatics analysis and network analysis. Furthermore, to discover the relevance of functional genes encoding and proteins expression, we performed comparative analysis between genomic data from whole genome sequencing (WGS) of 6 samples and proteomic data

Conclusion

Our in-depth quantitative FFPE tissue proteomic analysis platform will measure the levels of 7000 proteins with highly reproducibility from quite small amounts of individual human cancer or normal tissues. Our platform can easily be implemented in other type of cancer to analyze large numbers of pathologically relevant proteins in clinical specimens

TP01-22

including FFPE tissues and biopsy samples.

Keywords: Secretory Breast Carcinoma, Tandem Mass Tag, FFPE tissue, Quantitative proteomics

TP01-23

An in-depth proteomics investigation of the perturbed mechanisms underlying pemetrexed and paclitaxel treatments in non-small-cell lung cancer

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Introduction and Objectives

Non-small-cell lung cancer (NSCLC) is the most common type of lung cancer worldwide. Currently, pemetrexed, combining with cisplatin, has been used as the first-line treatment for patients carrying wild-type EGFR with locally advanced or metastatic NSCLC. Paclitaxel is an alternative drug for chemotherapy. In previous studies, our team found that patients received pemetrexed treatment showed better clinical outcome and immune response; however, the underlying mechanism has not yet been examined in the proteomic scope of view. Thus, we aim to unravel the perturbed mechanism upon pemetrexed and paclitaxel treatments on patient immune systems and cancer cells in NSCLC.

Methods

Purified PBMCs from 6 patients were collected before and after pemetrexed or paclitaxel treatments and subjected to quantitative membrane proteomics analysis. The membrane protein fractions were purified, subjected to gel-assisted digestion, labeled with iTRAQ reagents, fractionated by high-pH reversed phase StageTip, and each fraction was analyzed in duplicate by LC-MS/MS. For proteomics and secretome analysis on A549 cells, the total cell lysate and serum-free medium were collected after treated with pemetrexed and paclitaxel separately for 24 hrs and applied iTRAQ-based quantitation in comparison with untreated A549 cells.

Results and Discussion

A total of 2,166 proteins were identified in the PBMC membrane proteome. The Pearson correlation coefficient analysis of the expression profiles of PBMC membrane proteomes revealed diverse and patient-specific patterns in regulated proteins. We also investigated the immunological-related signaling pathways and correlated with patient outcome. Patient with better progression free survival (PFS) of 14.5 months showed more suppressive cytotoxic T cells with down-regulation of AP1 and AP2, leaving CTLA4 on the surface of cytotoxic T cells. Patients with 2-month PFS expressed up-regulated CD3, MHC class I, and perforin which suggested an active immunological status. In the proteome and secretome analyses of A549 cells, we identified 370 and 169 differentially expressed proteins after treatments respectively. Under pemetrexed treatment, cancer cells executed elevated cell migration and proliferation and released signals for movements of myeloid cells, whereas paclitaxel negatively regulated tumor cell proliferation and released factors inducing synthesis of reactive oxygen species,

apoptosis of tumor cell lines and angiogenesis signaling.

Conclusion

The differential expressions in PBMC membrane proteomics as well as proteome and secretome of A549 cells indicate that pemetrexed and paclitaxel comply opposite regulations on both immune cells and cancer cells. By integration of these proteome data, we may have better understanding toward the regulated mechanisms of pemetrexed and paclitaxel that may subsequently facilitate the better usage of these chemotherapeutic drugs and discovery of new drugs for combination treatment.

Keywords: Quantitive proteomics, pemetrexed, paclitaxol, NSCLC

TP01-24

Understanding male infertility after SCI through quantitative proteomics

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Introduction and Objectives

The study of male infertility after spinal cord injury (SCI) has enlarged the understanding of seminal plasma (SP) as an important regulator of sperm functionality. Most SCI patients present a seminal profile characterized by normal sperm count but extremely impaired sperm motility. The most important factors leading to this condition remained unknown. Thus, to explore the correlation between SP and low sperm motility, accessing the molecular mechanisms underlying infertility after SCI, we used mass spectrometry-based quantitative proteomics.

Methods

SP was obtained from 12 SCI patients and 11 controls. Control samples were grouped together in a protein pool whereas SCI samples were either grouped together ("pool experiment") or analyzed individually ("individual experiment"). Samples were subjected to a Lys-C/Trypsin combinatorial digestion; the resulting peptides were dimethyl labeled, mixed and finally submitted to an off-line fractionation on a hSAX column. The collected fractions were analyzed by LC-MS/MS on an LTQ-Orbitrap Elite ("pool experiment") or on a LTQ-Orbitrap XL ("individual experiment").

Results and Discussion

Over 2,800 unique proteins were described. The mass differences of the dimethyl labels were used to compare the peptide abundance in different samples demonstrating a differential SCI proteome. A strong activity of the immune system, which does not seem to be triggered by microbial infection, was observed. We also found a striking induction of protease inhibitors and simultaneous loss of some proteases. Moreover, evidences of an important prostate gland failure, i.e. diminished abundance of metabolic enzymes related to ATP turnover, secreted via prostasomes, were observed.

Conclusion

Our data described SCI related infertility on a proteomic level, suggesting the molecular pathways hindering fertility and providing evidence for the possible factors leading to impaired sperm functionality, notably sperm motility. Moreover we shed new light on other causes of male infertility.

TP01-24

Keywords: Seminal plasma, spinal cord injury, male infertility

TP01-25

Clinical application of plasma biomarkers for early detection of pancreatic cancer identified from proteomic profile

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Introduction and Objectives

We previously reported that circulating apolipoprotein AII (apoAII) isoforms apoAII-ATQ/AT (C-terminal truncations of the apoAII homo-dimer) decline significantly with pancreatic cancer and thus might serve as plasma biomarkers for early detection of this disease. Our previous study used matrix-assisted laser desorption/ionization (MALDI-MS) mass spectrometry to measure the concentration of each apoAII-isoform. We report here the development of novel enzyme-linked immunosorbent assays (ELISAs) for measuring apoAII-ATQ/AT and their clinical applicability to early detection of pancreatic cancer for application to clinical settings.

Methods

Plasma and serum concentrations of apoAII-ATQ/AT were measured in three independent cohorts, comprising healthy control subjects and patients with pancreatic cancer or gastroenterologic diseases (n = 1156, two Japanese cohorts and one US cohort). These cohorts included 151 cases of stage I/II pancreatic cancer.

Results and Discussion

Significant reductions in apoA2-ATQ/AT levels were recognized in patients with pancreatic cancer compared with healthy controls in both independent Japanese cohorts ($p=1.34\times10^{-18}$ and 5.09×10^{-39}). Areas under the receiver operating characteristic curve (AUCs) were >0.92 for distinguishing patients with stage-I/II pancreatic cancer from healthy controls in the Japanese cohorts. AUCs of apoA2-ATQ/AT to distinguish patients with pancreatic cancer from healthy controls were higher than those of CA19-9 in both Japanese cohorts. Better discrimination of pancreatic cancer was also observed with apoA2-ATQ/AT than with CA19-9 in blinded tests using the pancreatic cancer reference set from the Early Detection Research Network of the US National Cancer Institute. Combining apoA2-ATQ/AT with CA19-9 led to significantly improved AUC compared to CA19-9 alone. We have established a cancer screening network in Japan to confirm the clinical applicability of this biomarker, starting experimental cancer screening for pancreatic cancer using this biomarker.

Conclusion

ApoAII-ATQ/AT offers a potential biomarker in screening for early-stage pancreatic cancer and identifying patients at risk of pancreatic malignancy.

Keywords: ApoA2-isoforms, plasma biomarker, early detection for pancreatic cancer

Integration of SWATH and MRM for biomarker discovery of esophageal squamous cell carcinoma

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Introduction and Objectives

It is gradually recognized that biomarker discovery based on DDA may meet the technique challenges in acquiring peptide MS/MS and accurate quantification. Recently DIA has proven useful for combined qualitative/quantitative analysis to proteomes. SWATH is a novel DIA technique that aims to complement traditional MS technique for quantitative proteomics. Herein, we proposed an approach that integrates SWATH and MRM for biomarker discovery and verification. We selected the pooled samples that were prepared from the tissues of esophageal squamous cell carcinoma (ESCC) for discovering the ESCC-related proteins using SWATH, and analyzed the individual samples from the ESCC tissues and the corresponding sera for verifying the biomarkers through MRM. The results demonstrated the combination of SWATH/MRM is feasible for ESCC biomarker discovery.

Methods

The ESCC and the adjacent tissue lysates were tryptic digested, and all DDA and SWATH data were acquired by 5600 TripleTOF MS coupled with Eksigent400 NanoLC. The peptides identified from DDA were taken to build the ion library, and the analysis of SWATH data was implemented by Peakview V2.0. The differential proteins between ESCC and the adjacent tissues were evaluated through SRMstats. The transitions extracted from SWATH were used to develop MRM assay. The schedule MRM mode was employed, and the MRM data was acquired from the lysate prepared from individual tissue samples and serum obtained from pre- and post-operation.

Results and Discussion

With SWATH, 2157 unique proteins were identified in ESCC and the adjacent tissues, respectively. Total of 523 unique proteins were found with their abundance in the pooled ESCC tissue lysate significantly different from that in the pooled lysate of the adjacent tissue, including 294 up-regulated and 228 down-regulated. With stringent criteria to select the ESCC-related proteins for MRM assay, total of 146 unique proteins with 505 peptides were selected from the 294 up-regulated ones. MRM was employed to monitor these target proteins in pooled ESCC and adjacent tissue samples. The target proteomics resulted in 133 unique proteins with 353 peptides identified, in which most proteins appeared the similar abundance changes as detected in SWATH. These ESCC-related proteins were further compared with the databases of tumor protein biomarkers and of the ESCC protein biomarkers. The MRM method was conducted to detect the targets in the individual serum collected from the 10 ESCC patients who received surgery. Of the 133 target proteins, total of 46 were identified in the ESCC sera. More importantly, 28 proteins were found in lowering protein abundance after operation, indicating that these proteins in serum were tingly correlated with the tumor removal.

Conclusion

The result demonstrated that the combination of SWATH and MRM is feasible for ESCC biomarker discovery.

Keywords: SWATH, MRM, ESCC, biomarker

TP01-27

Quantitative analysis of wild-type and V600E mutant BRAF proteins in colorectal carcinoma using immunoenrichment and targeted mass spectrometry

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Introduction and Objectives

The BRAF V600E mutation is one of the most common mutations implicated in the development of several types of cancer including colorectal cancer (CRC), where it is associated with aggressive disease phenotypes and poor outcomes. The status of the BRAF V600E mutation is frequently determined by direct DNA sequencing. However, no previous study has sought to quantify the BRAF V600E protein in cancer specimens.

Methods

We evaluated immunoenrichment coupled with two MS-based quantitative techniques, namely multiple reaction monitoring (MRM) and single ion monitoring conjugated accurate inclusion mass screening (SIM-AIMS), to detect and precisely quantify wild-type (WT) and V600E mutant BRAF proteins in DNA sequence-confirmed CRC tissue specimens. WT and V600E BRAF proteins were immunoprecipitated from a CRC cell line (HT-29), and their representative peptides (592IGDFGLATVK601 and 592IGDFGLATEK601, respectively) were confirmed by LC-MS/MS analysis and then quantified by MRM or SIM-AIMS with spiked stable isotope-labeled peptide standards.

Results and Discussion

The MRM and SIM-AIMS assays worked well for measuring WT BRAF from different amounts of HT-29 cell lysates, but the MRM assay was more sensitive than SIM-AIMS assay for quantifying lower levels of V600E BRAF. In protein extracts (2 mg) from 11 CRC tissue specimens, the MRM assay could measure WT BRAF in all 11 cases (0.32 - 1.66 ng) and the V600E BRAF in two cases (0.1 - 0.13 ng; mutant-to-WT ratio, 0.16 ~ 0.17). The SIM-AIMS assay could also detect WT and V600E BRAF in CRC specimens, but the measured levels of both targets were lower than those determined by MRM assay.

Conclusion

Collectively, this study provides an effective method to precisely quantify WT and V600E BRAF proteins in complex biological samples using immunoenrichment-coupled targeted MS. Since the V600E BRAF protein has emerged as an important therapeutic target for cancer, the developed assay should facilitate future BRAF-related basic and clinical studies.

Keywords: Oncogene, BRAF V600E mutation, Protein quantification, Immunoenrichment, Targeted mass spectrometry

TP01-28

Quantitative tissue proteomics analysis of primary lung adenocarcinoma for potential biomarker discovery

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Introduction and Objectives

Lung cancer is the leading cause of cancer death worldwide. Lung adenocarcinoma (ADC) is the predominant histological type of lung cancer, comprising approximately 40% of non-small cell lung cancer (NSCLC) cases. The stage as well as lymph nodes (LNs) involvement in lung cancer is highly correlated to prognosis and mortality. Thus, it is emergent to identify and characterize the stage and/or metastasis-related biomarker for lung cancer diagnosis/prognosis and rational design of therapeutics.

Methods

We aimed to identify potential biomarkers for the diagnosis of early-stage lung ADC without LN metastasis using isobaric tags for relative and absolute quantification (iTRAQ) labeling combined with 2D-LC-MS/MS. The potential biomarkers were selected based on pathway and network analyses, protein expression profiles released in the public Human Protein Atlas database, literature search and novelty. The clinical significance of potential biomarkers was verified by western blot and immunohistochemistry staining (IHC). The cell viability, migration and invasion assays in gene-knockdown cells were used to examine the biological function of potential biomarkers in ADC progression.

Results and Discussion

We identified 133 candidates with 1.5-fold increase in expression in ADC tumors without LN metastasis compared with adjacent normal tissues. Six potential biomarkers were verified by Western blotting and IHC. We observed that the protein levels of all potential biomarkers were overexpressed in cancer tissues as compared with adjacent normal tissues. The levels of ERO1L and NARS were significantly higher in tumor cells from metastatic LNs than in corresponding primary tumor cells. Importantly, ERO1L overexpression in patients with early-stage ADC was positively correlated with poor survival, suggesting that ERO1L overexpression in primary sites of early-stage cancer tissues indicates a high risk for cancer micrometastasis. Moreover, both ERO1L and NARS modulated the mobility and viability of ADC cells.

Conclusion

Collectively, our study provides a potential useful biomarker dataset for lung ADC and

reveals novel roles of ERO1L and NARS in lung cancer progression.

Keywords: lung cancer; adenocarcinoma; lymph node metastasis; biomarkers; ERO1L, NARS, iTRAQ

TP01-29

Identification of Potential Biomarker Related to EGFR Mutation by Functional Proteome Profiling in Primary Non-small Lung Cancer

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Introduction and Objectives

Non–small-cell lung cancer (NSCLC) accounts for approximately 85% of lung cancers which the leading cause of cancer-related death worldwide. Recently, epidermal growth factor receptor (EGFR) activating mutations have been proved relevant to NSCLC and let treatment of EGFR tyrosine kinase inhibitors (EGFR-TKIs) become an alternative therapy for NSCLC patients. Despite excellent clinical response to EGFR-TKIs, the intrinsic and acquired resistance to EGFR-TKI created challenges in clinical practice. Although several mechanisms may contribute to solve EGFR?TKIs resistance, there still have 20% to 30% of these patients that how the resistance developed remain unclear. Studies indicated that the increment of membrane efflux may be associated with drug resistance. In this study, we use differential membrane proteomic analysis to identify potential biomarkers for therapeutics of EGFR-TIKs resistance in NSCLC patients.

Methods

Several primary NSCLC cell lines with different EGFR status were be isolated by our laboratory. Identification of modulators on membrane of primary cancer cells with different EGFR status was analyzed by membrane proteomic assay using LC-MS/ MS. The expression patterns of EGFR mutation cells would compare with that of wild type groups and the potential candidates would be selected by cross the results of interactome profiles of EGFR mutation cell lines. The expression patterns and functional role of these candidates were be examined in NSCLC cells by several molecular, cellular and biochemical analysis; and demonstrated the clinical relevance of these specific targets with EGFR mutation.

Results and Discussion

The expressions of 100 modulators up-regulated in EGFR mutation cells compared with that of wild type group. After cross reacted with the 474 interaction proteins of mutated EGFR, 8 proteins were selected as potential candidates that related to EGFR-TKI resistance, especially the expression of a protein we called SHB in EGFR mutation cells. We demonstrated the real expression levels of SHB in different lung cancer cells, and performed network analysis of SHB and EGFR in translational level.

Conclusion

The analysis of differential membrane proteomic found several novel biomarkers and potential regulation mechanisms that related to the development of EGFR-TKIs resistance in NSCLC. These identification may provide us new direction for exploiting therapeutic strategy for NSCLC treatment in the future.

Keywords: NSCLC, EGFR, membrane proteome, interactome

TP01-30

Proteomic Study on Pancreatic Cancer Patients with Long and Short Survivalswith Samples from the South Swedish Biobank

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Introduction and Objectives

Pancreatic cancer (PC) is the fourth lethal cancer with a 5-year survival of 6 percent. Proteome characterization of pancreatic tissues allows identification of potential protein biomarkers for early detection and progression of PC, which can improve the survival of PC. Using deep sequencing by shotgun proteomics, this study is aimed to find potential protein biomarkers for both diagnosis and prognosis of PC.

Methods

We collected formalin-fixed paraffin-embedded tumor tissues from the South Swedish Biobank, incuding nine PC patients with short survival (<12 months), ten PC patients with long survival (>45 months), seven locally metastasized lymph node tissues from these PC patients and six normal tissues from patients with benign serous cystadenoma. Proteins were extracted from tissues and digested into peptides, which were then fractionated into 5 fractions by strong cation exchange and analyzed on a platform of nano-liquid chromatography coupled with Quadrupole-Orbitrap mass spectrometry. Parallel reaction monitoring was conducted to confirm candidate prognostic proteins in the 19 PC patients. Proteome Discoverer software was applied for the identification and quantification of proteins. Comparison of the proteins in the patient groups were conducted by t-test based on median normalized intensity.

Results and Discussion

Around 20,000 peptides, which mapped to 3000 proteins, were identified in each sample. A total of 5750 proteins were identified from the 32 tissues. There were 1205 proteins differentially expressed in tumor tissues compared to the normal controls (>2 or <0.5 folds change, $P<0.01$), including 632 proteins and 573 proteins upregulated in tumor tissues and normal controls respectively. Furthermore, 171 proteins were more than 2 folds differentially expressed between patients with short survival and long survival ($P<0.05$), of which 24 proteins were up-regulated in the short survival group and 147 proteins in the long survival group, respectively. Besides, 284 proteins showed different expression between the original tumor tissues and the paired metastasized tissues (>2 or <0.5 folds change, $P<0.05$). Preliminary data on a targeted proteomic study by parallel reaction monitoring confirmed that seven proteins were upregulated in patients with long survival compared to short survival.

Conclusion

A number of protein biomarker candidates for diagnosis and prognosis of pancreatic

cancer have been identified on tissue level. Further studies in a larger sample size, using targeted LC-MS/MS or immunohistochemistry, are needed to validate these protein biomarkers.

Keywords: Proteomics, Biomarkers, Pancreatic cancer

TP01-31

Study on sputum cell proteomics for screening early detection biomarkers of lung cancer

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Introduction and Objectives

Lung cancer is the leading cause of cancer death in China and America. Currently, no available noninvasive biomarkers to detect early stage lung cancer. Sputum is directly contacted with the lungs and respiratory tract, allowing the pathogenic changes that occur in lung cancer tumor tissue to be transferred into sputum. In addition, sputum could be obtained noninvasively, making its collection feasible for population-based screening. Molecular study of sputum could detect the cells containing lung tumor-associated molecular aberrations, thus providing a noninvasive approach for diagnosis of lung cancer.

Methods

Proteomes of sputum samples from 15 patients with lung cancer and 13 controls with chronic obstructive pulmonary disease (COPD) were assayed using LC-MS/MS analysis. Sputum cytology was performed for each samples.

Results and Discussion

Totally, we identified 6605 proteins in lung cancer sputum and 6159 proteins in COPD sputum using the MaxQuant software. Overlap of proteins is 90.5%, and 540 proteins included exclusively in lung cancer. These proteomic profiles identified functional differences between lung cancer and COPD, related to translational elongation, translation and antigen processing and presentation of peptide or polysaccharide. We identified 4 candidate proteins, expression higher in lung cancer sputum. The expression level of candidates 1 in lung cancer tissues was significantly higher than that in normal lung tissue ($P<0.001$), which showing a promising clinical application.

Conclusion

Conclusion, in this study, we focused on tumor-related proteins in sputum of lung cancer patients as biomarkers.

Keywords: Lung cancer/Sputum/ Biomarkers/ Early Detection

TP01-32

Novel circulating peptide biomarkers for esophageal squamous cell carcinoma revealed by a magnetic bead-based MALDI-TOFMS assay

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Introduction and Objectives

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignant neoplasms worldwide. Patients are often diagnosed at advanced stages with poor prognosis due to the absence of obvious early symptoms.

Methods

We applied a high-throughput serum peptidome analysis to identify circulating peptide markers of ESCC. Weak cationic exchange magnetic beads coupled to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was used for two-stage proteotypic peptide profiling in complex serum samples collected from 477 patients and healthy controls.

Results and Discussion

We established a genetic algorithm model containing three significantly differentially expressed peptides at 1925.5, 2950.6 and 5900.0 Da with a sensitivity and specificity of 97.00% and 95.92% in the training set and 97.03% and 100.00% in the validation set, respectively. These peptides were identified as fragments of AHSG, TSP1 and FGA by linear ion trap-orbitrap hybrid tandem mass spectrometry. Notably, increased tissue and serum levels of TSP1 in ESCC were verified and correlated with disease progression. In addition, tissue TSP1 was an independent poor prognostic factor in ESCC.

Conclusion

In conclusion, the newly established circulating peptide panel and identified proteins could serve as potential biomarkers for the early detection and diagnosis of ESCC.

Keywords: Circulating peptide markers / ESCC / TSP1 / AHSG / FGA

TP01-33

Investigating the Platelet-derived Growth Factor B regulation network in Gastric Cancer with a label free quantitative proteomics approach

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Introduction and Objectives

Gastric cancer is one of the most common cancers in Asian countries and is the fourth most commonly occurring cancer worldwide (9% of all cancers). In the past decade, numerous groups have attempted to profile the expression changes in gastric cancer tissues using proteomic approaches, in search of diagnostic and prognostic biomarkers. However, investigating signalling network with proteomics approach is rarely reported. In this study, We aimed to investigate the PDGFB regulation network in gastric cancer with a label free proteomics approach.

Methods

Six Pairs of gastric tumor tissue and adjustment normal gastric tissues were obtained from surgical resection and analyzed with QExactive Plus coupled with nanoLC system with a label-free quantitative approach. The differentially expressed proteins were validated by Western blotting analysis. the PDGFB regulation network was predicted from differenctially expressed proteins with Ingenuity IPA. To validate the hypothezed network, the PDGFB was silenced in gastric cancer cells with shRNA based approach. The predicted proteins were validated with western blot in the PDGFB knockdown cells. To evaluate the effect of PDGFB on Gastric cancer cells, Cell proliferation was measured in PDGFB knockdown cells with xCELLigence Real-Time Cell Analyzer (RTCA)-DP system.

Results and Discussion

Conclusion

Our studies suggested that PDGF-B signaling pathway plays an important role in the regulation of gastric cancer proliferation and may be a reasonable approach for treatment of gastric cancer.

Keywords: Gastric cancer, quantitative proteomics, label free, PDGFB

TP01-34

Proteomic analysis of novel targets associated with simvastatin-induced cell death in DU145 human prostate cancer cells

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Introduction and Objectives

Docetaxel is the most commonly prescribed anti-cancer agent in the standard first-line chemotherapy for metastatic castration-resistant prostate cancer (CRPC). Interestingly, it is known that simvastain enhances docetaxel-induced cell death in PC3 human CRPC cells, indicating a role of simvastatin-regulated targets in the improvement of cancer cell death. Until now, simvastatin-regulated targets associated with cell death in CRPC cells have not been identified by 2D proteomic analysis. To better understand a prostate cancer cell death mechanism caused by simvastatin, here we investigated novel targets associated with simvastatin-induced cell death in DU145 human CRPC cells by 2D proteomic analysis.

Methods

1. Cell morphology analysis2. Detection of dead cells3. Cell viability analysis 4. Sample preparation, 2D electrophoresis and CBB staining 5. Image analysis and statistical significance 6. Mass spectrometric analysis and database search 7. Western blot analysis

Results and Discussion

Recently, simvastatin is known to improve the effectiveness of chemotherapeutic agents in cancers. In this study, we showed that simvastatin enhanced the downregulation of cell viability by anti-cancer agent docetaxel in DU145 human prostate cancer cells by inducing morphological change and cell death. To better understand the molecular mechanisms associated with simvastain-induced cell death, we identified 7 simvastatin-regulated protein spots through CBB-stained 2D proteomic analysis and MALDI-TOF/TOF mass spectrometric analysis. Except for vimentin, we proved that the heterogeneous nuclear ribonucleoprotein K (hnRNP K), thioredoxin domain-containing protein 5 (TXNDC5), cytoplasmic hydroxymethylglutaryl-CoA synthase (cHMGCS), N-myc downstream-regulated gene 1 (NDRG1), ras-related protein Rab-1B (RAB1B) and isopentenyl-diphosphate Delta-isomerase 1 (IDI1) proteins were either upregulated or downregulated by simvastain from the results of Western blot analysis on the each protein spot, 2D samples, and whole cell extracts. Moreover, our results demonstrated that cHMGCS, IDI1 and NDRG1 were significantly upregulated by simvastatin during docetaxel-induced c-Jun N-terminal kinase (JNK) activation, whereas TXNDC5 was downregulated by simvastatin. We suggest here that simvastatin could alter cell morphology and induce cell death associated with JNK signaling through the molecular mechanisms by the simvastatin-regulated targets, which could be targeted for the efficient therapy of chemotherapeutic agents in various cancers.

Conclusion

TP01-34

We identified 5 novel simvastatin-regulated targets, cHMGCS, IDI1, TXNDC5, hnRNP K and RAB1B, as well as 2 already known targets, vimentin and NDRG1, in DU145 CRPC cells, and these simvastatin-regulated targets were implicated in the docetaxel-induced cell death signaling associated with JNK activation, suggesting a new molecular mechanism for the efficient cancer cell death.

Keywords: Simvastatin, Docetaxel, DU145 cell death, Prostate cancer, 2D proteomic analysis

TP01-35

Verification of Prostate Cancer Genomics Biomarker Candidates at Protein Level Using SRM-MS

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Introduction and Objectives

Targeted MS-based proteomics such as selected reaction monitoring (SRM) provide an antibody-independent strategy for sensitive, specific and multiplexed verification of genomics biomarker candidates at the protein level. In this study, 52 biomarker candidates for prostate cancer derived from genomics data were selected and analyzed in tumor and control tissue samples using SRM to identify a panel of differentially abundant proteins with the potential to discriminate between aggressive and indolent forms of prostate cancer, based on their increased expression in tumors with advanced Gleason scores.

Methods

High-pressure, high-resolution separations coupled with intelligent selection and multiplexing (PRISM)-SRM assays have been developed for 52 prostate cancer biomarker candidates, derived from genomics data including: prostate cancer prognosis associated genes, prostate cancer associated genes that were up-regulated in transcriptomics studies, and other cancer related genes (including the ERG or ETV1 isoforms). Ten OCT-embedded high Gleason-score (7-9) prostate tumor and 10 benign prostatic hyperplasia (BPH) tissue samples were analyzed using PRISM-SRM with heavy isotope-labeled synthetic peptides as internal standards.

Results and Discussion

PRISM-SRM analysis of patient tissue samples as well as a VCaP xenograft tissue sample enabled the detection of 46 out of 52 biomarker candidates, suggesting extremely low level of expression of the remaining 6 genes (HXC6, OSTP, PAFA, TPM2, TWST1, and ERG8); regular LC-SRM can only detect 21 of these candidates at protein level. In the 10 high Gleason-scores tumors and 10 BPH controls, a total of 42 proteins were quantified and compared, among which 13 proteins were differentially abundant in tumors versus controls, with a p value <0.05 (HPN, AMACR, MYO6, NCOA2, PK3CA, STAT3, CAMKK2, HSPB1, SERPINI1, FOLH1, TGFB1, RAF1, and MMP2). Although PSA and ERG levels were increased in tumors by 2.2- and 4-fold, respectively, the calculate p values were slightly greater than 0.05 (0.056 and 0.064, respectively). These promising biomarker candidates will be further evaluated in independent, larger cohort for their potential prognostic applications.

Conclusion

TP01-35

PRISM-SRM provides a highly sensitive method for multiplexed quantification of potential biomarker candidates at the protein level, enabling rapid screening of multiple candidate biomarkers at the protein level. This approach holds great potential for rapidly translating genomics-based discovery candidates into protein-based biomarkers.

Keywords: Prostate cancer; biomarker; selected reaction monitoring; quantitation; PRISM-SRM.

PROTEOMICS IDENTIFIED THE PROTEINS ASSOCIATED WITH TUMOR INVASION IN MYXOFIBROSARCOMA

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Introduction and Objectives

Myxofibrosarcoma (MFS) is a rare mesenchymal malignancy with a high prevalence in the elderly. MFS is refractory to both chemotherapy and radiotherapy, and the surgical operation is the only curative treatment. Recurrence after surgery was observed in 50-60% cases, and there is no effective treatment for those cases. MFS cells are highly invasive, and the elucidation of the molecular mechanisms of the invasive behaviors of MFS cells has been required for the better treatment. We aimed to identify novel candidate proteins responsible for highly invasive activity of MFS.

Methods

We examined the proteomic profiles of the 11 biopsied tumor tissues obtained prior to treatments. Based on the pathological observation, the samples were classified into two groups: the invasive MFS group that exhibited the tumor cell invasion in neighboring tissues (n=6), and the non-invasive MFS group (n=5). Proteomic profiling was achieved using large format two-dimensional difference gel electrophoresis (2D-DIGE) and mass spectrometry.

Results and Discussion

The protein expression profile produced by 2D-DIGE consisted of 3453 protein spots. Among them, 59 protein spots had the intensity with >2-fold difference between the invasive and non-invasive MFS groups with statistical significance ($P<0.01$). Mass spectrometric protein identification showed that these 59 protein spots corresponded to 47 distinct proteins. Those included cell motility regulators, cytoskeleton organizers and cell cycle regulators. Literature mining and functional classifications revealed that those proteins were relevant to clinically important events such as poor prognosis and tumor invasion in the other malignancies, but not in MFS. After extensive immunohistochemistry, one protein was expressed on the membrane of tumor cells, and correlated with frequent invasion in the additional 21 MFS cases with statistical significance ($P<0.05$).

Conclusion

Proteomic investigation resulted in the identification of proteins, whose expression was significantly associated with the frequent invasion in MFS patients. These proteins will be an innovative seeds for novel treatment for the better prognosis of MFS patients.

TP01-36

Keywords: myxofibrosarcoma, 2D-DIGE, invasion, immunohistochemistry

TP01-37

Development for Lung Cancer Diagnostic Meta-markers using Multiple Reaction Monitoring

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Introduction and Objectives

Lung cancer has the low symptomatic its specific characteristic and lack of diagnosis tools in early stage with high sensitivity. To develop clinical diagnostic tools for lung cancers in early stage, robust meta-markers, which are combination of multiple markers with conventional assay platform, are on high demand. In this study, 50 healthy control and 50 lung cancer clinical samples were subjected for LC-MRM-MS analysis on multi lung cancer biomarker candidates and modeling for generating meta-marker was conducted.

Methods

4 targets were selected from proteome profiling in the lung cancer sera and secreted media on primary cultured lung cancer cell line. Quantitative analysis of these targets were conducted on albumin and IgG depleted serum samples (healthy=50, lung cancer=50) using LC-MRM-MS. The data was analyzed using logistic model, a type of probabilistic statistical classification model.

Results and Discussion

Quantitative analysis on patient samples using MRM showed various serum levels of target proteins in a recurrent time dependent manner. To develop robust diagnostic model, which called ‘meta-marker’, logistic regression model was used. Finally, we found a best combination of three target proteins reflecting disease status and showing best predictive diagnostic capability.

Conclusion

Our results suggested that the combination of potential several biomarkers via statistical model provide better diagnostic specificity and sensitivity than a single biomarker for the diagnosis in lung cancer patients.

Keywords: Lung Cancer, Biomarker, Multiple Reaction Monitoring, Combination

TP01-38

Phosphoproteomic analysis aimed at elucidating the mechanisms underlying the high malignancy of ovarian clear cell carcinoma

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Introduction and Objectives

Cancer cells accumulate various mutations and sometimes acquire novel features related to malignancy, such as metastasis, limitless proliferation, and antitumor drug tolerance. Ovarian clear cell carcinoma (OCCC) is a highly malignant cancer that frequently exhibits resistance against first-line antitumor drugs (e.g., platinum-based agents) and a high recurrence rate, resulting in poor prognosis relative to other types of ovarian cancers. To obtain insight into the mechanisms involved in malignant transformation of OCCC, we performed comparative phosphoproteomic analysis, Selected Reaction Monitoring (SRM)/MS-based quantification of protein phosphorylation levels, and analyses of cell lines with a mutation in the detected phosphorylation site.

Methods

Comparative phosphoproteomic analysis was performed using six OCCC and four non-OCCC ovarian cancer cell lines. The set of proteins with significant up/downregulation of phosphopeptides was subjected to pathway enrichment analysis using DAVID Bioinformatics Resources. Phosphorylation levels of detected proteins were verified by the SRM/MS-based detection of phospho- and non-phosphopeptides in the same LC/MS run. Trypsin digests obtained from SDS-PAGE bands of immunoprecipitated proteins were analyzed by Qtrap 5500 LC-MS/MS. Protein and phosphoprotein levels were also confirmed by immunoblot analysis using 10 OCCC and five non-OCCC cell lines. A phosphorylation-site serine of Brg1 was replaced with either alanine or aspartic acid to delete or mimic phosphorylation by site-directed mutagenesis using HEK293T cells. Stable clones were constructed, analyzed for various phenotypes related to cancer malignancy, and subjected to comparative proteomic/phosphoproteomic analyses.

Results and Discussion

Among 620 phosphopeptides with ANOVA p value <0.01, the phosphopeptide levels of five components of the SWI/SNF chromatin remodeling complex (Brg1, Arid1a, Baf155, Baf170, and Rpb1), were significantly downregulated in OCCC cells. SRM/MS analysis revealed that the phosphorylation level of Brg1 at Ser1452 was 10 times lower in OCCC cells than in other non-OCCC cells. This result was confirmed by immunoblot analysis using an antibody raised against the phospho-Brg1 peptide, while there was no significant difference in Brg1 levels between OCCC and non-OCCC strains, as shown using anti-Brg1 antibody. By contrast, protein levels of Arid1a, a tumor suppressor protein mutated in >50% of OCCC, were significantly downregulated in many OCCC cell lines. A mutant strain lacking the phosphorylation site of Brg1 proliferated more rapidly than wild-type or phosphorylation-mimic strains. Comparative proteomic/phosphoproteomic analyses revealed marked quantitative differences in proteins/phosphoproteins related to histone modification, transcription, nuclear transport, and DNA binding, as well as some

oncoproteins, in phosphorylation-deleted strains. Taken together, these results demonstrate the crucial roles of Brg1 phosphorylation in the controlled expression of various cancer-related proteins via chromatin remodeling by the SWI/SNF complex.

Conclusion

Our results suggest that reduced phosphorylation of Brg1 is a novel molecular signature of OCCC that might be related to the high malignancy of OCCC through reduced antitumor activity of the SWI/SNF complex.

Keywords: phosphorylation, ovarian cancer, malignancy, Brg1, SWI/SNF complex, SRM

TP01-39

Potential diagnostic value of protein biomarkers in albumin-depleted pleural effusions for detection of NSCLC malignancy and EGFR mutation status

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Introduction and Objectives

Pleural effusion (PE) is caused by a variety of medical conditions, including benign causes such as cardiopulmonary disease, pneumonia, and tuberculosis, and malignant causes such as lung cancer metastasis. Thus, prompt aetiological diagnosis of malignant pleural effusion is important for treatment and prognosis of the patients. In this study, we aim to identify potential protein biomarkers in PE that may be used for differential diagnosis of malignant and benign PE, as well as for indication of EGFR mutation status in malignant PE. However, detection of lower abundant protein biomarkers in the presence of high abundant albumin is challenging. Standard multiple antibody affinity-based depletion approach is difficult to translate to clinical practice; an effective and reproducible depletion method remains unmet.

Methods

Three pooled PE samples: benign control (2 cardiopulmonary, 5 pneumonia, 1 tuberculosis), malignant EGFRwt (8 NSCLC with wide-type EGFR) and malignant EGFRmt (8 NSCLC with mutant EGFR), were selectively albumin-depleted by trichloroacetic acid (TCA)/acetone precipitation and the remaining proteins were relatively quantified using Tandem Mass Tag (TMT) isobaric tags. A basic reversed-phase fractionation of the labelled peptides was carried out to enhance the dynamic range of proteome coverage prior to LC-MSMS analysis.

Results and Discussion

The results showed that TCA/acetone precipitation is a simple and reproducible method for the selective depletion of albumin in PE, with more than 90% of protein abundances in technical duplicates of all three samples having percentage standard errors of 20% or less. A number of proteins implicated in lung cancer: polymeric immunoglobulin receptor (PIGR), peroxiredoxin-2 (PRDX2) and alpha-enolase (ENO1), were detected at ratios greater than 2:1 malignant to benign PE. In addition, apolipoproteins, complement components and fibrinogen chains were found to be present at higher concentrations in EGFRmt compared to EGFRwt PE samples; the association of serum C3 complement and fibrinogen a chain to NSCLC have been reported. This observation was consistent with the key findings of a parallel metabolomics study, which noted the elevation of several lipid classes in malignant PE.

Conclusion

Our results suggest that proteome analysis of albumin-depleted PE is a promising diagnostic test for NSCLC in clinical practice, and may also be useful for assessing EGFR mutation status in NSCLC.

Keywords: Pleural effusions, protein biomarker, albumin-depletion, NSCLC malignancy and EGFR mutation

TP01-40

Identification of cerebrospinal fluid markers for recurrent malignant brain tumor by quantitative proteomics.

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Introduction and Objectives

Malignant brain tumor was generally diagnosed by means of magnetic resonance imaging (MRI), however MRI can't distinguish between early recurrence and pseudoprogression. Therefore, as alternative diagnosis, the biomarker for detecting recurrent malignant brain tumor has been required. Nevertheless, biomarker for recurrent malignant brain tumor has not been identified yet. The purpose of the present study was to identify diagnosis marker for detecting recurrent malignant brain tumor by comparing the cerebrospinal fluid (CSF) collected from preoperative, postoperative and recurrence of same patient with malignant brain tumor, and those with no brain tumor by quantitative proteomics.

Methods

CSF specimens were digested by trypsin. Expression of proteins in the CSF was compared by quantitative proteomics analysis with data independent acquisition (SWATH-MS) for biomarker discovery. Analysis object are peptides that false discovery rate is under 10% and peak area is over 10000 in more than 90% of all specimens.

Results and Discussion

Tryptic digest of CSF (0.5 mg protein) from preoperative (14 specimens), postoperative (14 specimens) and recurrence (5 specimens) of same malignant brain tumor patients and CSF from no brain tumor patients (5 specimens) were analyzed by global quantitative proteomics. As a result, we identified 18 tryptic digest peptides and 11 proteins as marker candidates discriminating between preoperative and postoperative patients or postoperative and recurrent patients. The maximum of AUC discriminating preoperative specimens from postoperative specimens was 0.91 and recurrence specimens from postoperative specimens was 0.81 by ROC analysis with single peptide or protein. Multivariate analysis with all candidate peptides could distinguish group of preoperative from group of postoperative with AUC=0.96 and group of recurrence from group of postoperative with AUC=0.89 by ROC analysis. Diagnosis ability was improved by using all candidate peptides in comparison with using one candidate peptide.

Conclusion

In conclusion, we identified peptides and protein in CSF as potential marker candidates for discriminating among preoperative, postoperative and recurrent patients.

Keywords: malignant brain tumor, quantitative proteomics, biomarker, cerebrospinal fluid

TP01-41

Changes in Protein Expression between Primary Breast tumour and lymph node or distant metastases

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Introduction and Objectives

Breast cancer is a very heterogeneous disease and some patients are cured by the surgical removal of the primary tumour whilst other patients suffer from metastasis and spreading of the disease, despite adjuvant therapy (1). A number of prognostic and treatment predictive factors have been identified such as tumour size, oestrogen (ER) and progesterone (PgR) receptor status, human epidermal growth factor receptor type 2 (HER2) status, histological grade, Ki67 and age. Lymph node involvement is also assessed during surgery to determine if the tumour has spread, which requires dissection of the axilla and adjuvant treatment. The prognostic and treatment predictive factors assessing the nature of the tumour are all routinely based on the status of the primary tumour (2).

Methods

We have analysed a unique tumour set of fourteen primary breast cancer tumours with matched synchronous axillary lymph node metastases and a set of nine primary tumours with, later developed, matched distant metastases from different sites in the body. We used a pairwise tumour analysis (from the same individual) since the difference between the same tumour-type in different patients was greater (3). We used a specific glyco-capture technique developed by Berndt Wollschied (4).

Results and Discussion

One clinically very important observation was the change in receptor status between primary tumour and metastasis. For all three receptor types, we often saw a switch from either positive to negative upon metastasis or vica-versa. This has important consequences for treatment.We show that the expression of a large number of glycosylated proteins change between primary tumours and matched lymph node metastases and distant metastases, confirming that cancer cells undergo a molecular transformation during the spread to a secondary site.

Conclusion

The proteins are part of important pathways such as cell adhesion, migration pathways and immune response giving insight into molecular changes needed for the tumour to spread.We are currently analysing changes in the expression of enzymes involved in the changes in glycoprotein synthesis and decoration and will present a detailed description of the steps involved in the progression from primary tumour to metastasis.

Keywords: Breast cancer, Lymph node metastases, Distant metastases, Biomarkers, Secreted proteins,Membrane proteins, Mass spectrometry

TP01-42

2D Proteomic profile following Agrimonia Pilosa Ledeb roots extract treatment in oral squamous cell carcinoma

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Introduction and Objectives

Oral squamous cell carcinoma (OSCC) is known as the most common malignancy and a major cause of worldwide cancer mortality. Oral squamous cell carcinomas (OSCCs) are characterized by a marked tendency for local assault, so the identification of agents inhibiting the inception and progression of OSCC has recently gained notice. Agrimonia pilosa is the aerial parts of the Agrimonia pilosa Ledeb, which belongs to the Rosaceae plant family; it is mainly used in the treatment of various hemorrhage, malaria, fatigue, etc. In the present study, we investigated the antitumor activity of the methanol extract from roots of Agrimonia pilosa Ledeb (ARE), improving the experimental basis for the in-depth study of pharmacological effects of roots of Agrimonia pilosa Ledeb .

Methods

Cancer Cell Strains YD-10B cells (received from Dept. of Oral Pathology and Pusan National University)Preparation of ARE 500g of dry Agrimonia pilosa was weighed and added with water 15 times its volume; the solution was extracted for three times using ultrasonic extraction with 1 h each.Determination of Cell viability Fresh culture medium was added again and single cell suspension was prepared, after cell counting, the concentration of suspension was prepared as 1×104/ml. Different concentrations of drugs (0, 10, 20, 25, 30, 40, 50 ?/?) were added in the cell culture microplate, with at least three parallel wells for each concentration; solvent control-treated cells were prepared as the control group.Two-Dimensional gel electrophoresis (2D-GE) Secreted proteins (40 ?) were first cleaned using the 2D clean-up kit.Image and data analysis Gel images were analyzed using the ImageMaster 2D Platiumum V 7.0 software. Briefly, the 2D gel images were subjected to spot detection and quantification in the differential in-gel analyses module.

Results and Discussion

In this study we have followed proteomic approach to investigate the effects of ARE treatment in YD-10B OSCC cells. 2D based quantitative proteome studies showed that among totally quantifiable many proteins and bioinformatic analysis further revealed that those quantifiable proteins were mainly involved in multiple metabolic and enzyme-regulated pathways as anti-cancer drugs. Here, we found that ARE inhibited cell proliferation in YD-10B OSCC cells.

Conclusion

Most of identified proteins that were differently altered in ARE treated cells were involved in regulating the many biological processes in the human body.

TP01-42

Keywords: Oral squamous cell carcinoma, Agrimonia Pilosa Ledeb Roots, 2D based quantitative proteome

TP01-43

Anchorage-independency altered tubular phenotype of melanoma tumors through downregulation in ANPEP/SDC1/beta4-integrin axis

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Introduction and Objectives

Detachment of tumor cells from extracellular matrix and survival under anchorage-independency was recognized as initial step of malignant tumor metastasis. In our previous studies, the phenotypes of anoikis-resistant melanoma cells were characterized and alterations of specific gene expression were found to explain the loss of cell invasiveness under cell suspension through downregulation of SDC1 expression , but enhanced malignancy in subcutaneous xenograft tumors.

Methods

Quantitative proteomic analysis by stable isotope dimethyl labeling at cytosolic and nuclear compartments of adherent, suspended, and reattached melanoma cells were done and the relative protein expression were compared. Suppression of protein expression at melanoma cells was done upon gene-specific shRNA transfections and were analyzed by qPCR, western blot, and flow cytometry. The effect of suppression in protein expression at melanoma cells were investigated by cell proliferation, transwell migration ability, and animal xenograft. Histological phenotypes or specific protein expression of subcutaneous tumors were visualized by H/E stains or immunohistochemistry, respectively.

Results and Discussion

Histological phenotypes of subcutaneous tumors inoculated with adherent melanoma cells showed networks of vascular mimicry. In contrast, they were not found in the tumors inoculated with suspended melanoma cells. Proteomic analysis showed downregulation of many protein associated with tubular formation. Of them, aminopeptidase N (ANPEP) was downregulated in both suspended and reattached melanoma cells. The suppression of ANPEP expression by shRNAs showed reduction in cell invasiveness, inhibition of SDC1 expression, but promoted tumor malignancy, which was consistent with previous observation in suspended melanoma cells. Downregulation of SDC1 expression also lead to suppression of beta4-integrin expression. These proteins associated with tubular-vascular formation (ANPEP, SDC1, beta4-integrin) were also downregulated in xenograft tumors inoculated with suspended melanoma cells.

Conclusion

We proposed the regulatory axis of ANPEP / SDC1 / beta4-integrin upon cell detachment of melanoma cells would exhibit critical role in alteration of vascular phenotype and malignancy of melanoma cells.

Keywords: Anchorage independency, melanoma, dimethyl labeling, aminopeptidase N, tubular formation

TP01-44

The roles of AKR1C1 and AKR1C2 in ethyl-3, 4-dihydroxybenzoateinduced esophageal squamous cell carcinoma cell death

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Introduction and Objectives

The aldo-keto reductase (AKR) superfamily of enzymes is critical for the detoxification of drugs and toxins in the human body; these enzymes are involved not only in the development of drug resistance in cancer cells but also in the metabolism of polycyclic aromatic hydrocarbons. Our previous studies have shown that ethyl-3, 4-dihydroxybenzoate (EDHB) can effectively induce esophageal cancer cell autophagy and apoptosis, and the AKR1C family represents one set of highly expressed genes after EDHB treatment. Here, we demonstrated that AKR1C1/C2 increased the metabolism of EDHB in esophageal squamous cell carcinoma (ESCC) cells.

Methods

To explore the cytotoxic effects of EDHB, esophageal cancer cells with higher (KYSE180) or lower (KYSE510) AKR1C expression levels were evaluated by cell proliferation assay. The effective subunits of the AKR superfamily were quantitatively identified using multiple reaction monitoring (MRM) assays. AKR1C1/C2 was knocked down by siRNA in ESCC cells and apoptosis and autophagy related proteins were further investigated.

Results and Discussion

The proliferation of KYSE180 cells was inhibited more effectively than that of KYSE510 cells by EDHB treatment. Furthermore, among the effective subunits of the AKR superfamily, AKR1C1/C2 that were determined by MRM assays was found to be elevated in KYSE 180 cells, but no obvious change in KYSE 510 cells after EDHB treatment. The sensitivity of esophageal cancer cells to EDHB was significantly attenuated by the siRNA knockdown of AKR1C1/C2. We also demonstrated that AKR1C1/C2 increased the metabolism of ethyl-3,4-dihydroxybenzoate (EDHB) in esophageal squamous cell carcinoma (ESCC) cells. Moreover, the expression of autophagy inducers (Beclin, LC3II and BNIP3) and NDRG1 was significantly elevated in KYSE180 cells, but not in KYSE510 cells, after EDHB treatment. When autophagy was inhibited by 3-methyladenine, KYSE180 cells exhibited an increased sensitivity to EDHB.

Conclusion

These results indicated that ESCC patients with high AKR1C1/C2 expression may be more sensitive to EDHB, and AKR1C1/C2 may facilitate EDHB-induced autophagy and apoptosis, thus providing potential guidance for the chemoprevention of ESCC.

Keywords: Aldo-keto reductase 1C1/C2 / Ethyl-3, 4-dihydroxybenzoate / MRM / ESCC

TP01-45

Identification of hypoxia-induced splicing variants in cancer cells using proteomics approach

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Introduction and Objectives

Almost 70% of human proteins are alternatively spliced – a phenomenon involved in not only life-sustaining processes such as metabolism and tissue composition but also diseases such as cancer.In the adaptation of cancer cells to a hypoxia environment, alternative splicing is implicated in helping them grow in the harsh environment. The resultant cancer-specific splicing variants may be turned into diagnostic, prognostic, and predictive biomarkers. Genome-wide analysis of alternative splicing is typically pursued on the genome level using high-throughput approaches such as DNA microarray or deep sequencing. However, these methods examine the alternatively spliced transcripts instead of the protein products. In the current study, a novel proteomics approach was developed to confirm the existence of alternative spliced proteins by mass spectrometry. We have identified a few novel alternative spliced variants using this new approach and the consequent biological evaluation is undergoing. We believe that our novel method will provide a new direction for alternative splicing-omics studies in the future.

Methods

The human cancer cell lines (MCF7), grown in DMEM medium, were incubated for 24 h to attach and then were incubated for 48 h at normaxia or hypoxia. For protein identification, tryptic peptides were analyzed using Thermo ScientificTM LTQ Orbitrap. We constructed a Generic peptide database, which contains many different types of alternative spliced isoform through bioinformatics tool. MS data were searched on a Generic peptide database using MASCOT MS/MS ion search.

Results and Discussion

We exploit an alternative splicing proteome analysis technique to identify and quantify alternative splicing isoform. We compare hypoxia data with normaxia data to find their unique peptides, which may be a key point about how cancerous organs' cells respond to hypoxia. To validate our newly developed method, we use RNA-seq technology to confirm that the mRNA, the peptide corresponding to, is real in cancer cells. In the future, we hope that such an integrative systems approach can provide a method about development and validation of alternative splicing isoform.

Conclusion

not applicable

Keywords: alternative splicinghypoxiacancer

TP01-46

Quantitative analysis of HER2 protein expression in multiple cancer indications

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Introduction and Objectives

Aberrant HER2 activity due to gene amplification and consequent protein overexpression results in a HER2-driven oncogenic phenotype. HER2 is amplified or overexpressed in approximately 20% of breast cancers and 22% of gastric cancers as assessed by immunohistochemistry (IHC) and/or in situ hybridization (ISH). Patients with these cancers have responded remarkably to anti-HER2 therapies, prompting ongoing trials of such therapies in other tumor types where HER2 is overexpressed. HER2 positivity (IHC 3+ and/or amplification) rates >10% have been reported in bladder (5%-15%), ovarian (6%-19%), and gallbladder (12.8%) cancers. Variability of HER2 positivity rates is due in part to the lack of standardized HER2 assessment methods for cancers other than breast and gastric. We previously reported that quantitative HER2 measurement by targeted MS is superior to IHC and ISH in predicting outcome after treatment with anti-HER2 therapy. Here, we applied targeted MS to quantify HER2 protein and other clinically-actionable proteins from FFPE patient biopsies of various tumor types.

Methods

FFPE tissue blocks (n=3,444) representing a range of cancer indications were obtained and tumor area of >=8mm^2 from 1 or 2 sections of each block were marked by board-certified pathologists. Following laser microdissection of marked areas, tumor cell proteins were extracted using the Liquid Tissue process and subjected to selected reaction monitoring MS to quantify 27 protein targets in each patient sample.

Results and Discussion

Quantitative proteomic analysis yielded a wide range of HER2 protein expression (from <LOD to 24,671 amol/ug). HER2 overexpression rates were 20.0% in breast (121/605), 19.4% in esophageal cancers (6/31), 12.5% in gall bladder (1/8), 12.0% in bladder/genitourinary system (3/25), 5.9% in liver (1/17), and 3.4% in pancreas (3/88). Lung, head and neck, colorectal, and gynecological cancers yielded HER2 overexpression rates of 2.9% (20/696), 1.2% (1/82), 3.0% (4/134), and 11.9% (5/42), respectively. Co-expression of other actionable target proteins, such as PD-L1, EGFR, and MET, were detected in some samples. Targeted proteomics analysis of HER2 protein expression in 3,444 samples of various tumor types was performed in a single, accredited laboratory. Unlike traditional methods, targeted MS offers objective quantification of HER2 and other clinically-relevant protein markers from a single microgram of tumor biopsy, thus identifying patients with various cancer types who may benefit from targeted therapies.

Conclusion

Targeted MS analysis provides clinicians with valuable diagnostic information to ensure

TP01-46

that all patients have the opportunity to receive treatment tailored to their clinical characteristics, in order to avoid unnecessary toxicities. By providing absolute quantitation of multiple actionable proteins from patient biopsies, targeted proteomics is delivering personalized cancer care.

Keywords: Targeted proteomics, HER2 quantitation, FFPE tumor tissue, Mass Spectrometry, cancer diagnosis

TP01-47

Iron deprivation perturbs ribosome biogenesis on protein synthesis and cell growth in MCF-7 human breast cancer cells

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Introduction and Objectives

Perturbation of ribosome biogenesis has been shown to activate p53 expression and cause translational arrest, inhibition of cell growth and apoptosis. Iron deprivation using with iron chelator DFO has demonstrated efficacy in promoting p53 activation and inhibiting of cell growth in several cancer cells. Therefore, in this study our aim was investigated whether inhibition of cell growth and p53 activation by iron chelator are associated with the function of ribosome biogenesis.

Methods

Results and Discussion

Combined two replicate of stable isotope dimethyl labeling-quantitative proteomic experiments, 114 proteins were up-regulated and 68 down-regulated proteins were identified in DFO-treated nuclear faction for 48hr, respectively. Furthermore, a total of 144 up-regulated and 96 down-regulated were identified in DFO-treated post-nuclear fractions, respectively. Using a hypergeometric probability distribution, “ribosome biogenesis” including rRNA processing and modification, ribosme assembly and transport were found to be overexpressed ($p < 0.05$) for the upregulated proteins in DFO-treated nuclear fraction. DFO treatment can led to a reduction in the fractions of ribosomes that are engaged in polysomes. The SUnSET results also support that a decrease in protein synthesis was observed in DFO-treated cells. DFO treatment led to the accumulation of cells arrested in G0/G1 phase, with concomitant decreased BrdU incorporation and induce cell apoptosis. The member of AP1 activator c-Jun and c-Fos, and p53 levels were high expressed in DFO treated nuclear fraction. In contrast, the protein levels of ribosome export adaptor NMD3 was reduced in both of DFO-treated nuclear and post-nuclear fractions.

Conclusion

In summary, perturbation of ribosome biogenesis by iron chelator suggests causes inhibition of mRNA translation, and trigger AP1 and p53 activation to inhibit cell growth.

Keywords: Iron chelator, ribosome biogenesis, cell growth, breast cancer

TP01-48

Quantitative Proteomics Investigation of Frataxin Over-Expression in Colorectal Cancer Cells

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Introduction and Objectives

Metabolic alterations such as Warburg effect are essential for cancer cells to maintain aggressive and proliferative behavior. Although Frataxin (FXN) deficiency is embryonic lethal and associated with reduced mitochondrial ATP production and increased tumor progression, the molecular mechanisms behind FXN function are largely unknown. To elucidate the role of FXN in tumor cells, we investigated the proteome changes induced by stable FXN over-expression in colorectal cancer cell line HCT116 (OF).

Methods

Frataxin cDNA was cloned into Vivid Colors™ pcDNA™ 6.2/EmGFP-Bsd/V5-DEST mammalian expression vector backbone (Invitrogen) and transfected into Human colorectal carcinoma cell line HCT116. Stably transfected cells were selected and maintained under 7.5µg/ml Blasticidin (Invitrogen). Three different FXN over-expressing clones (OF1, OF5 and OF6) were chosen as biological triplicates for subsequent experiments. Control cells were made to express bacterial Chloramphenicol acetyltransferase (CAT) as a control gene. iTRAQ quantitative proteomics analysis was conducted to compare the FXN-overexpressing cell lines with the control. The selected proteins showing differential expression were validated with SWATH. Metabolite analysis was conducted to validate some of the findings. Functional analysis of the cells including measurements of mitochondrial membrane potential and ROS scavenging and cell cycle analysis were also performed.

Results and Discussion

Conclusion

Keywords: Frataxin; overexpression; colorectal cancer; HCT116; iTRAQ; SWATH-MS

TP01-49

Using SWATH to analysis Peripheral Blood Mononuclear Cells of Pancreatic cancer

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Introduction and Objectives

Understanding how the immune system affects during cancer development and progression has been one of the most challenging questions in immunology. Owning to the complex and unrevealed mechanism of cancer, tumor immunologic diagnosis which are mostly based on tumor antigens, antibodies and radio immune image are not developed much for years. To observe proteins expression related to immune response may be an optional way for us to detect tumor at an early stage. Peripheral Blood Mononuclear Cell (PBMC) cells including T cells, B cells, natural killer cells, mononuclear cell, granulocytes, play important roles in innate system and adaptive immunity system. And the method for acquiring of PBMCs is non-invasive .In this study, We analyzed PBMCs of serum from normal people and patients bearing Pancreatic cancer (PC) to find some potential biomarkers and mechanisms for the process of PC in immune system.

Methods

Protein of PBMCs of serum from normal people and patients bearing Pancreatic cancer (PC) were extracted and analyzed using SWATH technology based on 1D LC separation on a TripleTOF 5600 systems. Each group were analyzed triplicates. Bioinformatics analysis (Gene ontology analysis , KEGG analysis and IPA analysis) of differentially expressed proteins were performed .Two of interested proteins were validated by Western Blot.

Results and Discussion

SWATH spectrums with high reproducibility were obtained. BGAL protein digestion of Ecoli were Spiked-in the samples and showed a fold change of 1.18 in tumor samples which showed an high reproducibility as well. Among 2197 identified proteins,146 proteins were up regulated and 112 proteins were down regulated according to defined criteria(Ratio >0.8 or <1.2,P<0.05,ttest<0.05). Most differentially expressed proteins were related to immune activity and homostastatic process. Up-regulated protein Copper chaperone for superoxide dismutase(CCS)and down regulated protein APOA1 were verified with Western Blot which showed a similar results of SWATH.

Conclusion

PBMCs from healthy people and PC patients were analyzed using SWATH technology, and the results showed that most differentially expressed proteins were connected to immune activity which can help us to understand the mechanism of changes of immune system during the process of PC . CCS and APOA1 were validated to be potential biomarkers.

Keywords: PBMC SWATH APOA1 CCS

TP01-50

Quantitative Proteomics Analysis of a Primary Bladder Cell line treated with Ketamine, a narcotics and anesthesia medicine

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Introduction and Objectives

Chronic ketamine abuse is associated with physical harms such as unexplained deaths, acute cardiac risk, ulcerative cystitis, kidney dysfunction, and intense abdominal pain. There is an urgent need to study the mechanisms of ketamine-induced ulcerative cystitis and psychological dependence to promote effective treatments for these ketamine-related disorders. However, there is no report about the systemic (proteomics) study of ketamine cystitis. We speculate that ketamine may cause cell death of bladder epithelial cells, which in turn may lead to ulcerative cystitis and subsequent dysfunction of the bladder.

Methods

To further explore the molecular mechanism underlying ketamine cystitis, we used the iTRAQ-based LC-MS/MS technology to systemically quantify the levels of proteins in a primary bladder cell line treated with ketamine in time course.

Results and Discussion

A total of 4235 proteins were quantified from the total cellular proteome and revealed at least 146 regulated proteins among time course treatment when mean±2SD was used as a threshold. Network analysis of proteins altered in cells treated with ketamine revealed that the changed biological processes are related to apoptosis, signal transduction and inflammation processes.

Conclusion

Identification of proteins with altered levels among these changed biological processes may be helpful to elucidate the mechanism regarding ketamine cystitis. In addition, this information may discover novel biomarker(s) for ketamine cystitis.

Keywords: Ketamine, Bladder, iTRAQ, Quantitative Proteome Analysis

TP01-51

Discovery of potential colorectal cancer serum biomarkers through quantitative proteomics of the tissue interstitial fluids from two mouse models

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Introduction and Objectives

The proteins in tissue interstitial fluids (TIFs) can spread into blood and TIFs are thus reasoned as ideal material to discover serum protein biomarkers. To more efficiently seek biomarkers for colorectal cancer (CRC), we performed dynamic and quantitative proteomic study on colon TIFs of AOM-DSS and APCMIN/+ mice, two well acceptable CRC mouse models.

Methods

TIFs were collected from AOM-DSS, APCMIN/+ and control C57BL/6J mice at four different time points, and analyzed by iTRAQ using Q-Exactive LC-MS/MS, respectively. Multiple reaction monitor (MRM) using QTRAP 5500 MS was used for verification and validation in individual mouse colon TIFs, mouse sera and human CRC sera.

Results and Discussion

A total of 252 proteins displayed significant changes in their abundances during tumor growth of two mouse models, including 60 consecutively increased, 41 consecutively decreased and 151 irregularly changed proteins. Of these consecutively changed proteins, 26 were verified using MRM in individual TIF samples. Eighteen proteins verified to be consecutively increased in TIFs were measured by MRM in individual mouse serum samples. The abundances of LRG1, TUBB5, IGJ and six serine proteases, CELA1, CEL2A, CTRL, CTRB1, TRY2, and TRY4 were significantly higher in CRC mouse sera than in control mouse sera. The quantitative verification of the nine proteins was further extended to the clinical sera, revealing significantly higher levels of LRG1, TUBB5, CELA1, CEL2A, CTRL, and TRY2 in CRC patient sera than healthy controls. The receiver operating characteristic analysis illustrated that the combination of CELA1 and CTRL reached the best diagnostic performance, with 90.0% sensitivity and 80.0% specificity.

Conclusion

Coupling dynamic TIF proteomics with targeted serum proteomics in animal models is a promising avenue for pursuing the discovery of tumor serum biomarkers. Six proteins, LRG1, TUBB5, CELA1, CEL2A, CTRL, and TRY2, are potential CRC serum biomarkers.

Keywords: Colorectal cancer biomarker, tissue interstitial fluid, quantitative proteomics, CRC mouse model

TP01-52

Targeted Quantification of N-1-(carboxymethyl) Valine and N-1-(carboxyethyl) Valine Peptides of β-hemoglobin for Better Diagnostics in Diabetes

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Introduction and Objectives

N-1-(deoxyfructosyl) valine (DFV) β-hemoglobin (β-Hb), commonly referred as HbA1c, is widely used diagnostic marker in diabetes, believed to provide glycemic status of preceding 90-120 days. However, the turnover of hemoglobin is about 120 days, the DFV-β-Hb, an early and reversible glycation product eventually may undergo irreversible advanced glycation modifications such as carboxymethylation or carboxyethylation. Hence quantification of N-1-(carboxymethyl) valine (CMV) and N-1-(carboxyethyl) valine (CEV) peptides of β-Hb would be useful in assessing actual glycemic status.

Methods

Fragment ion library for synthetically glycated peptides of hemoglobin was generated by using High Resolution-Accurate Mass Spectrometry (HR/AM). Using parallel reaction monitoring (PRM), deoxyfructosylated, carboxymethylated and carboxyethylated peptides of hemoglobin were quantified in clinical samples from healthy control, pre-diabetes, diabetes and poorly controlled diabetes.

Results and Discussion

For the first time, we report N1-β-valine undergoes carboxyethylation and mass spectrometric quantification of CMV and CEV peptides of β-hemoglobin. Carboxymethylation was found to be the most abundant modification of N1-β-Valine. Both CMV-β-Hb and CEV-β-Hb peptides showed better correlation with severity of diabetes in terms of fasting glucose, postprandial glucose and microalbuminuria.

Conclusion

This study reports carboxymethylation as a predominant modification of N1-β Valine of Hb, and quantification of CMV-β-Hb and CEV-β-Hb could be useful parameter for assessing the severity of diabetes.

Keywords:

Diabetes, diagnosis, glycation, glycemic control, HbA1c, mass spectrometry

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Multicentric Study of the Effect of Pre-analytical Variables in the Quality of Plasma Samples Stored in Biobanks by Complementary Proteomic Methods

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Introduction and Objectives

Analytical proteomics has experienced an exponential progress in the last decade and it is expected to lead research studies on diagnostic and therapeutic biomarkers in the near future. The development of this type of analysis requires the use of a large number of human samples with a minimum of quality requirements and the hospital biobanks facilities will be directly involved in the collection, processing and storage of those samples. We pursued to identify appropriate indicators for quality control of plasma samples stored in biobanks for research in proteomics.

Methods

Plasma samples from 100 healthy donors were obtained and processed according to different pre-analytical variables: a) Time delay in the first centrifugation of the original blood sample (4 or 24 hours) and b) freeze-thaw cycles (1, 2 or 3) of the processed plasma samples. Analysis of samples under different pre-analytical conditions was performed by different and complementary methods such as SPE MALDI-TOF, DIGE, shotgun (iTRAQ, nLC MALDI TOF/TOF) and targeted nLC MS/MS proteomic techniques (SRM and MRM). Selected proteins were orthogonally validated by Western-blot.

Results and Discussion

In general, there were not clear indications of protein degradation that correlated with the delay in the first centrifugation or with the number of freeze-thaw cycles since the

distribution of proteins in all samples was very similar. Only minor differences could be observed in the expression of Hemoglobin related to a delayed centrifugation. Results indicate that the presence of up to three freeze/thaw cycles have little or no effect on the stability of proteins in the plasma samples obtained. Only, we can find very slight differences in the amount of ApoB100 that were confirmed by western but not by MRM studies. Our data suggest the stability of plasma proteins under expanded clinical laboratory storage conditions (4 °C for up to 24 h prior to sample processing) so acceptable timeframes for plasma collection could be expanded from typical standard operating procedures (processing within 4 h of collection), and also the potential utility of plasma samples subjected to up to three freeze-thawing cycles, at least in the case of controlled thawing times of no more than one hour.

Conclusion

In summary, although it is evident that pre-analytical variables may influence clinical proteomic studies with plasma and these must be taken into account, the results of the present work indicate that blood proteins are broadly insensitive to pre-analytical variables such as delayed processing or freeze-thaw cycles for plasma when analyzed at the peptide level. These findings do not mean that hereby described variables do not affect other analyses directed at for instance intact proteins as immunoassays in which collection protocols may have different requirements.

Keywords: Plasma, biomarkers, biobanking, sample thawing

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Tissue proteomic analysis to validate differentially expressed markers associated with meningioma pathobiology

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Introduction and Objectives

Meningiomas are one of the most commonly diagnosed brain tumors, which originate from the outermost layer of the brain and spinal cord accounting to around 33.8% of all primary CNS tumors. Although meningiomas are primarily benign by nature but recent reports have pointed out to its inter- and intra-grade heterogeneity as well as increased instances of recurrence, which calls for identification of grade-specific biomarkers for better prognosis of the tumor.

Methods

A comprehensive tissue-based proteomic analysis was done on meningioma patient cohort using ESI-Q-TOF and Q-Exactive mass spectrometry, which enabled identification of 2376 differentially expressed proteins (>= 2 peptide and >= 1.5-fold in at least one grade) in meningiomas. Further, in silico analysis was done using multiple bioinformatics tools namely IPA®, DAVID and STRING elucidated role of several key signaling pathways in meningiomas. We have used immunoassay and targeted proteomics to validate few of the differentially expressed targets namely Vimentin, AnnexinA2, S100-A10, Cellular Retinoic Acid Binding Protein from peptides isolated from meningioma patients using Multiple Reaction Monitoring (MRM)-based approach for quantitation of the peptides. Furthermore, phosphorylation status of several differentially expressed proteins were investigated using TiO2 based enrichment to elucidate alterations in the several dysregulated pathways.

Results and Discussion

Transitions for several of the differentially expressed proteins that were found to be differentially expressed (>= 2 peptide and >= 1.5-fold in at least one grade) were monitored across meningioma patients using MRM assay this substantiated our preliminary findings. The Integrin signaling, Notch and EGFR signaling pathway were some of the key pathways that were found to be perturbed among many others. Key components of these pathways like Filamin-B, Vimentin, Annexin A2 were screened in individual patients. Vimentin being a type III intermediate filament protein, which is also an established IHC based marker for meningiomas was found to be highly abundant in MGI patients. Annexin A2 which has been reported to mediate angiogenesis and tumor progression in several malignancies including brain cancers was also found to be upregulated in MGI tumors. S100 A10 another target that was found to be differentially expressed is one of the binding partners of AnnexinA2 and has been reported to act together. Cellular retinoic acid binding protein is primarily expressed during

embryogenesis and nerve development; however, up regulation in CRABP1 in meningiomas could be an indicator of sub-arachnoid haemorrhage.

Conclusion

To the best of our knowledge, this is one of the first initiatives where comprehensive quantitative and targeted mass spectrometric approach using MRM has been used in meningiomas to validate potential biomarkers followed by in-depth phosphorylation screening. The identified biomarkers would be beneficial in designing discovery to validation pipelines.

Keywords: cancer

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Serum Proteomic Biomarkers of Hepatocellular Carcinoma Associated with Nonalcoholic Fatty Liver Diseases in Pigs

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Introduction and Objectives

Nonalcoholic fatty liver disease (NAFLD) is recognized as the hepatic manifestation of the metabolic syndrome. The spectrum of the disease ranges from simple steatosis to nonalcoholic steatohepatitis (NASH) that can progress into hepatocellular carcinoma (HCC). Noninvasive biomarkers are of urgent necessity for the optimal management of NAFLD. Because it is a non-primate mammal that closely resembles man in terms of its anatomy, physiology, and genetics, the pig is expected to become an ideal model animal for biomedical research. In the present study, using the microminipig, the smallest pig in the world, we chronologically traced serum proteome to identify biomarkers of HCC associated with NAFLD.

Methods

A 4-month-old male microminipig weighing 5 kg was fed a high-fat diet with chemical intervention for 60 weeks to induce HCC in association with NAFLD. Liver biopsy and blood sampling were performed every 12 weeks under general anesthesia. Liver tissues were pathologically characterized into simple steatosis, NASH or HCC according to the extent of lipid accumulation, fibrosis and the presence of cancerous lesions. With this model, two microliter of serum, which corresponded to each phase of NAFLD were separated by two dimension blue native polyacrylamide gel electrophoresis (2D BN-PAGE). Differentially stained spots on BN-PAGE gels were excised and identified under standard peptide mass fingerprinting (PMF) protocol.

Results and Discussion

The pig showed features of the metabolic syndrome such as obesity, hypertension, and hyperlipidemia. Histologic features of NASH consisting of hepatocyte ballooning and lobular inflammation were observed at 24 weeks, and hepatic fibrosis became more prominent at 48 weeks. Multiple liver tumors that had histopathological characteristics of human well-differentiated HCC were developed at 60 weeks. Blood chemistry also supported the histological findings and the corresponding serum proteome was analyzed with 2D BN-PAGE based strategy. Of note, the acute reactant proteins such as haptogloblin were differentially detected on this 2D platform among control, simple steatosis and HCC.

Conclusion

We have succeeded in generating the pig HCC model in association with NAFLD. The

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current model proposed a novel biomarker strategy combining liver histology and serum proteome. Here we identified the altered serum multiple-protein complex features corresponding to the development of HCC in association with NAFLD. It would be of great importance for understanding the complex biological process of HCC and NAFLD as well as surrogate marker detection.

Keywords: nonalcoholic fatty liver disease, hepatocellular carcinoma, two dimension blue native polyacrylamide gel electrophoresis, microminipig

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A novel model system and approach for identification of biomarkers of response to heat shock protein 90 inhibitors in prostate cancer

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Introduction and Objectives

The primary treatment for men with advanced prostate cancer is to suppress the production of androgen by surgical or medical castration, termed androgen deprivation therapy. While these strategies are initially effective in castrate-sensitive prostate cancer, patients relapse within 18-24 months. A promising approach to reduce AR expression and function in PC cells is to interfere with the maturation of the AR protein. Hsp90 is a chaperone that is essential for AR function via folding and stabilization of the receptor. Inhibiting Hsp90 results in degradation of AR along with many other prostate cancer onco-proteins. First-in-class Hsp90 inhibitors, such as 17-allylamino-demethoxygeldanamycin, were ineffective in clinical trials. The new generation Hsp90 inhibitor AUY922 has greater affinity to Hsp90 than any inhibitor reported to date and shows greater efficacy in prostate cancer cells than 17-AAG. In this study, we applied a HRM-DIA mass spectrometry-based proteomic strategy combined with a patient-derived prostate cancer explant (PDE) model to identify

Methods

A unique PDE model that retains structure, stromal-epithelial interactions, and inter-tumor heterogeneity was utilized as it better mimics the native tumor microenvironment. PDEs (n=16) were cultured in growth medium containing: (1)vehicle, (2)Hsp90 inhibitor AUY922; 500nM, and (3)HSP inhibitor 17-AAG; 500nM. Samples were analysed by LC-MS/MS in hyper-reaction monitoring data independent acquisition (HRM-DIA) on a Thermo Scientific Q Exactive Plus mass spectrometer. Quantitative data were extracted from a retention-time-normalized spectral library using the software Spectronaut. Differentially expressed proteins between treatment groups were identified using repeated measure analysis of variance ($p<0.05$) based on the top three fragment ions of proteotypic peptides for each protein.

Results and Discussion

202 differentially expressed proteins were identified that segregated PDEs into vehicle, 17-AAG, and AUY922 treatment groups. 112 proteins significantly decreased in expression in samples treated with Hsp90 inhibitors compared to vehicle and showed a reduction of HSP90 client proteins, ribosomal proteins, histone deacetylase, and glycolysis. 74 proteins significantly increased in expression in samples treated with AUY922 compared to both vehicle and 17-AAG. HSP70 proteins, the standard pharmacodynamic biomarker of Hsp90 inhibition, was identified along with pathways associated with tissue development and cytoskeletal organization.

Conclusion

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These findings confirm that our research strategy can identify markers that are selectively associated with AUY922 activity. Next, the 202 differentially expressed proteins will be validated in an independent set of treated versus untreated prostate cancer explants (n=30). These results will identify and validate a protein signature to reliably monitor prostate cancer patient response to treatment with AUY922.

Keywords: Data independent acquisition,heat shock protein 90 inhibitor,ex vivo, prostate cancer

TP01-57

Protein panel for monitoring the progression of Keratoconus- Clinical application of mass spectrometry

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Introduction and Objectives

Keratoconus (KC) is a corneal ectatic condition characterized by thinning and steepening of the cornea leading to astigmatism and thereby affecting vision. The disease progresses over a couple of decades with incidence at puberty into the third decade of life. Based on the severity of the disease measured by topographical parameters, Amsler-Krumeich classification was devised for categorizing keratoconus into four grades (Grade I-IV). In the current study we aim to identify the changes in the tear proteome of KC patients from grades I-III and validate a panel of differentially expressed proteins by label-free parallel reaction monitoring (PRM) for progression of keratoconus.

Methods

Tear protein extracts collected from control (n=40) and grades I-III (n= 33,25 and 21) KC patients were differentially labeled with iTRAQ reagents post proteolytic digestion. Proteins identified with iTRAQ ratios in atleast 50% of the patients in each grade of KC are considered for further analysis. Proteins with a ratio fold of >= 1.5 and one-way ANOVA p value <= 0.05 between the grades of comparison are considered statistically significant. An independent cohort of patients, control (n=28) and grades I-III (n=23, 25 and 13), were selected for label free quantitation for proteins displaying either an increasing or decreasing trend in higher grades of KC by PRM. Tear protein digest from each patient was spiked with 5 fmol/µl of beta-galactosidase digest from E.coli as an internal reference. The peptides and their transitions of altered proteins for PRM analysis were selected using Skyline targeted analysis tool.

Results and Discussion

Proteins involved in glycolysis, Rho GTPase cytoskeletal regulation, FGF signaling and p38/MAPK pathways were observed to be significantly altered in keratoconus. Thirty-five proteins were observed to be displaying either increasing or decreasing trend in their levels in grades II and III of KC tears compared to grade I. Proteins lacritin (LACRT), cystatin-S (CST4), S100P, lactoperoxidase (LPO), Ig alpha 1 chain (IGHA1) were identified to be elevated whereas tear proteins lipocalin1 (LCN1), lysozyme (LYZ) besides superoxide dismutase 1 (SOD1), lactoperoxidase (LPO), galectin 3 binding protein (LGALS3BP) and glutathione synthetase (GSS) were among the proteins that were identified at reduced levels in grades II and III compared grade I. Besides identifying proteins associated in other ocular surface conditions as dry eye syndrome, eg. LACRT, LCN1 and LYZ, our results also illustrate novel molecular markers like S100P, CST4, GSS, LAGLS3BP, LPO unique to KC.

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Conclusion

Using quantitative proteomics we propose a set of proteins showing consistent elevated/ reduced levels in correlation with the severity of keratoconus which can serve as a panel for monitoring the progression of the disease.

Keywords: Tear Proteomics, iTRAQ, PRM, Cornea, Keratoconus

TP01-58

Proteomic profiling of serum exosomes to identify novel early detection biomarkers for gastric cancer

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Introduction and Objectives

In the recent past, biological importance and clinical utility of extracellular vesicles (exosomes) have been extensively investigated. Particularly exosomes are considered as ideal targets of biomarker discovery due to their molecular characteristics reflecting those of original cells. Recently, We developed 3 key technologies (CD9-MSIA tips, ExoTrap columns, and EV-Second columns) allowing rapid isolation of high-purity exosomes from serum/plasma or cell culture medium by simple procedures. We integrated these new devices with our leading-edge mass spectrometric technology to perform proteome-wide exosomal biomarker screening for gastric cancer (GC).

Methods

Serum samples were collected from 58 individuals (normal donors; n = 10, early stage GC patients; n = 16, advanced stage GC patients; n = 18, and scirrhous type GC patients; n = 14). Following isolation of exosomes by EV-Second columns based on mixed mode of size exclusion and weak hydrophobic interaction, exosomal proteins were subjected to comprehensive LC/MS analysis using LTQ-Orbitrap-Velos mass spectrometer. Protein identification, label-free quantification, and subsequent statistical analysis were performed on Expressionist proteome server platform (Genedata AG, Swiss). Exosomal biomarker candidates showing significant upregulation in GC patients' sera were further validated by independent MRM quantification analysis.

Results and Discussion

The LC/MS analysis identified 822 exosomal proteins in which 13 proteins showed significant up-regulation in GC exosomes (t-test, $p < 0.05$, fold change > 2.0, and valid value > 80%). Subsequently we performed replication study for 13 exosomal biomarker candidates using independent sample set (45 healthy controls, 186 gastric cancer patients, 40 colon cancer patients, and 35 pancreatic cancer patients) by means of MRM absolute quantification analysis. As a representative result, exosomal PN-1 protein showed 64.2% sensitivity and 91.7% specificity when comparing even clinical stage I gastric cancer patients to healthy controls. Importantly the sensitivity and specificity were 60.0% and 91.7% in comparison of scirrhous gastric cancer patients with healthy controls. IHC experiments demonstrated that PN-1 strongly expressed in gastric cancer cells, whereas no expression was observed in normal gastric cells. In addition, tissue microarray analysis confirmed high expression rate in a subset of GC samples (59.1% for adenocarcinoma and 80.0% for undifferentiated gastric carcinoma), suggesting that PN-1+ exosomes were generated and secreted by GC cells but not by normal gastric cells. Further functional analyses indicated that potential contribution of PN-1+ exosomes

to apoptosis prevention and growth promotion in GC cells.

Conclusion

These data suggested that blood test measuring PN1+ exosomes could greatly enhances a chance for early detection of lethal type of gastric cancer and also helpful for revealing novel mechanisms underlying tumor progression or metastasis.

Keywords: exosome, serum, gastric cancer

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Proteomic Analysis of TGF-β–Induced Cancer Metastasis

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Introduction and Objectives

Lung adenocarcinoma patients account for approximately 50–60% of primary lung cancer patients, and many are non-smokers and women. The prevalence of lung adenocarcinoma is increasing yearly. The majority of lung adenocarcinoma cases are of a peripheral type that occurs in the peripheral lung. When advanced, it metastasizes easily to the lymph nodes and develops distant metastases. Were the mechanism of progression or metastasis of cancer to be elucidated, new therapeutic targets can be identified, and novel therapeutics developed. We focused on TGF-β (β-type mutant growth factor), which is reportedly related to metastatic or recurrent cancer. TGF-β is involved in the induction of the epithelial–mesenchymal transition (EMT). EMT is a phenomenon in which epithelial cells change into mesenchymal-like cells. EMT-induced cells acquire motility and invasive potential, and EMT acquisition may be involved in cancer cell invasion. TGF-β is often overexpressed in tumor tissues, and it facilitates cancer progression by enhancing cell invasion and metastasis. In this study, we performed relative quantitation of tyrosine-phosphorylated peptides in both untreated and TGF-β–treated human alveolar adenocarcinoma (A549) cells. We detected elevated levels of three tyrosine-phosphorylated proteins in TGF-β–treated cells and identified them by LC-MS/MS. Two of these proteins are receptor proteins, and the concentrations of their respective ligands were elevated in TGF-β–treated cells. We focused on these two receptor proteins, and analyzed the mechanism of TGF-β–induced malignant transformation.

Methods

We performed relative quantitative analysis of tyrosine phosphorylation of proteins from both untreated and TGF-β–treated A549 cells. We also determined the levels of protein tyrosine phosphorylation and of ligand proteins by western blotting.

Results and Discussion

We detected and identified a total of 145 unique tyrosine-phosphorylated peptides belonging to 94 tyrosine-phosphorylated proteins in our samples. Among these, two receptor proteins showed greatly elevated tyrosine phosphorylation levels in response to TGF-β treatment, and were therefore chosen for subsequent verification. We determined the amounts of protein tyrosine phosphorylation and total protein by western blotting and MRM analysis. Elevated levels of tyrosine phosphorylation on two receptor proteins were observed in TGF-β–treated cells. However, we observed no significant difference in total protein levels. Moreover, we determined the levels of

receptor ligand proteins by western blotting, and found that these were elevated, suggesting that TGF-β may enhance intracellular signaling via these two receptors.

Conclusion

Analysis of the post-translational modification levels of proteins in EMT cells will likely become an important method for identification of diagnostic biomarkers.

Keywords:

tyrosine phosphorylation, epithelial-mesenchymal transition (EMT), transforming growth factor-β (TGF-β)

TP01-60

Comparison of protein expression profiles between human crown and root dentins by label-free quantitative proteomics analysis

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Introduction and Objectives

The human tooth is the hardest part of the human body possessing a discrete structure and distinct composition. Dentin is the intermediary part of the tooth, being located between the enamel and the pulp. Mature dentin is approximately 70% mineral, 20% organic matrix and 10% water. The organic matrix are mainly secreted by odontoblasts which are the key cells responsible for dentin formation and maintenance. Recently, mass spectrometry-based proteomics has become one of the most vital and central tools for various biological studies. Owing to the fast-evolving MS instrumental improvement, the study of dentin proteomics has become more amenable to more rigorous analysis. However, human crown dentin (relative early formation of dentin) and root dentin (relative new formation of dentin) proteomes has yet been comprehensive studied by proteomics scale. In this study, label-free quantitative proteomics has been applied to compare the different protein expression profiles between human crown and root dentins.

Methods

Healthy human third molars from three adults were cut into crown and root parts and then the corresponding dentin for each part was isolated, demineralized and subjected to protein extraction. The extracted proteins were fractionated by SDS-PAGE, subjected to in-gel digestion and then analyzed by LC-MS/MS.

Results and Discussion

Spectral counting-based label-free quantitative proteomics approach has been applied to evaluate the differences in protein profiles between human crown and root dentins. Hierarchical cluster analyses were performed for the crown and root dentin proteomes from three healthy teeth based on spectral counting method. The results reveal two major cluster groups. One consisted of three crown dentin proteomes and the other consisted three root dentin proteomes, indicating that the distinct protein profiles exist between crown dentin (relative early formation of dentin) and root dentin (relative new formation of dentin). These results further suggest that dentin is a metabolically active tissue.

Conclusion

In this study, label-free quantitative proteomics has been applied to compare the different protein expression profiles between human crown and root dentins, resulting that the distinct protein profiles exist between crown and root dentins. It had long been believed that dentin is a relative inert tissue with no significant extracellular matrix turnover. It has recently been proposed that the structure and composition of dentin may be more heterogeneous than was previously thought. This study is the first comprehensive study

of crown and root dentin proteomes, providing the evidence of that dentin is a metabolically active tissue. Understanding different protein expression profiles in proteomes between crown and root dentins would shed some light on the mechanism(s) underlying the dentin formation and regeneration.

Keywords: Label-free quantitative proteomics, spectral counting, human dentin, crown dentin proteome, root dentin proteome

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Data independent acquisition-based targeted proteomics for plasma analysis – in the context of cancer biomarker detection

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Introduction and Objectives

Data independent acquisition (DIA)-based targeted proteomics affords enormous multiplexing capacity and is particularly suitable for clinical biomarker studies. However, DIA-based quantification of clinical plasma samples is a daunting task due to the high complexity of clinical plasma samples, the high biologic dynamic range of plasma proteins, and the diversity of peptides within the samples. Here, we present a DIA-based targeted proteomics pipeline and assessed its utility for clinical plasma biomarker detection.

Methods

Global proteomics was applied to analyze fractionated plasma samples for peptide and protein identification to establish a peptide spectra library for DIA analysis. Clinical plasma samples were processed using a robust sample preparation protocol. The DIA-based targeted analysis was carried out using an Orbitrap QEplus mass spectrometer. Skyline software was used for data processing.

Results and Discussion

A pancreatic cancer relevant plasma spectral library was established by extensively profiling clinical samples from patients with PDAC, chronic pancreatitis and healthy controls. The library consists of over 14000 confidently identified peptides derived from over 2300 plasma proteins. A set of criteria based on the characteristics of the peptides were introduced for the minimal variations. Empirical parameters, including retention time deviation and intensity CV based on replicate analysis, provided reasonable guidance in selecting quantifiable peptide for precise DIA analysis. The sensitivity of quantification was evaluated with different levels of spiked-in standard, suggesting that sample complexity is a significant factor in determining the limits of quantification, justifying the optimal selection of DIA isolation windows. The technical validation of the assay using clinical plasma samples confirmed its robustness for study of large scale clinical samples. The empirical data presented in this study also suggests an intrinsic link between peptide physicochemical properties and their robustness in DIA quantification, which warrants further investigation.

Conclusion

A DIA-based targeted proteomics pipeline was developed and assessed for large scale quantification of plasma proteins in the context of cancer biomarker detection. Empirical parameters were developed to optimize the selection of quantifiable proteotypic peptides for DIA quantification.

Keywords: Proteomics, mass spectrometry, data independent acquisition (DIA), plasma, quantification, pancreatic cancer

TP01-62

Ubiquitin specific protease 19 involved in transcriptional repression of retinoic acid receptor

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Introduction and Objectives

Methods

Results and Discussion

The transcription levels of USP19, USP42, and USP54 were changed during adipogenesis. USP19 is most significantly suppressed in adipocyte differentiation. CORO2A was identified as a novel binding partner for USP19 by immunoprecipitation and MALDI-TOF-MS analysis. USP19 regulates the ubiquitination of CORO2A and stabilizes CORO2A through its deubiquitinating activity. Interaction between USP19 and CORO2A mediates the regulation of transcriptional repression of RAR.

Conclusion

Regulation of CORO2A through the deubiquitinating activity of USP19 affected the transcriptional repression activities of RAR, suggesting that USP19 may be involved in the regulation of RAR-mediated adipogenesis.

Keywords: Adipogenesis, CORO2A, Deubiquitinating enzyme, USP19

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Proteomic and bioinformatic analysis of overactive bladder in a rat model

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Introduction and Objectives

The overactive bladder (OAB) syndrome is characterized by urgency, with or without incontinence, frequency and nocturia, in the absence of urinary tract infection (UTI) or any other obvious pathology. Although the exact mechanism of OAB is not completely understood, OAB has been suggested to be related to neurotransmitters and other receptor systems in the urothelium. While the urothelium has been historically viewed primarily as barrier, it is increasingly recognized to be a responsive structure that is capable of detecting physiological and chemical stimuli and of releasing a number of signaling molecules.

Methods

To investigate the potential difference in protein expressions between normal bladder urothelium and OAB urothelium, urothelium of bladder was carefully removed from smooth muscle layer and extracted proteins were analyzed by using LTQ Orbitrap Velos mass spectrometer. The identified proteins were analyzed using Ingenuity Pathway Analysis (IPA) tool.

Results and Discussion

A total of 507 proteins were identified in normal rat urothelium and 380 proteins in OAB rat urothelium. The urothelium of the normal and OAB rat shared 306 (52.7%) proteins. In contrast, 201 and 74 unique proteins were expressed in normal and OAB urothelium, respectively. In OAB urothelium, pathways involved in inflammation, such as complement system, acute phase response signaling, LXR/RXR activation, and p38 MAPK signaling, were notably up-regulated. In contrast, signaling related to cytoskeletal organization, which includes ILK signaling, RhoA signaling, and remodeling of epithelial adherens junctions, were commonly down-regulated in OAB urothelium. To investigate the potential causes of OAB, we performed further bioinformatics analysis using IPA. As a result, 17 putative upstream regulators were identified. These regulators are mainly involved in inflammation and cytoskeletal organization.

Conclusion

Our findings suggest a new target of accurate OAB diagnosis thus it can provide essential information to drug treatment strategies and it can give a help to the establishment of criteria for screening patients for prescription of the drug in the clinical environment.

Keywords: Over active bladder, urothelium, proteomics, rat

TP01-64

The surveillance and early diagnosis of hepatocellular carcinoma using multiple reaction monitoring

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Introduction and Objectives

Hepatocellular carcinoma (HCC) represents approximately 90% of all cases of primary liver cancer. HCC is the second leading cause of cancer-related deaths globally and has an incidence of approximately 850,000 new cases per year. The major risk factors, such as hepatitis B and C virus infection, for developing HCC are well known, so the patients at high risk are recommended to be screened. Currently, ultrasound examination is used for surveillance of HCC, yet its low sensitivity and specificity have been a problem (AUC=0. 65). The biomarker for HCC, Alpha-Fetoprotein (AFP) is also unsatisfactory in diagnosing HCC. Consequently, there is an unmet need for detection system in early-stage HCC with high sensitivity and specificity. So, we aimed to develop novel single biomarker or multi-marker panel for HCC surveillance.

Methods

In this study, we performed a verification and validation of known biomarker candidates in large clinical samples by multiple reaction monitoring (MRM) assay. The HCC-related protein marker candidates were included by data-mining of databases, profiling of liver tissues, and previous experiments. The detectability of proteins were checked by 6490 Triple Quadrupole LC-mass spectrometry in the pooling sample of 150 serum. Then, the detected targets were analyzed in the individual samples of 50 liver cirrhosis (LC), 50 hepatitis B (HBV), and 50 hepatocellular carcinoma (HCC). Through the multivariate analysis, differentially expressed proteins were tested as a biomarker of HCC.

Results and Discussion

The total of 2189 HCC-associated candidate proteins were listed from the data-mining. The targets were narrowed down by secretion prediction tools, peptide spectrum library, and MRM assay, and 681 proteins were verified in 150 plasma samples (50 LC, 50 HBV, and 50 HCC). To develop a novel biomarker, we performed a comparative analysis of HBV vs.LC, HBV vs. HCC, and LC vs. HCC samples by 6490 Triple Quadrupole LC-mass spectrometry. Internal standard was an iRT peptide or an empirically derived dimensionless peptide-specific value that allowed for highly accurate RT prediction. The raw data was processed by Skyline program. Then, the receiver operating characteristic (ROC) curve and area under the curve (AUC) for each groups were derived. As a result, 24 proteins, 2 proteins, and 40 proteins differentiated HBV and HCC, LC vs. HCC, and HBV vs. LC, respectively with AUC >0.7, which showed the better diagnostic performance than the conventional tests. This study still needs a further verification and validation in an independent samples.

TP01-64

Conclusion

This study might lead to more reliable and easy detection of early stage HCC in long-term surveillance.

Keywords: Multiple reaction monitoring (MRM); hepatocellular carcinoma (HCC); multi-marker panel

TP01-65

Characterization of the discoid lateral meniscus tissues using mass spectrometry-based proteomics

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Introduction and Objectives

The discoid lateral meniscus (DLM) is an intra-articular knee disorder. The cause of DLM is not clear. But, impaired regeneration of extracellular (ECM) involved in meniscus formation is likely to affect a meniscus tear. It is important to investigate ECM proteins related to DLM for their function in ECM modeling of meniscus. Therefore, we characterized the DLM tissues to discover the DLM specific proteins using mass spectrometry-based techniques and measured several proteins related to the ECM formation in DLM patients using the multiple reaction monitoring (MRM) assay.

Methods

We optimized the easy protocol of protein extraction from patients' meniscus, which are insoluble. In brief, the protein extracts were prepared by cutting and grinding the meniscus tear frozen with liquid. Here, we employed isobaric chemical tag, Tandem Mass Tag (TMT) to identify the proteins related to a meniscus tear using LTQ-Orbitrap Elite mass spectrometry equipped with EASY-spray source and nano-LC UltiMate 3000 (Thermo Fisher, San Jose, CA). After doing in solution trypsin digestion of proteins, peptides were labeled with the TMT Reagents according to the manufacturer's instructions followed by proteomic analysis. Moreover, to characterize the alterations of ECM formation in 30 DLM of patients, we achieved MRM-based assay of the main fiber-forming ECM proteins (such as collagens, fibronectins, elatstins, etc.) using Q-TRAP 5500 (AB Sciex, Foster City, CA, USA).

Results and Discussion

We identified a total of 628 proteins with a 1% false discovery rate (FDR) by LC-ESI-MS/MS. A large number of ECM-associated proteins were detected in the DLM protein extracts, such as collagens, elastins, fibronectins and laminins etc. Interestingly, the expression of type I, II, III collagens, the main fibrillar component of the meniscus were impaired in a meniscus tear. We established the MRM assays enable to quantify the these proteins and confirmed their altered expression in patients' meniscus with DLM.

Conclusion

We present here the proteomic characterization of the DLM with mass spectrometry based comparative proteomics analysis and developed MRM-based assay of several ECM proteins.

Keywords: Discoid lateral meniscus, Proteomics, MRM

TP01-66

Bioactive Dietary Compounds (BDCs)-mediated anti-cancer effects on colorectal cancer cells

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Introduction and Objectives

Numerous epidemiological studies reveal an inverse correlation between intake of bioactive dietary compounds (BDCs) and incidence of cancer. BDC-mediated epigenetic changes have been suggested to be one of the key mechanisms mediating its anti-cancer property. Among the BDCs, PEITC (phenethyl isothiocyanate) is one of the major bioactive chemical in cruciferous vegetables which been reported to exert anti-cancer effects. However, global analyses of epigenetic changes upon PEITC treatment have not been carried to provide comprehensive understanding on the anti-tumorigenic effects of this compound.

Methods

We investigated PEITC-mediated anti-cancer effects by multidimensional approach including DNA methylation array and TMT-isobaric tag labeling-based quantitative proteomic analysis of chromatin-binding proteins. An in vitro cell line model was established to allow determination of long-term effects of PEITC treatment on cancer cells and characterize primary cellular targets of PEITC.

Results and Discussion

PEITC treatment led to stable changes on epigenetic markers, such as DNA methylation and histone modulators. In particular, changes of Polycomb-group (PcG) complex and histone deacetylases (HDACs) were found to be significant following long-term rather than short-term PEITC treatment. Further analysis using DNA methylation array showed that PEITC mediated hypomethylated genes overlapped with PcG targets or frequently methylated genes in cancer. In addition, PEITC-mediated activation of pro-apoptotic genes was partly reverted by overexpression of BMI-1, a key component of PcG complex, indicating a positive correlation between PEITC-mediated anti-cancer effects and PEITC-mediated modulation of PcG complex. Furthermore, PEITC treatment blocked the binding of HDACs onto euchromatin region, suggesting potential role of PEITC as a HDAC inhibitor derived from natural sources.

Conclusion

Our results showed PEITC induced cellular responses at DNA and protein levels and give an insight of coordinated regulation between epigenetic modifiers and their correspondent changes on epigenetic marks for anti-cancer effects. Elucidating the molecular mechanisms that underlie this process could shed light on utilizing these bioactive agents as possible tools in cancer prevention.

Keywords: BDCs, PEITC, Epigenetic regulation, DNA methylation Array, Quantitative proteomics, Chromatome

TP01-67

Quantitative Analysis of AKT/mTOR Pathway using Multiplex-Immunoprecipitation and Targeted Mass Spectrometry

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Introduction and Objectives

A major bottleneck in the quantitation of signaling pathway proteins is the lack of rigorously validated methods/reagents and a reliance on semi-quantitative results from current immunoassay technologies (Western blot, ELISA and Luminex). Mass Spectrometry (MS) is increasingly becoming the detection methodology of choice for proteins and their post-translational modifications (PTMs). Immunoprecipitation (IP) is commonly used upstream of MS as an enrichment tool for low-abundant proteins. The objective of this study was to determine the efficacy of multiplex IP to targeted MS (mIP-tMS) technique for measurement of the total and phosphorylated AKT/mTOR pathway targets and to evaluate whether mIP-tMS assays are as effective as the current singleplex immunoassay (WB and ELISA) and multiplex Luminex assays.

Methods

Serum starved HCT116, MCF7 and A549 cells were stimulated with IGF-1. mIP-tMS assays were developed and validated for absolute quantitation of 12 total and 11 phosphorylated AKT/mTOR pathway targets. Validated mIP-tMS assays were benchmarked against currently available WB, ELISA and multiplex Luminex immunoassays across three unstimulated and IGF-1 stimulated cell lysates.

Results and Discussion

We validated multiple antibodies for 12 total and 11 phosphorylated AKT/mTOR pathway targets using the optimized IP-MS workflow. Immunoprecipitation using MS-Compatible Magnetic IP Kits (Protein A/G and Streptavidin) for MS applications resulted in a higher yield of AKT-mTOR pathway target proteins and less non-specific binding proteins. mIP-tMS assays allowed absolute quantitation for all 12 total and 11 phosphorylated targets in low to sub nanogram concentrations from unstimulated and IGF stimulated A549, HCT116 and MCF7 cell lysates. Enrichment was necessary for identification and quantitation of low abundant signaling pathway proteins, interacting partners & PTMs. The benchmarking of mIP-tMS assays showed high correlation for quantitation of total target relative abundance compared to WB, ELISA and Luminex assays. However, for some phosphorylated targets, mIP-tMS assays had low concordance to the other immunoassays possibly due to differences in the specificity of anti-phospho antibodies used for each assay.

Conclusion

Overall, mIP-tMS assays were used to quantitate 12 total and 11 phosphorylated AKT/mTOR pathway targets and benchmarked against current immunoassay techniques.

Keywords: IP-MS, AKT, PTMs, pathway analysis, PRM, multiplex, targeted assays

TP01-68

A Serum Based Combinatorial Proteomic Biomarker Assay is Unaffected by the Density of Breast Tissue

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Introduction and Objectives

Breast density is associated with reduced imaging sensitivity and specificity for breast cancer. Women with dense breasts are at a four- to six-fold increased risk of developing breast cancer. A biochemical approach that is not affected by density would provide an additional tool to health-care professionals who are managing women with dense breasts and suspicious imaging findings. Videssa® Breast, a combinatorial proteomic biomarker assay, comprised of SPBs and TAAbs integrated with clinical characteristic data to produce one diagnostic score that reliably detects BC was recently developed as an adjunctive tool to imaging. The goal of this study was to determine whether the diagnostic performance of Videssa® Breast was impacted by breast density.

Methods

Provista-001 enrolled 351 participants under the age of 50 years with no prior history of breast biopsy, and Provista-002 cohort one enrolled 210 participants under the age of 50 years with no history of breast biopsy within six months; all participants were assessed as BI-RADS 3 or 4. Breast density status was retrospectively obtained for participants; the four American College of Radiology breast density categories (A, B, C, and D) used for clinical reporting were applied. Serum was collected and tested with Videssa® Breast. Women were stratified into Dense, which included categories C and D, and Non-dense, which included categories A and B, groups.further anxiety in this higher risk patient population.

Results and Discussion

To understand the performance of Videssa® Breast in women with dense breasts, the clinical sensitivity, specificity, NPV and PPV were evaluated in the dense and non-dense groups from the comprehensive Provista-001 and Provista-002 set (n=545). Of these 545, breast density information was available for 454; 62.6% (n=284) were categorized as having dense breasts (C and D) and 37.4% (n=170) were categorized as having non-dense breasts (A and B). The sensitivity of Videssa® Breast in the non-dense and dense groups was 92.3% and 88.9%, respectively, and the specificity in the non-dense and dense groups was 86.6% and 81.2%, respectively. No significant differences were observed in the sensitivity (p=1.0) or specificity (p=0.1783) of Videssa® Breast in detecting breast cancer in participants with non-dense breasts and dense breasts. The NPV in both groups exceeded 99%, and the PPV was comparable across both groups.

Conclusion

In summary, this study demonstrates that Videssa® Breast demonstrates comparable performance in women with dense and non-dense breasts. Videssa® Breast demonstrates high sensitivity and specificity for detecting breast cancer, irrespective of

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density status. Thus, Videssa® breast provides an additional tool for health-care providers when women with dense breasts present with challenging imaging findings. In addition, Videssa® breast provides assurance to a women with dense breasts that she does not have breast cancer, reducingfurther anxiety in this higher risk patient population.

Keywords: BI-RADS, Density

TP01-69

Proteomic study of thyroid cancer metastasis using 3D culture system

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Introduction and Objectives

The incidence of thyroid cancer is steadily increasing in all age groups, worldwide. Local recurrence and metastases are the main causes of death in thyroid cancer patients, especially with anaplastic thyroid carcinoma (ATC). ATC usually metastases to other organs and there is currently no effective treatment available, thus ATC is one of the most aggressive human cancers. Metastasis includes multiple processes such as invasion of neighboring tissues, cell survival under detachment (anoikis resistance) and colonization at distant sites. Anoikis or anchorage-dependent cell death is a key barrier against cancer metastasis so in order to survive, malignant cancer cells develop anoikis resistance. As the molecular mechanism of ATC metastasis is still not well understood, specifically anoikis resistance, we investigated the proteomic profiles of an anaplastic thyroid carcinoma cell line, ARO, in a 3D culture (polyHEMA) system as compared to that of attached cells.

Methods

The metastasis potential of ARO cells was studied using Transwell invasion assay, MTT assay, flow cytometry and Western blot analysis of anti-apoptosis proteins. Two-dimensional SDS-PAGE with LC/MS/MS was used to detect proteins with differential expression between the two systems.

Results and Discussion

Even though the viability of ARO cells in 3D culture decreased at 24 hours, its invasiveness increased when compared to cells grown in monolayer. Flow cytometry showed no significant differences in total cell death or cell survival, and expression of anti-apoptosis proteins, XIAP, Mcl-1, Bcl-XL and survivin, increased, suggesting anoikis resistance in 3D culture at 24 hours. Twenty-seven protein spots had altered expression, eleven of which were identified to be macrophage-capping protein (CapG), RNA-binding protein 4B isoform 1 (Hlark), tumor susceptibility gene 101 protein (TSG101), keratin 8 (CK8), ATP synthase (H+ transporting, mitochondrial F1 complex), 60kDa heat shock protein (HSP60), heterogeneous nuclear ribonucleoprotein L (hnRNP-L), lamin A/C, 14-3-3 zeta/delta, GMP synthase and cofilin-1 (CLF1). At present, the expression of HSP60, TSG101 and CK8 was validated using Western blot, and interestingly, TSG101 had a significant increase in expression in 3D culture. Further identification of remaining spots and functional validation of proteins are underway.

Conclusion

Our study revealed TSG101 to have the potential to be a diagnostic biomarker or a therapeutic target for metastasizing anaplastic thyroid carcinoma.

Keywords: thyroid carcinoma, proteomics, metastasis, anoikis resistance, biomarker

TP01-70

Plasticizer induced tumor migration by regulating of sialyltransferase expression in colon cancer cells

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Introduction and Objectives

Plasticizers (di (2-ethylhexyl) phthalate/mono (2-ehylhexyl) phthalate (DEHP/MEHP)) exposure can cause many potential health effects in humans. However, the effects of plasticizers in tumorigenesis, cancer progression, migration or, invasion are still unclear. Abnormal glycosylation changes are a universal feature of malignant transformation and tumor progression and play roles in a variety of biologic processes such as tumor cell adhesion, migration, invasion and cell-cell communication. In our previous study, we found that abnormal sialylation is highly correlated with oral cancer metastasis. Hence, the aims of this work is to investigate the effects of plasticizer exposure and roles of abnormal sialylation in colon cancer cells.

Methods

Firstly, we established two long term DEHP or MEHP treated colon cancer cell lines (HCT116-D and HCT116-M, respectively). The effect of plasticizer on migration and EMT biomarkers were evaluated by wound healing, transwell migration assays and western blotting. Using quantitative reverse transcriptase-PCR (qRT-PCR), the expression of sialyltransferases were also examined. We also detected relevant substrates by immunoprecipitation with siglec 7 which bind alpha-2,8-linked disialic acids and analyzed candidate proteins by protein identification.

Results and Discussion

A long term exposure of plasticizer to colon cancer cells increased cell migration ability through changing the expression of EMT markers including E-cadherin, N-cadherin and vimentin. The qRT-PCR results also showed the differential expressional profiles of sialyltransferase genes in the both HCT116-D and HCT116-M cancer cell lines. We discovered the mRNA expression of alpha-2,8-sialytransferase 6 (ST8SIA6) is decreased in HCT116-D and HCT116-M. In addition, silencing of ST8SIA6 increased cell migration ability and the EMT biomarkers. Furthermore, disialylated glycoproteins of HCT116, HCT116-D and HCT116-M cells were purified by lectin pull down assay. Several glycoproteins were identification and most of them played key players in tumor malignancy. Our results indicate that ST8SIA6 is involved in DEHP/MEHP-induced cancer migration.

Conclusion

Disialylated glycoproteins were discovered and suggested to be potential novel therapeutic targets for tumor metastasis.

Keywords: sialyltransferase, plasticizer, migration, colon cancer

TP01-71

Proteomic analysis of the oral squamous cell carcinoma

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Introduction and Objectives

In oral carcinomas in-situ (CISs), which are pre-invasive lesions of squamous cell carcinomas (SCCs), differences in protein expressions like heat shock protein, alpha B-crystalline, myosin or keratin subtypes between CISs and non-cancerous epithelium (NCE) have been reported previously. To find differences more comprehensively, we did proteomic analysis of the oral CISs and NCE.

Methods

The formalin-fixed paraffin-embedded (FFPE) tissues (5 surgical cases) were subjected for preparation of CISs and NCE sections by laser microdissection and the sections were digested with trypsin and peptides were purified by using C-18 spin column (OSDD method) as reported previously. The peptides were analyzed by 5600+ (SCIEX) and proteins were identified by ProteinPilot® software. The proteins identified were quantitated by summing the peptide intensity. We compared the amounts of the proteins between CISs and NCE and found differences in their proteomes.

Results and Discussion

In this study, 2205 proteins were identified in the SCC tissues and 1669 proteins in the NCE tissues. The proteins, which had been reported previously as cancer-related proteins, were identified in the CISs tissues. In addition several new proteins were newly identified as unique proteins in CISs and as significantly increased or decreased proteins in oral cancer.

Conclusion

We analyzed oral CISs by proteomics and demonstrated characteristics of CISs proteome in this study, which may help to understand CISs for future development of new treatments.

Keywords: Proteome, Cancer, Health, Biomarker

TP01-72

Proteomic analysis of metastatic colorectal cancer cell and verification by SRM/MRM analysis.

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Introduction and Objectives

Early detection of cancer metastasis is crucial to improve the prognosis. Cancer progression and acquisition of metastatic potential are influenced by surrounding tissue environment. Orthotopic implantation of human cancer cells into nude mice provides a valuable in vivo model for cancer metastasis research. In this study, we performed differential proteome profiling of metastatic HCT-116 cells for identification of colorectal cancer metastatic biomarker candidates.

Methods

We established metastatic colorectal cancer cells by surgical orthotopic implantation of HCT-116 human colon cancer cells stably expressing green fluorescent protein (GFP). Matrigel invasion assay was performed to examine the invasive potential of metastatic cell.Then, multiplex peptide stable isotope dimethyl labeling for relative quantification were used for differential proteome profiling. Identified biomarker candidate proteins were validated by selected reaction monitoring (SRM) assay.

Results and Discussion

Matrigel invasion assay demonstrated that these established cells were highly invasive. As the result of quantitative proteomic analysis, 4593 proteins were identified by quantitative proteomic analysis and 123 proteins were differentially expressed between metastatic and non-metastatic cancer cells. In these biomarker candidates, 7 proteins were validated by SRM analysis. Down-regulation of 2 candidate proteins expression by siRNA decreased cell invasion.

Conclusion

We have discovered a new colorectal cancer biomarker candidates. These two proteins were seemed to be involved in tumor cell invasion.

Keywords: Colorectal cancer, Metastasis

TP01-73

Salivary proteins from pre-malignant and malignant lesions of the oral cavity and their translational potential for early diagnosis

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Introduction and Objectives

Oral cancer (OSCC) constitutes 40% of the cancers in India with a survival rate of only 50%. Early diagnosis using saliva based molecular markers would be a non-invasive solution for down -staging the disease to improve the outcome. The aim of the present study is the first-stage identification and validation of protein biomarkers present in the saliva from patients with pre malignant lesions or from OSCC patients.

Methods

The study subjects were leukoplakia patients with dysplastic lesions (n=15), lymph node negative (N0; n=15) and lymph node positive (N+; n=15) patients with carcinoma of buccal mucosa and healthy controls (n=15). The cell free saliva from 5 patients of each group were pooled and the pools analyzed using iTRAQ based quantitative proteomic analysis on Orbitrap Velos high resolution mass spectrometer. Differentially altered proteins in above consecutive stages were assed for their translational potential.

Results and Discussion

We identified a total of 1319 proteins from triplicate experiments, 178 of them were found with altered levels from various paired comparisons, 92 being up-regulated (>=1.5 fold). A scoring system was made for these 92 proteins based on their tumor and biological relevance and secretability assessed using prediction tools (Exocarta, Signal P and Secretome P). Thirty proteins were thus short listed that included members between any two or all three consecutive stages – leukoplakia, N0 and N+. The annotated list of 30 priority proteins along with their proteotypic peptides is provided. We verified 4 representative molecules (S100A7, CD44, COL5A1, and COL1A1) by ELISA in the saliva specimens from independent cohorts of OSCC patients. S100A7, CD44, COL5A1 levels were high in early stage leukoplakia and tumor; COL1A1 and CD44 can differentiate lymph node negative tumor from lymph node positive tumors. Our results provide a high confidence resource of salivary proteins for targeted investigations for use in early diagnosis of dysplastic lesions/cancers of the oral cavity.

Conclusion

The study shows the potential of salivary protein markers to be a promising approach to develop saliva based diagnostic methods. S100A7, CD44 and COL5A1 have been verified for diagnosis at leukoplakia stage. Validation of these and more candidates using targeted mass spectrometry or immunoassays, in larger independent patient cohorts

would further establish the clinical feasibility and utility of these markers.

Keywords: Saliva, Oral Cancer, Proteomics, Mass Spectrometry, Biomarkers

TP01-74

Quantitative Analytical Method for Measuring the Levels of PIVKAII in Human Serum Using Multiple Reaction Monitoring-Mass Spectrometry

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Introduction and Objectives

Vitamin K absence or antagonist (PIVKAII) is known as an effective tumor marker for hepatocellular carcinoma (HCC). Our goal was to develop an analytically sensitive, robust MRM-MS method for quantification of PIVKAII in serum samples. Using this approach, we were able to quantify PIVKAII in serum for the first time by a mass spectrometry-based method.

Methods

The first part of our work is to identify the surrogate peptide for determining the PIVKAII level in human serum by narrowing down candidate peptides in accordance with criteria. The second part of our work is to set up MRM-MS transitions, linear range and to optimize parameters of mass spectrometry to measure the absolute concentrations of chymotryptic peptide by using SIS peptides. The third part of the work is to evaluate the optimized MRM-MS based method using 250 human serum comprised of 5 groups, normal control, chronic hepatitis, liver cirrhosis, before HCC treatment (HCC) and after HCC treatment (recovery).

Results and Discussion

The multiple reaction monitoring (MRM)-MS assay for determining the concentration of PIVKAII showed a limit of detection (LOD) value of 21.39 ng/mL and a limit of quantification (LOQ) value of 44.46 ng/mL. A good correlation (R2 = 0.912) was observed for the measurable PIVKAII concentrations between MRM-MS and immunoassay. In the cohort of normal control (n=50), chronic hepatitis (n=50), liver cirrhosis (n=50), HCC (n=50), and recovery (n=50) subjects, the area under the receiver operating characteristic curves (AUROCs) value of 0.870, 0.766, 0.740 and 0.706 in HCC versus normal control, chronic hepatitis, liver cirrhosis and recovery, respectively.

Conclusion

MRM-MS assay serves as a complementary and/or alternative technique to antibody-based immunoassays especially when high-quality antibodies for sensitive immunoassays are not available. Quantification of PIVKAII at the ng/mL level (LOD = 21. 39 ng/mL, LOQ = 44.46 ng/mL) was achieved. In addition, good correlation (R = 0.912) of PIVKAII concentration was obtained between the MRM-MS assay approach and the immunoassay results for several clinical specimens, demonstrating the reliability of this assay. Although the AUROC value from MRM-MS assay was lower (AUROC 0.058) than that from immunoassay, we achieved the objective of this study to develop a novel method based on mass spectrometry.

Keywords: Mass spectrometry, PIVKA II, MRM-MS, Quantification

TP01-75

Method Validation of the Protein Biomarkers Approved by FDA and LDT following CPTAC Assay Development Guidelines using Multiple Reaction Monitoring

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Introduction and Objectives

Development of disease biomarkers has been attempted numerously to be approved by official organizations like FDA. Although each of approved biomarkers, especially a protein, shows a good performance, different quantitation methods were used to quantify single or few targets, which makes hard to conduct high-throughput analysis of protein biomarkers. Here, we developed the assay which could quantify some of these protein biomarkers simultaneously using Multiple Reaction Monitoring (MRM-MS). Following CPTAC assay guidelines (v1.0), we also validated that our MRM-MS assay was applicable to constructing quantitative Laboratory Developed Test (LDT).

Methods

We performed the absolute quantitation using semi-high purity stable isotope standard (SIS) peptides (about 90%) for 93 target biomarkers approved by FDA. We used the FDA approved normal serum and plasma as a matrix of sample to evaluate the category number 3 of CPTAC assay guidelines which provides a list of experiments for more reliable data. All of the data were processed by Skyline software (Maccoss lab, v3.5).

Results and Discussion

The 53 of 93 target biomarkers were selected to develop an assay based on detectability and availability of right standard peptide. We built calibration curves (> 8 points) for these 53 protein targets through serial dilution of each heavy peptide, and all of the targets showed good linearity and performance. Also, almost all targets were very reproducible for intra- and inter- day assays. These results mean that our quantitative MRM-MS assay might be useful to quantify overall dynamic concentration range of the target proteins in serum samples. Along with 2 categories of guidelines, we evaluated selectivity, stability and repeatability which showed satisfying data.

Conclusion

We finally developed the quantitative MRM-MS assay which could quantify many FDA approved LDT targets and validated the assay through the official CPTAC assay guidelines. Furthermore, more FDA approved biomarkers will be evaluated to construct a larger MRM-MS assay, so the validated assay can be qualified as a medical device to diagnose several diseases including cancers.

Keywords: Method validation, FDA approved biomarkers, MRM-MS, CPTAC assay guidelines, Absolute quantitation

TP01-76

Serum biomarker panels for the early detection of pancreatic cancer

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Introduction and Objectives

Pancreatic cancer is a deadly disease for which the available biomarkers, such as CA19-9, lack the desired sensitivity and specificity for early detection. The majority of patients present with unresectable disease leading a median survival of 6 months and an overall 5-year survival of less than 5%. Combination of individual serum biomarkers could improve their performance in early detection of pancreatic cancer. This study is aimed to develop a serum biomarker panel for the early detection of pancreatic cancer.

Methods

Bioinformatical analysis of publicly available gene, protein, and PUBMED databases was performed to identify candidate biomarkers under a weighted scoring system based on either fold changes and number of publications or sensitivity/specificity and study sample sizes. Magnetic bead-based multiplex immunoassays were developed for the selected candidate serum biomarkers using a Bio-Plex 200 suspension array system (Bio-Rad). Briefly, monoplex assays of individual candidates were first developed, cross-reactivity checked, and multiplex assays validated and optimized. All of these proteins plus CA19-9 (Tosoh) were analyzed in sera of patients diagnosed with pancreatic ductal adenocarcinoma (PDAC: IA/IB/IIA/IIB, 13/19/17/48; III/IV, 19/73), benign pancreatic conditions including intraductal papillary mucinous neoplasm (IPMN, 63) and chronic pancreatitis (68), and healthy controls (89). The performances of these candidate markers were evaluated individually or in combination on their capacity to complement CA19-9 in early detection of pancreatic cancer.

Results and Discussion

A 6-plex immunoassay of OPN, MIA, CEACAM-1, MIC-1, SPON1 and HSP27 was in-house developed with negligible cross-reactivity, recovery of 89-104%, and intra-assay or inter-assay precision of 2.1-15.4% or 3.7-21.5% for QC samples, respectively. The 6-plex assay demonstrated wide dynamic ranges for the target measurements, and was significant correlated with their respective monoplex assays and/or commercial ELISAs ($p<0.05$). Individually, the best biomarkers to separate PDAC early stage from pancreatitis or IPMN based on the ROC analysis were CA19-9 (AUC=0.77, [0.70-0.84]), MIC-1 (0.64, [0.55-0.73]), CEACAM-1 (0.60, [0.51-0.69]) & MIA (0.57, [0.49-0.66]) or CA19-9 (AUC=0.81, [0.74-0.88]), MIC-1 (0.73, [0.65-0.81]), CEACAM-1 (0.67, [0.59-0.75]) & OPN (0.64, [0.55-0.73]), respectively. Logistic regression modeling and ROC analysis selected a five-marker panel of CA19-9, MIC-1, CEACAM-1, MIA & OPN with an AUC=0.84 (0.78-0.90) for PDAC early stage versus pancreatitis or AUC=0.86 (0.80-0.91) for PDAC early stage versus IPMN, which greatly improved the individual biomarker performance.

TP01-76

Conclusion

The multiplex immunoassay workflow provides sufficient analytical performance to evaluate serum biomarker panels that complement CA19-9 in early detection of pancreatic cancer. The biomarker panels identified in this study warrant additional clinical validation to determine their role in early detection of pancreatic cancer.

Keywords:

TP01-77

Study on sputum cell proteomics for screening early detection biomarkers of lung cancer

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Introduction and Objectives

Lung cancer is the leading cause of cancer death in China and America. Currently, no available noninvasive biomarkers to detect early stage lung cancer. Sputum is directly contacted with the lungs and respiratory tract, allowing the pathogenic changes that occur in lung cancer tumor tissue to be transferred into sputum. In addition, sputum could be obtained noninvasively, making its collection feasible for population-based screening. Molecular study of sputum could detect the cells containing lung tumor-associated molecular aberrations, thus providing a noninvasive approach for diagnosis of lung cancer.

Methods

Proteomes of sputum samples from 15 patients with lung cancer and 13 controls with chronic obstructive pulmonary disease (COPD) were assayed using LC-MS/MS analysis. Sputum cytology was performed for each samples.

Results and Discussion

Totally, we identified 6605 proteins in lung cancer sputum and 6159 proteins in COPD sputum using the MaxQuant software. Overlap of proteins is 90.5%, and 540 proteins included exclusively in lung cancer. These proteomic profiles identified functional differences between lung cancer and COPD, related to translational elongation, translation and antigen processing and presentation of peptide or polysaccharide. We identified 4 candidate proteins, expression higher in lung cancer sputum. The expression level of candidates 1 in lung cancer tissues was significantly higher than that in normal lung tissue ($P<0.001$), which showing a promising clinical application.

Conclusion

Conclusion, in this study, we focused on tumor-related proteins in sputum of lung cancer patients as biomarkers.

Keywords: Lung cancer/Sputum/ Biomarkers/ Early Detection

Effects of Apigenin on growth inhibition and apoptosis induction of human cholangiocarcinoma cell line

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Introduction and Objectives

Nutraceuticals are compounds considered to provide medical and health benefits. Apigenin, an interesting nutraceutical, was recently shown to have biological activity in inhibiting several cancers. The effects of apigenin on the growth inhibition and apoptosis of cholangiocarcinoma cell line (HuCCA-1) were studied. The proteomic approach was used to study protein alterations after treatment with apigenin.

Methods

The values of IC20, IC50, and IC90 were determined by MTT cell viability assay. Apoptotic cell death was detected by using two different methods, a flow cytometric analysis (Muse Cell Analyzer) with Annexin-V and dead cell assay kit, and DNA fragmentation assay. Various conditions including attached and detached cells were selected to perform two-dimensional gel electrophoresis (2-DE) to study the alterations in expression of treated and untreated proteins and identified by LC/MS/MS.

Results and Discussion

The IC20, IC50, and IC90 values of apigenin after 48 h treatment in HuCCA-1 cells were 25 μM, 75 μM, and 200 μM, respectively, indicating cytoxicity of this compound. Apigenin induced cell death in HuCCA-1 cells via apoptosis as detected by flow cytometric analysis and confirmed with DNA fragmentation characteristic of apoptotic cells. We identified 67 proteins with altered expression from the 2-DE analysis and LC/MS/MS.

Conclusion

Cleavage of proteins involved in cytoskeletal/mobility (cytokeratin 8, 18, and 19) and high expression of S100-A6 and S100-A11 suggested that apoptosis was induced by apigenin via the caspase-dependent pathway. Two interesting proteins, heterogeneous nuclear ribonucleoprotein H and A2/B1 disappeared completely after treatment, suggesting the role of apigenin in inducing cell death. These studies indicate that apigenin shows induction of growth inhibition and apoptosis in cholangiocarcinoma cells and the apoptosis pathway was confirmed by proteomic analysis.

Keywords: cholangiocarcinoma, apoptosis, apigenin, proteomics

TP01-79

Discovery of ARV induced kidney-injury related protein biomarkers in urine using SWATH-MS.

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Introduction and Objectives

Antiretroviral therapy (ART) is made freely available to HIV positive patients in South Africa, with majority of patients having positive outcomes. However ART therapy is also known to cause severe side effects including kidney damage. The identification of new renal protein biomarkers will allow for early interventions to be made and potentially avoid further ART related complications. Urine has emerged as an attractive source for biomarkers due to the relative ease with which it can be obtained. Though it is readily available, its dynamic range spans approximately 10 orders of magnitude, with abundant proteins such as uromodulin preventing in-depth urine proteome characterization. Sample processing and data mining strategies are required to ensure that highest proteome coverage is obtained. The aim of this work is to develop a workflow for in-depth analysis of the urinary proteome that can be applied in a clinical setting. A variety of depletion and fractionation strategies in will be tested in combination with microflow liquid chromatography coupled to SWATH MS data acquisition.

Methods

Urine samples were preserved and concentrated using a Norgen Urine kit. Uromodulin depletion was performed using (i) lectin affinity, Lens culinaris (Lens) or (ii) anti-uromodulin mAb where Lens and anti-uromodulin mAb were immobilised on MagReSyn® polymer microspheres. Undepleted samples were trypsin digested and spiked using iRT standards. Peptides were fractionated using high pH RP and fractions analysed using Dionex RSLC, in micro mode, coupled to an AB Sciex 6600 TripleTOF in DDA mode. Alternatively samples were analysed directly using the microLC-MSMS in DDA as well as SWATH modes. Data was searched using MaxQuant and spectral libraries as well as SWATH runs processed using SpectronautTM.

Results and Discussion

In comparison to 1D, 2D-microLC-MSMS generated more than double identified proteins (400 1D vs 700 2D; 1% local FDR cut-off). This resulted in a more comprehensive spectral library with close to 600 quantifiable proteins (CV <= 13%). Changes in expression of known kidney damage markers were detected. In order to improve the depth of urine proteome cover as well as reduce analysis time we are also testing two uromodulin depletion workflows. Currently the efficiency and specificity of the two depletion methods is being evaluated.

Conclusion

The 2D-microLC-MSMS resulted in more comprehensive urine proteome coverage. This was achieved at the expense of increased MS time, not conducive to processing of large clinical sample sets. The use of uromodulin depletion strategies compacts the dynamic

range and results in deeper proteome characterization using a shorter 1D workflow. Application of magnetic microspheres for affinity depletion allows full automation of sample preparation with parallel sample processing. In combination with microLC this provides a robust, high-throughput workflow useful for clinical sample analysis.

Keywords: Urinary Proteomics, SWATH-MS, mirco LC, Depletion, Fractionation, MagReSyn®

TP01-80

MDC1-SDTD repeats as a potential target to block DDR and promote radiosensitization in cancer cells

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Introduction and Objectives

DNA Damage Response (DDR) guards genomic stability by transducing signals of DNA repair, cell cycle arrest, and apoptosis. It is initiated by MRN (MRE11, Rad50, and NBS1) complex to sense DNA double strand breaks (DSBs) and recruit ATM in the NBS1 C-terminus-dependent manner, allowing the phosphorylation of adjacent histone H2AX by ATM. Tandem BRCT domain of MDC1 recognizes phosphorylated H2AX (rH2AX), and phosphorylated SDTD repeat region of MDC1 associates with FHA domain of NBS1, thereby recruiting more MRN complexes to the DSB sites to amplify DDR signals. We proposed that these signaling events can be targeted to provide therapeutic efficacy in conjunction with conventional radiotherapy.

Methods

We performed clonogenic cell survivals upon X-ray irradiation in the presence of ectopic expressions of the NBS1 C-terminus ATM binding site, MDC1-FHA domain, MDC1-SDTD repeats, MDC1-tandem BRCT domain, rH2AX-s139 site, and CHK2 SCD domain, and selected the most effective target. Immunofluorescence and comet assays were also conducted to evaluate the kinetic of DDR signal and subsequent DSB repair efficiency through the targeting. The immunoprecipitation was then used to validated interacted signaling factors by the targeting approach.

Results and Discussion

We showed that ectopic expression of MDC1-SDTD repeats most effectively provides radio-sensitivity in cells, suggesting that DDR signal pathway is able to be targeted between NBS1 and MDC1. We also observed a delayed signal amplification of rH2AX and less efficient chromosome repairation in cells through this targeting. More specifically, we found that ectopic expressed MDC1-SDTD repeats are able to bind endogenous NBS1, leading to a decreased interaction between NBS1 and CtP1.

Conclusion

Our results demonstrated that ectopic expression of MDC1-SDTD repeats can target DDR signals and promotes radio-sensitization in cell, and this targeting may provide therapeutic potential as an adjuvant in radiotherapy to treat cancers.

Keywords: DNA damage response, radiotherapy, MDC1, NBS1

TP01-81

Novel hypoxia-driven oncogenic pathways are revealed by tandem quantitation of the tumor cell proteome, chromatome, and secretome.

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Introduction and Objectives

The rapid growth of solid tumors depletes microenvironmental oxygen and exerts hypoxia stress on the constituent cancer cell clones. We hypothesized that hypoxia-induced pathways of clonal evolution represent promising targets for effective new cancer treatments. Our objective was to identify novel mediators of hypoxia-driven tumor progression that can be targeted for cancer therapy.

Methods

To assess the influence of hypoxia on cancer progression, we designed an in vitro model that facilitates the application of variable hypoxia stress to cultured cancer cells and then conducted quantitative proteomic analyses of their differentiation. Human tumor-derived cells were initially grown under standard culture conditions before being subjected to controlled oxygen deprivation, with or without subsequent re-oxygenation. The influence of this simulated tumor microenvironment on clonal evolution was then assessed using iTRAQ quantitative proteomics to determine how hypoxia modulates the tumor cell proteome, secretome, and chromatome. This holistic strategy revealed that hypoxia stimulates multiple pathways that promote cancer cell survival, evolution, angiogenesis, tissue invasion and host immune suppression.

Results and Discussion

Cancer cells are known to secrete soluble and exosomal proteins that promote disease progression by inducing angiogenesis, promoting metastasis, and suppressing host immunity. Accordingly, our quantitative analysis of the secretome revealed that low-oxygen conditions stimulate tumor cell expression of multiple mediators of host immune suppression that could represent novel targets for immunotherapy. Moreover, we identified that hypoxia up-regulates the NHEJ pathway which mediates repair of DNA double-strand breaks, likely accounting for hypoxic tumor resistance to radiotherapy and chemotherapy as observed in clinical settings. In addition, we determined that hypoxic conditions induce integrin glycosylation at specific residues and alter cellular distribution of adhesion molecules that promote cancer cell detachment from the primary tumor and increase metastatic potential. Our results further demonstrate that hypoxia modifies chromatome composition, DNA methylation patterns, and histone modification profile to enhance cancer cell ability to establish new tumors with improved survival characteristics. This work has led to the identification of several potential new drug targets including HP1BP3, which appears to be a key switch in promoting cancer stem cell formation in both in vitro and in vivo settings. Various biochemical assays have since been used to validate our finding that HP1BP3 is a promising epigenetic drug target for the treatment of hypoxic solid tumors.

Conclusion

TP01-81

Quantitative proteomic analyses identify multiple novel hypoxia-induced proteins and pathways that are promising new targets for effective treatment of human cancers.

Keywords: Cancer clonal evolution, hypoxia, immunotherapy, angiogenesis, quantitative proteomics, secretome, chromatome, proteome.

TP01-82

Analysis of human high-grade serous ovarian carcinoma by mass spectrometry using data-dependent and data-independent acquisition

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Introduction and Objectives

Although proteomics technologies have revolutionized cell biology and biochemistry by providing powerful new tools to characterize complex proteomes, multiprotein complexes and post-translational modifications, there is a critical need to verify the disease-associated proteins that are gained from large-scale discovery phase proteomics studies using data-dependent acquisition (DDA). Towards this end, SWATH-MS has emerged as a quantitatively accurate method that is well suited for the orthogonal verification of protein targets using data-independent acquisition (DIA). Previously, we conducted an iTRAQ-based integrated proteogenomic characterization of >100 human ovarian tumors using DDA as part of the Clinical Proteomic Tumor Analysis Consortium (CPTAC) effort to systematically identify proteins derived from alterations in cancer genomes and related biological processes (H. Zhang et al. 2016. Cell. In press). Here, we describe the analytical performance of SWATH-MS for the orthogonal verification of the biological insights gained from this global proteomics study, namely the identification of functional protein modules that are associated with homologous recombination deficiency (HRD) and the identification of proteomic subtypes.

Methods

106 clinically annotated ovarian high-grade serous carcinomas (58 HRD, 48 non-HRD) previously characterized by The Cancer Genome Atlas (TCGA) were processed for proteomic analysis. SWATH-MS analysis was conducted using a chip-based nanoLC system interfaced with a 5600+ Triple-TOF mass spectrometer. SWATH-MS data were acquired using a variable window acquisition strategy for improved specificity. A sample-specific spectral library was generated from the DDA analysis of a bRPLC-fractionated pooled sample and the DDA analysis of the individual samples. OpenSWATH was used for feature, peptide and protein identification, and mapDIA was used for data quality analysis, data normalization and differential analysis.

Results and Discussion

After applying the abundance re-quantification algorithm of OpenSWATH, 1,945 proteins were quantified across all 106 tumors. The intra-tumor protein dynamic range was 4 orders of magnitude. The overall peptide- and protein-level CVs were <20%. As an indication of the quantitative performance of SWATH-MS in this study, the median CV of the low abundance proteins was 15% and the median CV of the high abundance proteins was <5%. Differential expression analysis of the Immunoreactive, Differentiated, Mesenchymal and Proliferative TCGA subtypes indicated the up-regulation of proteins that were representative of the respective subtypes. Additionally, functional annotation analysis of the proteins up-regulated in the HRD tumors verified protein classes that were identified from the iTRAQ-based DDA analysis of the tumors.

TP01-82

Conclusion

Protein changes associated with genomic alterations identified from the analysis of >100 human ovarian high-grade serious tumors using DDA discovery phase proteomics were successfully verified by an orthogonal SWATH-MS DIA approach.

Keywords: SWATH, ovarian cancer, DDA, DIA, proteogenomics

TP01-83

Quantitative proteomics of transgenic prostate cancer mice reveals that PDGF-B regulatory network plays a key role in prostate cancer progression

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Introduction and Objectives

Prostate cancer (PCa) remains the second most frequent cancer and the second cause of cancer-related death in men. Although several effective therapy options are available, PCa is still one of the most intriguing challenge in oncology due to the lack of knowledge of disease progression mechanisms on the molecular and cellular levels. Transgenic cancer animal models have provided a fundamental contribution to the investigation and understanding tumor growth. Among the PCa animal models, Transgenic adenocarcinoma of the mouse prostate (TRAMP) mice is one of the most commonly used models in clinical research.To investigate the regulatory network associated with PCa progression, we performed a label free quantitative proteomics analysis combined with a careful bioinformatics analysis on the entire prostate protein extraction from TRAMP mice and compared with WT littermates.

Methods

Prostates were isolated from four pairs TRAMP mice and Wild Type mice and individually analyzed with a mass spectrometry platform of QExactive Plus coupled with nanoLC system. The raw files were quantified with Maxquant 1.5.0.1, and significantly altered proteins were validated with western blot and qRTPCR in both animal models and clinical samples. novel regulation networks were predicted with Ingenuity IPA and validated in vivo and in vitro.

Results and Discussion

From totally 2400 identified proteins, we here presented a modest mice prostate reference proteome containing 919 proteins. Biostatistics analysis of 515 selected proteins that were identified in all biological replicates indicated that 61 proteins presented a significant expression difference between the two groups. The subsequent integrative bioinformatics analysis based on both qualitative and quantitative proteomics results predicted the overexpression of the platelet-derived growth factor B (PDGF-B) in tumor tissue and supports the hypothesis of the PDGF-B signaling network as a key upstream regulator in PCa progression. Importantly, the overexpression of PDGF-B and the associated proteins were experimentally validated in both animal model tissues and clinical human samples. PRDX2, PDIA3, and HNRNPL were confirmed as three novel PDGF-B-regulating proteins with immunoblot in shRNA-based PDGF-B knockdown PCa cells. The other six novel PDGF-B-regulating proteins were validated with qRT-PCR. We also proved that inhibiting PDGF-B signaling suppressed PCa cell proliferation with shRNA knockdown or induction of a PDGFR inhibitor. Furthermore, we demonstrated that Crenolanib, which is a novel PDGF receptor inhibitor, inhibited PCa cell proliferation in a dose-dependent manner.

TP01-83

Conclusion

we revealed the importance of PDGF-B regulatory network in PCa progression, which will assist to understand the role and mechanisms of PDGF-B in promoting the cancer growth and provide valuable knowledge reference in the future research on anti-PDGF therapy.

Keywords: Prostate cancer, animal model, quantitative proteomics, PDGFB

TP01-84

Global proteome analysis of carcinoma associated fibroblasts and dystrophic epidermolysis bullosa fibroblasts in 3D cell culture

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Introduction and Objectives

Dystrophic epidermolysis bullosa (DEB) is a skin blistering disease, induced by mutations in the gene COL7A1, which encodes type VII collagen, causing a disruption of dermal-epidermal adhesion. Patients suffering from DEB frequently develop metastasizing squamous cell carcinoma (SCC). Carcinoma associated fibroblasts (CAFs) are able to remodel the ECM of the dermis and can promote SCC tumor invasion. Gene expression profiles of DEB skin fibroblasts and CAFs are similar, leading to the hypothesis that DEB fibroblasts play a role in promoting tumor cell invasion. The aim of our study is to shed a light on the molecular mechanisms deregulated in DEB fibroblasts, which might be involved in promoting tumor cell invasion. For this we compare proteomes of DEB fibroblasts to CAFs in 3D cell culture.

Methods

The proteome of fibroblasts from 3D cultures were quantified by stable isotope labeling by amino acids in cell culture (SILAC)-based mass spectrometry (MS). For the quantitative comparison of primary normal human fibroblasts (NHF), DEB fibroblasts, DEB CAF and CAF from head and neck SCC with each other, we generated a super SILAC mix of all cell types. The cells were cultured under fetal calf serum (FCS) deprivation, and seeded directly into the collagen I/matrigel mixture before it polymerized. After 5 days of 3D cultivation, the cells were lysed and mixed in a 1:1 ratio with the respective super SILAC mix. Samples were prepared by in-gel digestion prior to LC- MS/MS analysis.

Results and Discussion

With our approach we were able to acquire accurate, quantitative proteomic data of NHFs, DEB fibroblasts, DEB CAFs and CAFs from 3D cell culture. We revealed intracellular and extracellular proteomic differences and similarities of NHF to DEB fibroblasts, DEB CAF and CAF, which gives us further understanding of deregulated mechanisms in DEB and SCC cells.

Conclusion

Up to date the molecular mechanisms behind aggressive SCC invasion in DEB and the specific role of loss of collagen VII in tumor progression has not been fully understood. With our approach we are able to get a global view on the proteomic deregulation of DEB fibroblasts and CAFs in the context of an organotypic cell culture system.

Keywords: Dystrophic epidermolysis bullosaSquamous cell carcinoma3D cell culture

TP01-85

Integration of urine and tissue proteomes for biomarker verification of bladder cancer using targeted proteomics

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Introduction and Objectives

Despite considerable developmental efforts, currently, FDA-approved BC biomarkers fail to deliver sufficient sensitivity and specificity for clinical diagnosis. Therefore, discovery of potential biomarkers for cancer detection is a crucial challenge. Recently, multiple-reaction-monitoring mass spectrometry (MRM-MS) has become a powerful tool in targeted proteomics due to its high performance in large-scale verification. In this study, our aim is to establish MRM assay of potential biomarker candidates discovered from bladder cancer (BC) tissue proteome of previous study and further validate them in clinical urine and tissue samples after prioritizing the protein candidates in urine by LC-MRM-MS.

Methods

In previous study, we had identified potential BC biomarker candidates in clinical tumor tissues. Our targeted candidates of this approach were selected from the discovery phase of previous study. MRM software was used to select suitable Q1/Q3 transition pairs for each candidate for MRM assay development. Digested peptide mixtures of BC cell lysate and synthetic peptides were labeled through dimethylation for establishment of optimized MRM assay. Each MRM transitions were determined the retention times, top-3 transitions, and optimized collision energy. The verification of 122 candidates was performed by LC-MRM-MS analysis in a large cohort of clinical urine specimens. Validation of potential candidates was performed by western blot (WB), immunohistochemistry (IHC) and enzyme-linked immunosorbent assay (ELISA).

Results and Discussion

130 candidates from previous study were selected for verification in this study. Total 118 candidates, 2 BC biomarkers and 2 house-keeping genes were successful established final MRM assay and verified by LC-MRM-MS in clinical urine specimen of 31 hernia (H), 30 BC, 30 hematuria (HU), and 28 urinary tract infection (UTI). The MRM signals of 29 targets were detected in at least one urine sample. Among these 29 targets, 8 targets could be detected in at least 50% of all specimens. We also noticed 6 targets only detected in BC urine but not in control urine. Therefore, BC15 and BC17 were selected for further validation due to their higher ranking of detectable sample number. Urinary BC15 and BC17 exhibited higher protein levels than hernia in WB. Urinary BC17 of BC showed the significant difference with H ($p<0.01$), HU ($p<0.05$) and UTI ($p<0.05$) in ELISA. The IHC of BC17 in tumor tissues also showed the significant difference with adjacent normal and normal tissues ($p<0.05$). These data indicate that BC17 is a potential biomarker for further investigation and clinical application.

TP01-85

Conclusion

In this study, we successful integrated tissue and urine proteome by using targeted approaches, and performed primary validation of tumor-specific biomarker candidates in clinical urine specimens. Our findings provide valuable information for future validation of potential biomarkers.

Keywords: Bladder cancer, tissue proteomics, MRM-MS, targeted proteomics, biomarker verification

TP01-86

The Effects of Dietary Polyunsaturated Fatty Acids on Prostate Cancer: Unbiased Proteomic and Phosphoproteomic Studies

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Introduction and Objectives

Prostate cancer (PCa) is one of the most common cancers in men. Because of the slow progression of this disease, early intervention methods, especially, dietary fatty acid interventions are considered very important to control the disease in early stages. A line of evidence has suggested that early inference and dietary prevention are beneficial in PCa patient care. Fish oil (FO), which contains mostly omega-3 fatty acid, is one of the most widely studied candidate supplements for PCa prevention; however, the molecular mechanism is not thoroughly understood. The goal of this study is to evaluate the regulatory role of FO on PCa through a global unbiased proteomic analysis and a global phosphoproteomic profiling.

Methods

LC/MS-based label-free global proteomic and phosphoproteomic studies were carried out on PC3 cells with longitudinal treatment with fish oil or oleic acid.

Results and Discussion

With short-term fish oil treatment, sequestosome-1 was elevated. Prolonged treatment induced down-regulation of MSMP, a newly identified proinflammation factor, as well as proteins in the glycolysis pathway. In the global phosphoproteomic study, we confidently identified 828 phosphopeptides from 361 phosphoproteins. Quantitative comparison between fish oil and oleic acid treated groups and the untreated group suggests that the fish oil induces changes in protein phosphorylation of proteins involved in pathways associated with cell viability and metabolic processes specifically significant decreases in the levels of phospho-PDHA1(Ser232) and phospho-PDHA1(Ser300). The decrease in the phosphorylation of the PDHA1 protein was accompanied by an increase in PDH activity, suggesting a role for n-3 polyunsaturated fatty acids in controlling the balance between lipid and glucose oxidation.

Conclusion

This study confirmed that FO changed PCa cell function through diverse pathways including glycolysis, cell cycle, cytotoxicity induced stress, anti-inflammation and also provided useful details about the mechanism of these effects by identifying novel protein and phosphoprotein targets such as MSMP, phospho-sequestosome(S366) and phospho-PDHA1(S232)&(S300). Considering the success of the combination of global proteomic and phosphoproteomic studies to identify new important proteins altered by FA treatment in PC3 cells, a global proteome and phosphoproteome clinical study in PCa patients would validate and extend these findings to the patient population and potentially identify additional targets of FO action, providing novel opportunities for therapeutic development.

TP01-86

Keywords: Prostate Cancer, Proteomics, Phosphoproteomics, Mass spectrometry, Fish oil, Polyunsaturated fatty acids.

TP01-87

Proteomic analysis of cell response to photon and particle irradiation

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Introduction and Objectives

It is generally accepted that cancer patients benefit from radiation therapy. In contrast to photon irradiation which is well established in clinical therapy irradiation with particles is only recently introduced in clinical practice. In contrast to X-rays, particles deposit most of their energy to a small region within tissue, the so-called Bragg peak. This enables dose escalation in the tumor while sparing healthy tissues. To better understand the biology behind the radiation qualities we applied mass spectrometry based proteomics.

Methods

Human lung carcinoma (A549) cells were irradiated with 2 Gy carbon, 3.5 Gy proton and 6 Gy photon (radiobiological equivalent doses) and harvested 2 h after irradiation to uncover acute cell response. For quantification cells were metabolically labeled using SILAC. GeLC-MS/MS was used for protein expression analysis and enrichment of phosphopeptides with IMAC/Titanium dioxide for analysis of the phosphorylation status.

Results and Discussion

We were able to quantify 2634 proteins and 2818 phosphorylation sites in the irradiated samples. Only negligible changes occur at the protein expression level two hours after irradiation. In contrast, phosphorylation is a much faster event than synthesis or depletion of proteins. Thus we could identify 181 radiation regulated phosphorylation sites. Overall 55 of these sites show significant differences between the radiation qualities. We selected 28 candidates for validation using synthetic isotope labeled phosphopeptides in a targeted spike-in approach. With this experiment we were able to confirm the phosphorylation sites using fragmentation pattern as well as retention time.

Conclusion

This proteomic comparison of cells irradiated with particles versus X-rays clearly showed that phosphorylation sites respond in different ways to radiation qualities.

Keywords: proteomics, phosphoproteomics, particle irradiation, X-rays

TP01-88

Phosphoproteome profiling of isogenic cholangiocarcinoma exosomes reveal differential expression of a key metastatic factor

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Introduction and Objectives

Cholangiocarcinoma is an aggressive malignant tumor of bile duct with especially high-prevalent in Southeast Asian countries. In past decade, the incidence and mortality rates of cholangiocarcinoma have been increasing globally. Thus, continued efforts have been made to identify molecular mechanisms involved in the cholangiocarcinoma development, progression and metastasis. Tumor-derived exosomes have been demonstrated to play a key role in the multiple aspect of metastasis by serving as mediators of the intercellular communications. Increasing number of studies indicate that exosomes can promote the survival and outgrowth of disseminated cancer cells through pre-metastatic niche formation at the distant site. Protein phosphorylation has been reported to play an essential role in protein sorting into the multivesicular endosomes during exosome biogenesis. To gain insights into the molecular events of cancer metastasis, we compared the phosphoproteome profiles of exosomes released from two isogenic human cholangiocarcinoma cell lines.

Methods

Two isogenic human cholangiocarcinoma cell lines (low-invasive KKU-M213A5 cell and high invasive KKU-M213D5) were used a model study. Two-dimensional gel electrophoresis followed by LC-MS/MS and immunodetection of phosphoprotein were carried out to profile the phosphoproteome of exosomes derived from these two isogenic cells. Phos-tag western blotting was then performed to detect the stoichiometric protein phosphorylation in exosomes.

Results and Discussion

Phospho-proteomic of exosomal proteins revealed the significant increased level of phosphorylated proteins in the low invasive cancer cell-derived exosomes in comparison to those in the high invasive cancer cell-derived exosomes. Interestingly, most of them are involved in the cancer cell invasion and metastatic process. These data, for the first time, demonstrate a new aspect of phosphorylation involvement in the exosomal pathway.

Conclusion

The phosphorylation levels of exosomal proteins were potentially associated with the aggressive metastatic phenotype and this might be a novel regulation of cholangiocarcinoma progression.

Keywords: Phosphoproteomics, Cholangiocarcinoma, Metastasis, Exosome

TP01-89

GLP-1 was identified as a potential therapy target for colorectal cancer through label-free quantitative proteomic analysis

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Introduction and Objectives

Colorectal cancer is the third most frequent cancer worldwide. There is an urgent need to find novel biomarkers that can be applied for screening diagnosis or improving therapeutic effect and patient survival rates. In this study, we aimed to identify novel biomarkers involved in colorectal cancer and provide potential therapeutic targets through a label free quantitative proteomics approach.

Methods

A shotgun quantitative proteomic method was applied to identify the differentially expressed proteins in six pairs of colorectal cancer tissues and adjustment normal mucosal tissues of colorectal cancer patients. Proteins were identified and quantified in a label free approach with a QExactive Plus mass spectrometer coupled with a nanoLC system. The potential biomarkers were validated with western blotting and immunohistochemical staining. The molecular signaling machinery and therapy potential were further investigated with total internal reflection (TIRF) microscopy and a fluorescent translocation biosensor.

Results and Discussion

A total of 4,266 proteins were identified and quantified. Among them, 136 proteins presented a significant fold change. Differentially expressed proteins (DEPs) were subjected to further analyses. MPK14, EP300, CSRP1, CALR, GCG, CAMKII, NCOA, CAPG, PRDX1, ANXXA6 and TENC were selected for further verification by western blotting, and Glucagon(GCG) was confirmed significantly down-regulated in colorectal cancer tissues than in matched controls . We further confirmed the lower expression of GCG in 40 colorectal cancer tissues using immunohistochemical analysis, and found that the expression levels of GCG were correlated with tumor development stages and metastasis. The cancer therapy potential of glucagon receptor agonist was further validated with TIRF microscopy. GLP-1 significantly reduced the viability and migration ability of CRC cells.

Conclusion

Our findings suggest that glucagon will be a promising clinical biomarker in colorectal cancer and GLP-1 could be a potential therapy target.

Keywords: Colorectal Cancer, Quantitative proteomics, GLP-1, Biopsy

TP01-90

Development of phage display-based platform for discovery of cancer biomarkers and targeting ligands

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Introduction and Objectives

Cancer remains to be one of the leading causes of mortality worldwide. The development of new methods for early detection and effective treatments for cancer is contingent on the identification of biomarkers on the surface of cancer cells, as well as on the isolation of tumor-specific ligands with high binding affinity to such biomarkers.

Methods

We develop a platform for selecting specific peptide ligand to tumor cells and identifying its corresponding target protein. In vitro biopanning of a phage-displayed peptide library was used to identify specific peptides binding to human colorectal carcinoma (hCRC) cells. Based on the selection result, corresponding target protein of targeting ligand was identified by the platform consisted of functional peptide synthesis, chemical modification using cross-linking reagents, affinity trapping, and LC-MS/MS spectrometric analysis.

Results and Discussion

Conclusion

Keywords: Phage Display, Biomarker, Drug Delivery, Targeted Therapy

TP01-91

Comprehensive proteome analysis of fine needle aspiration cystic fluid and cancer cell secretome for papillary thyroid cancer biomarker discovery

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Introduction and Objectives

Papillary thyroid carcinoma (PTC) is the most common malignant neoplasm for thyroid. Although ultrasound guided fine needle aspiration (USG/FNA) biopsy and some thyroid tissue-specific markers (free-T3/T4 and thyroglobulin) have been currently used for diagnosis of PTC, their sensitivity and/or specificity remain to be further improved via combining other biomarkers.

Methods

To discover novel PTC biomarkers, we herein applied the GeLC-MS/MS strategy to analyze proteome profiles of serum abundant protein-depleted FNA cystic fluid from benign and PTC patients, as well as PTC cell line secretome.

Results and Discussion

From which 346, 488 and 2105 proteins were identified respectively. Further analysis revealed that 191 proteins were exclusively detected in the PTC FNAB samples, which may represent potential PTC biomarkers. Among them, 101 proteins could also be detected in PTC cell line secretome, and 7 out of the 101 proteins were reported with elevated mRNA expression in public oligonucleotide microarray. Immunoblot analysis illustrated the elevated expression of five proteins in FNAB of PTC patients versus benign lesions. Immunohistochemistry revealed that expressions of four proteins were significantly higher in PTC tumor tissue than adjacent non-tumor counterparts.

Conclusion

These 7 proteins representing novel candidates are worth to further explore in PTC diagnosis or management.

Keywords: papillary thyroid carcinoma, fine needle aspiration biopsy, GeLC-MS/MS, Hu-14 column, secretome, public oligonucleotide microarray

TP01-92

Uncovering the molecular mechanism of inhibitory activity of Taiwan local pomegranate against urinary bladder urothelial carcinoma cell

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Introduction and Objectives

Pomegranate (Punica granatum, Punicaceae), is an edible fruit cultivated in Mediterranean countries, Afghanistan, India, China, Japan, Russia, the United States, and few in Taiwan. Edible portions (80% of total fruit weight) of pomegranate fruit comprise 80% juice and 20% seed. Pomegranate contains crude ?bers, pectin, sugars, and polyphenols [tannins (mainly ellagitannins), ?avonoids, and anthocyanins] that provide the fruit potent antioxidant activity. Many well-documented evidences have showed that pomegranate fruit possesses anti-cancer potential.

Methods

Two-dimensional gel electrophoresis coupled with tandem spectrometry was used to identify the de-regulated proteins in pomegranate extract-treated bladder cancer cells.

Results and Discussion

Conclusion

Taiwan local pomegranate possesses the inhibitory effectiveness against urinary bladder uropthelial carcinoma cells.

Keywords: Pomegranate, bladder cancer, HSP90, two dimensional gel electrophoresis

TP01-93

Searching tumor-associated proteins for urinary bladder urothelial carcinoma in southwestern Taiwan using gel-based proteomics

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Introduction and Objectives

We try to search for specific serum or urinary biomarkers for the early detection, follow-up, and prediction of tumor recurrence, progression, and clinical outcome is a difficult task in individuals with bladder cancer.

Methods

In this study, urinary samples were dialyzed to remove any interfering molecules and concentration by lyophilization. The urinary proteome maps of 10 healthy volunteers and 10 urinary bladder urothelial carcinoma (UBUC) patients were explored through two-dimensional polyacrylamide gel electrophoresis coupled with mass spectrometry. With no fractionation, the proteome maps acquired in this study likely represented the total urinary proteins.

Results and Discussion

Comparative proteomics indicated that six proteins were down-regulated and five proteins wereup- regulated in BTCC patients as compared with normal. The down-regulated spots were identified ashuman haptoglobin precursor, human heparan sulfate proteoglycan perlecan, inter-alpha-trypsin inhibitorheavy chain H4 precursor, and AMBP protein precursor. The up-regulated spots were identified asperoxiredoxin 2, heparan sulfate proteoglycan perlecan, protease serine 1 fragment and AMBP proteinprecursor. Most of these de-regulated proteins were extracellular matrixeassociated proteins, whichmay play roles in regulating the immune response, signal transduction and tumor invasions.

Conclusion

In this paper, 11 de-regulated proteins were observed in the urinary specimens of BTTCpatients from the southwestern coast of Taiwan where Blackfoot disease is endemic and the unusuallyhigh incidence of BTTC in this area might attribute to high arsenic content in the drinking water. It ispossible that long-term arsenic-induced alteration of these de-regulated proteins, most of which wereextracellularmatrix e (ECM) related proteins which may play roles in regulating the immune response,signal transduction and tumor invasions, might be involved in BTTC development in southwesternTaiwan.

Keywords:

TP01-94

Salivary proteome and extracellular vesicles for the detection of cancer

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Introduction and Objectives

One of the main reasons for the high mortality of cancer is the delay in diagnosis. Non-invasive diagnostics are ideal approaches for the screening and early detection of cancer. Human saliva is such an early detection body fluid in our oral cavity, which has emerged as a novel molecular diagnostics medium and attracted broad attention. Extracellular vesicles (EVs) are important messengers for intercellular communications in the microenvironments, which play critical roles in signal transduction. Human saliva harbors plenty of salivary extracellular vesicles (SEVs) and is a unique target for clinical diagnostics. We intend to develop proteomics biomarkers from saliva and SEVs for the early detection of cancer.

Methods

Salivary proteins were extracted and analyzed by different quantitative proteomics technologies, including two-dimensional difference gel electrophoresis, tandem mass tags technology and iTRAQ technology. Potential biomarkers were further verified by western blot and ELISA. SEVs were prepared through conventional centrifugation method and a newly developed approach by removing high abundant proteins and viscous proteins before centrifugation. Obtained SEVs proteins were digested and analyzed by LC-MS/MS.

Results and Discussion

Through using quantitative proteomics approaches, candidate biomarkers were discovered for the detection of different cancers. Myoglobin, S100A8, and Cornulin were verified for the detection of oral cancer. Haptoglobin hp2, Zinc alpha2-glycoprotein, and Human calprotectin were verified for the detection of lung cancer and their over expression in cancer cell lines were also confirmed. Cystatin B, Triosephosphate isomerase, and Deleted in malignant brain tumors 1 protein were prevalidated for the detection of gastric cancer. The proteome of SEVs were analyzed, which indicated that EVs harbor unique proteins. We further applied the newly developed method to prepare SEVs for cancer biomarker discovery. Our results demonstrated that SEVs in human saliva could serve as a promising liquid biopsy for the detection of lung cancer.

Conclusion

Proteomics biomarkers have been discovered from human saliva and SEVs for the detection of different cancers. A saliva test could be easily conducted in the clinic and would have the potential to detect cancer at an earlier time point, when the likelihood of curative therapy would be greater.

Keywords: Salivary diagnostics / Proteomics biomarkers / Extracellular vesicles / Cancer / Early detection

TP01-95

Metastatic colorectal cancer cell-derived exosomes directionally promote the translation and protein degradation of macrophages

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Introduction and Objectives

Metastatic colorectal cancer cells (CRCs) hijack the immune cells at the homing sites by educating them with exosomes. We hypothesize that the proteome carried by exosomes released by CRCs with different metastatic capacities represent a phenotype directed molecular biosystem.

Methods

We analyzed the exosomes of human colon epithelial-like Caco-2 cells, as well as undifferentiated and highly metastatic SW620 and HCT116 cells by iTRAQ-based mass spectrometry (MS). After bioinformatics analyses, we used exosome treated macrophages to verify the functional proteomics findings.

Results and Discussion

A total of 126 and 162 significantly and differentially expressed proteins (DEPs) of exosomes ($P<0.05$) were detected in SW620/Caco-2 and HCT116/Caco-2 comparisons, respectively. Per pathway and gene ontology analyses, the upregulated proteins were predominantly focusing on the translation regulation and rRNA processing. To interrogate the biological impact of these proteins to macrophage, we performed deep sequencing on the total mRNA and ribosome nascent-chain complex bound mRNA (RNC-RNA) of macrophages treated with different CRCs exosomes, respectively. We found that CRC exosomes could up-regulate macrophage translation genome-wide; and interestingly, we observed preferential up-regulation of longer genes in macrophages treated with either SW620 or HCT116 exosomes, but not in those treated with Caco-2 exosomes. With translation initiation analyses, we found that highly initiated genes were focusing on the ubiquitin-proteasome system.

Conclusion

Metastatic CRC cells can secrete exosomes that significantly impact the translation and protein degradation of macrophages, which serves as a potentially new systems rule in the cell-cell communication of tumor microenvironment.

Keywords: exosomes; proteome; colorectal cancer; translation; RNC-mRNA

Predicting Ovarian Cancer Patients' Clinical Response to Platinum-based Chemotherapy by their Tumor Proteomic Signatures

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Introduction and Objectives

Ovarian cancer is the deadliest gynecologic malignancy in the United States, with most patients diagnosed in the advanced stage of the disease. Platinum-based antineoplastic therapeutics is indispensable to treating advanced ovarian serous carcinoma. However, patients have heterogeneous response to platinum drugs, and it is difficult to predict these inter-individual differences before administering medication.

Methods

In this study, we investigated the tumor proteomic profiles and clinical characteristics of 130 ovarian serous carcinoma patients analyzed by the Clinical Proteomic Tumor Analysis Consortium (CPTAC), predicted the platinum drug response using supervised machine learning methods, and evaluated our prediction models through leave-one-out cross-validation.

Results and Discussion

Our best classifier predicted platinum response status (platinum sensitive or platinum resistant) with area under receiver operating characteristic curve (AUC) 0.73 on cross-validation. We also built a least absolute shrinkage and selection operator (LASSO)-Cox proportional hazards model that stratified patients into early relapse and late relapse groups (P=0.00013). The top proteomic features indicative of platinum response were involved in ATP synthesis pathways and Ran GTPase binding.

Conclusion

Overall, we demonstrated that proteomic profiles of ovarian serous carcinoma patients predicted platinum drug responses as well as provided insights into the biological processes influencing the efficacy of platinum-based therapeutics. Our analytical approach is also extensible to predicting response to other anti-neoplastic agents or treatment modalities for both ovarian and other cancers.

Keywords: Bioinformatics; Cancer biomarkers; Drug resistance; Ovarian cancer; Tandem Mass Spectrometry

TP01-97

Targeted quantitative screening of Chromosome 18 encoded proteome in plasma samples of astronaut candidates

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Introduction and Objectives

Plasma is a valuable biological material which can be easily obtained from human subjects. Many proteins constituting the plasma proteome are either secreted proteins released into plasma or tissue leakage proteins which appear in circulation as a result of cell death or damage. Large-scale quantification of plasma proteins represents an important task needed for new biomarkers development. In this context, reference concentrations are known only for selected plasma proteins and most of them have been studied by the mean of routine clinical biochemistry. Approximate reference levels have been published for 150 proteins, representing about 1% of the putative plasma proteome. If some proteins are found in a disease and are suggested to serve as potential biomarkers, one should know the concentration range of such proteins in a healthy person to predict their applicability.

Methods

In this study we have determined the concentration ranges of plasma proteins encoded by genes Chr 18 in 54 plasma samples of astronaut candidates. All measurements were performed using SRM and SIS added to each individual plasma sample.

Results and Discussion

This work was aimed at estimating the concentrations of proteins encoded by human chromosome 18 (Chr 18) in plasma samples of 54 healthy male volunteers (aged 20-47). These young persons have been certified by the medical evaluation board as healthy subjects ready to space flight training. Over 260 stable isotope-labeled peptide standard (SIS) were synthesized to perform the measurements of proteins encoded by Chr 18. Selected reaction monitoring (SRM) with SIS allowed to estimate the levels of 84 of 276 proteins encoded by Chr 18. These proteins were quantified in whole and depleted plasma samples. Concentration of the proteins detected varied from 10^{-6} M (transthyretin, P02766) to 10^{-11} M (P4-ATPase, O43861). A minor part of the proteins (mostly representing intracellular proteins) was characterized by extremely high inter individual variations.

Conclusion

The chromosome-centric human proteome project seeks to detect all proteins in most human tissues. In this paper, we used selected reaction monitoring with labeled peptide standards to estimate the levels of 84 proteins encoded by Chr 18 in unique biological specimens, i.e. blood plasma samples from certified healthy donors. These donors were regarded as ready for space training by a special medical panel. Levels of the target proteins were quantified in the depleted plasma samples, most of them being close to the detection limit of the method, i.e. about 10^{-11} M. A minor part of the proteins studied was

characterized by extremely high inter-personal variance, mostly representing intracellular proteins. The results provide the background for biomarker plasma studies involving chromosome 18 encoded proteins.

Keywords: Plasma proteome, targeted proteomics, selected reaction monitoring (SRM), C-HPP, human chromosome 18 (Chr 18)

Multiple Post-translational Modifications Proteomes (PTMomes) Associated with TKIs in Non Small Cell Lung Cancer

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Introduction and Objectives

Sequential Enrichment of PostTranslational Modifications (SEPTM) allows characterizing dynamic changes of multiple posttranslational modifications on proteomes (PTMomes) under different physiological conditions from the same set of samples. We hypothesize that characterizing the regulation of PTMomes (phosphorylation, ubiquitination and acetylation) in receptor tyrosine kinases (RTKs) driving lung cancer by tyrosine kinase inhibitors (TKIs) allows deep insight into the drug mechanisms and identification of new combinational strategy for improving the therapeutic efficacy.

Methods

We have applied SEPTM to extend analyses of cancer cells and targeted therapeutics beyond phosphoproteomics to examining other key posttranslationalmodifications including ubiquitination and acetylation. We have conducted preliminary studies that examine crosstalk amongst these keyPTM in 10 NonSmall Cell Lung Cancers (NSCLC) cell lines driven by ALK, EGFR, ROS, HER2 and DDR2 and their response to kinase inhibitors. Statistics and network centered bioinformatics analysis were employed to mine their biological implications.

Results and Discussion

Total 10364 unique PTM sites (include 2446 UbK, 5818 pTyr, and 2810 AcK) on 3849 unique proteins were identified from quantitative SEPTM experiments. Dual and triple modification proteins and peptides plus network analysis among all identified proteins implied crosstalks among different PTMs. Further quantitative analysis revealed that kinase inhibition leads to numerous changes in both protein ubiquitination and acetylation as well. These results suggest new downstream effectors of RTK signaling and potential cotargeting strategies with emerging agents targeting protein ubiquitination (DUB inhibitors) and acetylation (HDAC inhibitors). Response of writers, erasers and readers of three PTMs within sub networks of new potential target proteins to TKI treatmentprovides insight into the mechanism and rationale for novel combinational therapy. For instance, Our study has disclosed the response of two E3 ligases to TKIs treatment, accompanying with the change of their phosphorylation modifiers including kinases, phosphatases and SH2/PTB domain containing proteins, these two E3 ligases are highly possible cotargets for combinational treatment with TKIs. Following up functional studies in both bother cell line and animal model levels will validate these findings.

Conclusion

In summary, this study applied SEPTM strategy to profile the triple PTMomes from human NSCLC cells samples and measured effects of TKIs on identified PTMomes. Early results have shown the ability of combining SEPTM with network analysis to identify the interplay among different types of PTMs, evaluate anticancer drug response, discover new drug

targets for developing new therapeutic therapies. Compare to single PTMome, SEPTM increases the capability of translational cancer research.

Keywords: Sequential Enrichment of PostTranslational Modifications (SEPTM)? cancer proteomics? TKI, crosstalk, combinational therapy

EBP50 suppresses the metastasis of breast cancer and HeLa cells by inhibiting matrix metalloproteinase-2 activity

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Introduction and Objectives

Ezrin-radixin-moesin-binding phosphoprotein-50 (EBP50) was reported to correlate with human breast cancer development, but its effects on metastasis of breast cancer and cervical carcinoma cells were not fully understood. Cell matrix interaction and cell motility are important for cancer cell metastasis. The present study was undertaken to investigate the potential effects of EBP50 on adhesion to matrix and migration/invasion abilities of breast cancer and cervical carcinoma cells.

Methods

EBP50 was stably overexpressed in MDA-MB-231 and HeLa cell lines. Meanwhile, EBP50 was stably knocked down in MCF-7 and HeLa cell lines. Cell adhesion to fibronectin matrix, migration and invasion abilities, matrix metalloproteinase activity of these stably expressed cell lines were examined by adhesion kit, wound healing and transwell assay, zymography assay, respectively.

Results and Discussion

MDA-MB-231 and HeLa cell lines stably overexpressed EBP50, MCF-7 and HeLa cell lines stably knocked down EBP50 were established. EBP50 overexpression significantly suppressed the ability of cell adhesion to fibronectin matrix in MDA-MB-231 and HeLa cell lines compared with vector control cells, and the knockdown of EBP50 significantly promoted cell adhesion ability. Additionally, EBP50 overexpression significantly inhibited the wound healing abilities of MDA-MB-231 and HeLa cells, and the knockdown of EBP50 promoted wound healing and invasion abilities. The results of zymography assay showed that EBP50 overexpression dramatically reduced the activity of matrix metalloproteinase (MMP)-2. While the knockdown of EBP50 enhanced the activity of MMP-2.

Conclusion

EBP50 could inhibit breast cancer and cervical carcinoma cell adhesion and migration and alter breast cancer and cervical cancer cell metastasis by reduction of MMP-2 activity. These findings provide a new perspective for therapeutics of breast cancer and cervical cancer.

Keywords: EBP50; adhesion; migration; matrix metalloproteinase-2

Osteoporosis Risk Protein Biomarkers Suggested by a Proteome Study in Chinese Males

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Introduction and Objectives

Circulating monocytes (CMs) participate in immune responses. They can give birth to osteoclasts to resorb bone. Low bone mineral density (BMD) is a risk factor of osteoporosis. Hip osteoporotic fracture (OF) is one of the most serious consequences of osteoporosis. With case-control design, the present study aims to identify osteoporosis risk proteins from human CMs.

Methods

We recruited hip OF patients (n=18, age mean: 77 yrs) at hospital entry day 0-20. We also recruited gender- and age- matched subjects without fracture history (abbreviated as NF, n=18, age mean: 73 yrs), one half with extremely low and the other half with high BMD (hip Z-score: -1.06 vs. +1.36). All the subjects are Chinese males. CMs were isolated from peripheral blood, and total proteins were extracted. Proteome-wide protein expressions were profiled by employing label-free quantitative proteomics methodology (Easy-nLC1000 and Q-exactive, Thermo). Proteomic data were processed by the softwares Maxquant and Perseus, to identify differentially expressed proteins (DEP, p<=0.05, FoldChange>=2) between subjects with low vs. high BMD, and between OF and NF subjects. Bioinformatic analyses, including Gene Ontology analyses, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses, and Protein Protein Interaction analyses, were conducted to annotate functions of DEPs.

Results and Discussion

Sixty one DEPs were identified between subjects with low and high BMD. These DEPs were not only interacted with each other, but also interacted with known osteoporosis susceptibility gene (OSG) reported by genome-wide association studies, generating a complext interaction network. For example, a key DEP, named AKT1, was exclusively expressed in high BMD subjects. AKT1 was located in the center of the interaction network, interacting with multiple DEPs (STAT5B, WNK1, BOLA2, TPM4, and DAP3), as well as multiple OSGs (CSF1, TNFSF 11, TNFRSF11B), and signifiantly enriched in the Osteoclast Differentiation pathway (KEGG ID 04380). In addition, fifty two DEPs were identified between OF and NF subjects.These DEPs were significantly enriched in biological processes, including “leukocyte migration” (p=6.73E-04), chemotaxis (p=0.01), inflammatory response(p=0.03), and would healing (p=0.03). Some DEPs, e.g., PRI2, were overlapped with that identified above in low vs. high BMD subjects. Specifically, low BMD subjects presented 4.8-fold higher PRI2 expression than high BMD subjects (p=0.006). OF subjects presented 10-fold (p=0.0003) higher expression than NF subjects.

Conclusion

The present study identified novel osteoporosis-related proteins, involved in biological processes related to bone resorption and fracture healing. Notably, PRI2 is a potential risk biomarker for both osteopenia and osteoporosis.Whether the DEPs are predictive of OF has yet to be evaluated in larger sample size and by longitudinal study. In-depth studies are being conducted to demonstrate functional mechanisms for key DEPs.

Keywords: Osteoporotic fracture, BMD, proteome, biomarker

TO02-01

In-depth analysis of human plasma glycoproteins by a combination of high-resolution native mass spectrometry and middle-down proteomics

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Introduction and Objectives

Many proteins in human blood plasma show complex combinations of post-translational modifications (PTMs). These modifications affect the functionality and clearance of the product and therefore need to be characterized in detail. Plasma glycoproteins also represent some of the most relevant protein biomarkers. Next to changes in abundance, changes in PTM/glycosylation profiles may provide even stronger biomarker signatures. Human blood serum-derived complement component proteins and other important glycoproteins were selected for detailed structural analysis combining two advanced mass spectrometry based methods; high-resolution native mass spectrometry and middle-down proteomics.

Methods

Firstly, the whole proteoform profiles of the intact glycoproteins were acquired on a modified Exactive Plus Orbitrap instrument with extended mass range which revealed the existence of a large variety of proteoforms. Subsequent middle-down analysis of the glyco-peptides provided site-specific quantitative profiles of all PTMs.

Results and Discussion

Our data reveal much new insights in the structural heterogeneity of biologically important plasma glycoproteins, exposing also several novel PTMs. For example, we discovered three unreported C-glycosylation sites on properdin and showed unexpected heterogeneity in occupancies of C-mannosylation on this protein. Next, we examined various components of the complement system and other glycoproteins. Except a detail specification of a composition of all modifications, a presence of new glycosylation sites was demonstrated for the first time. Furthermore, we introduce a new algorithm that allows a direct comparison of the data obtained from the two independent methods (native MS and middle-down proteomics). This algorithm can be efficiently used for defining biosimilarity between proteins, which would be beneficial for stratifying therapeutic proteins.

Conclusion

We applied middle-down proteomics, native MS and an integrative strategy to comprehensively assess the PTM profiles of human plasma proteins. Using high-resolution native MS, we obtained a qualitative and quantitative view of all co-appearing proteoforms. By using complementary middle-down proteomics, we in detail revealed PTM localizations, relative abundances and glycan structures in a site-specific manner. Therefore, we conclude that our presented combined approach uniting high-resolution native mass spectrometry and middle-down proteomics for the structural analysis of glycoproteins may find applicability in biotherapeutics as well as biotechnology.

TO02-01

Keywords: Plasma glycoprotein; post-translational modifications, native mass spectrometry, middle-down proteomics, biosimilarity

TO02-02

Confident, automated N-glycoproteomics profiling in enriched and unenriched cell samples

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Introduction and Objectives

Site-specific glycopeptide characterization and quantification increases the biological relevancy tied to glycan distributions across different biological states. However, global glycopeptide profiling using LC-MS remain challenging for data processing due to the complexity and dynamic range for glycoforms. In addition, performing unbiased sequencing against large databases is time consuming. We present a novel software routine for automated glycoprotein profiling that performs iterative searching based on HRAM MS and MS/MS modeling resulting in comprehensive identification, integration, and relative quantification across different biological states. Data analysis was performed on enriched as well as non-enriched samples demonstrating comprehensive profiling with significantly reduced processing time.

Methods

Glycopeptides were enriched from human serum and hela lysates digests using strong anion exchange columns. The enriched glycopeptides were analyzed using an EASY nLC 1000 with a C18 PepMap column (2um, 100A, 75umx50 cm) on an Orbitrap Fusion and Orbitrap Fusion Lumos mass spectrometer using HCD, ETD and EThcD. The samples were also analyzed without enrichment on the Orbitrap Fusion Lumos mass spectrometer using HCD, ETD and EThcD. The samples were also labeled with TMT-6plex to increase ionization and fragmentation quality. For data processing, Pinnacle utilized the Uniprot Human FASTA database containing more than 26,000 protein sequences, and an in-built N-glycan list. All data processing was done on a 12-core Xeon computing server.

Results and Discussion

The primary challenge associated with N-linked glycopeptides profiling is attributed to characterizing the glycan structure and peptide sequence in a complex mixture in an LC timescale. In addition, the resulting mixture of glycopeptides and corresponding glycoforms covers a wide range of peptide lengths and precursor charge state distribution. Therefore data acquisition incorporates various fragmentation modes to provide complementary evidence for peptide sequencing as well as characterization of the glycan Y-series. The Pinnacle software combines precursor isotopic fidelity with all corresponding fragment ion data including high resolution HCD/EThcD product ions and low resolution CID/ETD, along with chromatographic elution profiling between the various glycoforms.The samples were prepared in two ways to evaluate the processing routine. The first approach incorporated glycopeptides enrichment for exhaustive glycopeptides profiling. The initial results confidently identified over 500 glycoproteins. The same sample

TO02-02

was analyzed without glycopeptides enrichment. Despite the increased sample complexity and unbiased acquisition method, the data processing in Pinnacle confidently identified over 100 glycoproteins. The results show a high degree of overlap.

Conclusion

Pinnacle enables fast and high confidence glycopeptide search in unenriched as well as glycopeptide-enriched samples.

Keywords: glycoproteomics, enrichment, N-linked, glycoform

TO02-03

Identification of Intact Glycopeptides with In-Silico Deglycosylation Strategy for O-glycoproteomics Analysis

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Introduction and Objectives

O-GalNAc, as one of the important PTMs of proteins, plays important roles in tumor growth and metastasis, [1-2] and many of the O-GalNAc glycoproteins such as CA125 and CA15-3 were used as clinical biomarkers for diagnosis of cancers in clinics.[3] The analysis ofaberrant glycoforms of O-GalNAc glycoproteins could increase the specificity of biomarkers in cancer diagnosis.[4] Therefore, it is really important and urgent to investigate heterogeneity of the GalNAc-type O-glycoforms at the proteome level.

Methods

Herein, we fabricated an integrated workflow for analysis of O-GalNAc glycosylation by combining HILIC tip enrichment, Q-TOF MS detection and in-silico deglycosylation strategy for spectra interpretation. In this strategy, the O-GalNAc glycopeptides could be selectively enriched by using modified HILIC method, and the interference of N-linked glycopeptides could be eliminated by pretreatment using PNGase F. Additionally, the enrichment O-GalNAc glycopeptides were detected using Q-TOF MS detection, which employed beam-type CID for the decomposition of intact glycopeptides, containing sufficient information for identification both of peptide sequences and attached O-glycan structures. Finally, the original spectra were converted to de-glycosylated forms by using in silico strategy, which enabled database searching without setting glycosylation modifications. This could significantly decrease the search space and allowed the confident identification of peptide sequences and determination of glycan compositions.

Results and Discussion

By using this strategy, about 82 intact glycopeptides with 40 peptide sequences and 15 glycan compositions could be determined, which covered all of the glycosites of fetuin-A and -B, indicating the high efficiency of intact glycopeptide determination. Then comprehensive analysis of O-GalNAc glycosylation in human serum was performed, and 183 intact O-GalNAc glycopeptides from 55 glycoproteins were identified. Among them, 81% (148/183) of the glycoforms were sialylation, which could retain diverse the information sialylation, as well as the heterogeneity of O-GalNAc glycosylation. Furthermore, quantitative analysis of O-GalNAc glycoproteome between hepatocellular carcinoma (HCC) and normal human serum was completed, and about 55 glycopeptides were significant changed in 29 glycoproteins, including one fucosylated glycopeptide from APOE increased in HCC serum.

Conclusion

In summary, an integrated workflow for analysis of O-GalNAc was fabricated by combining HILIC tip enrichment, beam-CID detection and in-silico deglycosylation strategy for spectra interpretation. Taking the superior performance of intact glycopeptide identification, this strategy was utilized in the comprehensive analysis of O-GalNAc

TO02-03

between HCC and normal human serum, and several intact glycopeptides were significantly changed, which could increase the specificity of biomarkers in cancer diagnosis.

Keywords: O-GalNAc glycosylation; HILIC enrichment; In-silico deglycosylation; Intact glycopeptides;

TO02-04

Glycoproteomic Analysis of Human Plasma using SWATH-MS

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Introduction and Objectives

Glycoproteomic analysis aims to identify and quantitate glycan moieties at site specific manner to reveal the functional roles of protein glycosylation. Software assisted analysis generates hundreds of hits but still relies on good quality MS2 spectra of glycopeptide precursors and often requires manual validation to ensure confidence. Quantitation at MS1 level is potentially interfered by the presence of non-glycopeptide signals. In this study, we propose to use N-glycopeptide specific Y ions at MS2 level for N-glycopeptide identification and quantitation. MRM_HR of bovine fetuin digests demonstrated Y ions are good characteristic ions for glycopeptide identification and quantitation. To overcome inherent limitations of MRM_HR, we developed a SWATH analytical workflow that identifies N-glycopeptides and provides the opportunity to detect N- glycopeptides carrying unexpected glycan structures. The strategy was used to extract N-glycopeptide information from non-depleted human plasma tryptic digests that were acquired by different SWATH methods.

Methods

Tryptic digests of bovine fetuin and non-depleted human plasma were analyzed by several SWATH methods using 60 min gradients on a 6600 triple TOF (SCIEX). The variable windows (vW) SWATH method used 100 windows from 400–1200 m/z. 400–600 m/z with 4 Da fixed windows, 600–800 m/z with 4 Da windows, and 800-1200 m/z with 8 Da windows were chosen for GPF SWATH. Data were analyzed by Skyline.

Results and Discussion

MRM_HR of fetuin tryptic digests showed Y ions are useful for glycopeptide identification and quantitation. All N-glycosylation sites of fetuin could be robustly identified and relative abundance of each glycoform were similar to results from glycomic analysis. Although MRM_HR delivers reliable and robust results, this strategy is limited in the number of analytes to be analyzed in parallel and only collect MS data for predefined glyforms. To overcome the limitations, we developed a SWATH workflow to identify N-glycopeptides without pre-defined knowledge of precursor m/z and allows the potential to identify N-glycopeptides with unexpected structures. SWATH analysis of HILIC enriched human plasma digest identified 80 glycosylation sites from 45 glycoproteins. The method was then applied to analyze human plasma digests using different SWATH methods. Glycopeptides from major glycoproteins, such as HPT, A1AT, and IgG and their associated glycoforms could be identified and quantitated.

Conclusion

We developed SWATH-MS methods for glycopeptide identification. It does not require pre-defined glycopeptide precursors and bypass limitations of DDA based method. It also adds values to existing human plasma SWATH data by providing additional dimension of

TO02-04

information.

Keywords: SWATH, plasma, glycoproteomics

TO02-05

A suite of SWATH glycoproteomic approaches for easy global glycoprotein analysis

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Introduction and Objectives

Protein glycosylation is a critical post-translational modification that regulates the functions of diverse proteins. However, analysis of protein glycosylation is hampered by glycoprotein structural diversity both in site occupancy (macroheterogeneity) and glycan structure (microheterogeneity). Here, we developed variations of SWATH (Sequential Window acquisition of All THeoretical fragment ions) to enable straightforward yet powerful global and targeted analysis of glycoprotein structural diversity.

Methods

We developed a suite of complementary approaches to analyse the glycoproteome. To measure global site-specific macroheterogeneity and microheterogeneity we used SWATH glycoproteomics and systematically studied how mutations in the N-glycosy lation pathway led to defects in mature glycoproteins in Saccharomyces cerevisiae. For improved analytical performance we developed a variation of SWATH we term SWAT (Sequential Window Acquisition of Targeted fragment ions), which we used for targeted quantification of site-specific macroheterogeneity. For easy global profiling of the glycoproteome we used standard SWATH acquisition with measurement of glycan-specific oxonium fragment ions from N- and O-glycopeptides. We used this SWATH glycoprofiling approach in yeast N-glycosylation mutants and human saliva.

Results and Discussion

Global site-specific SWATH glycoproteomics of yeast N-glycosylation mutants showed decreased glycan occupancy and altered cell wall proteomes in all mutants, with defects in earlier steps of glycan biosynthesis showing more severe phenotypes. ER mannosyltransferase mutants showed global site-specific defects in microheterogeneity, with truncated glycan structures on mature proteins. Defects in macroheterogeneity did not correlate with defects in microheterogeneity, highlighting the importance of both aspects of glycoprotein structural diversity. Targeted SWAT measurement of glycosylation occupancy in selected yeast N-glycosylation mutants showed that this method provided improved analytical performance. To measure glycosylation even when site-specific identification was not possible, we used SWATH glycoprofiling with oxonium ion detection for global measurement of glycopeptides. These SWATH glycoprofiles of yeast N-glycosyl ation mutants were consistent with our detailed site-specific SWATH glycoproteomic analyses, and allowed easy global profiling of the yeast glycoproteome. SWATH glycoprofiling of human saliva showed a diverse N- and O-linked glycoproteome, with oxonium ion ratios allowing profiling of glycopeptide-specific glycan structures across the global glycoproteome.

Conclusion

The complementary approaches of SWATH glycoproteomics, SWAT, and SWATH

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glycoprofiling allow easy relative quantitative glycoproteomics for analysis of glycoprotein structural diversity. Importantly, these methods do not require specialised sample preparation or MS acquisition. All SWATH experiments can now also be glycoproteomic experiments.

Keywords: Glycoproteomics, SWATH, Yeast, Saliva

TO07-01

Identification of intact glycopeptides at a proteome scale

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Introduction and Objectives

Protein glycosylation is a heterogeneous post-translational modification (PTM) that generates greater proteomic diversity than any other PTMs. Mass spectrometry (MS) is often the method of choice in site-specific glycoproteomic study. However, MS-based profiling of intact glycopeptides yet remains challenging due to the enormous complexity of glycosylation such as macro- and micro-heterogeneity, low abundance and low ionization efficiency of glycopeptides relative to peptides. The overall throughput, data quality and accessibility of intact glycopeptide identification are overwhelmingly lower than those of routine proteomic studies.

Methods

Here we propose an integrated workflow that robustly identifies intact glycopeptides at a proteome scale. Our approach is based on the application of stepped-energy MS/MS that generates abundant fragment ions of both glycan and peptide of a glycopeptide in one single spectrum, together with a dedicated search engine, pGlyco+, which allows rapid glycopeptide identification with strict and comprehensive quality control for both the glycan and peptide matches. The major contributions of the purposed workflow are its comprehensiveness and robustness in the phases of a relatively simple MS-based measurement and data interpretation, which gives the highest identifications of glycopeptides or site-specific glycans. Our method can be easily incorporated into routine glycoproteomic research.

Results and Discussion

To demonstrate the throughput and accuracy of our workflow for intact glycopeptide analysis, we carried out a large-scale site-specific N-glycosylation study on five mouse tissues. We have identified 24,381 glycopeptide spectra under 1% FDR for both the glycan and peptide matches, corresponding to 5,564 distinct site-specific glycoforms on 1, 478 glycosylation sites of 809 glycoproteins in 20 hours of mass-spectrometry analysis. Global FDR for glycopeptide-spectrum matches was 2%. Although the glycosylation difference between mouse tissues have been reported before, our data displayed deeper information in the site-specific level: the overall glycosylation patterns were significantly different between tissues. Brain showed the most distinctive glycosylation compared to other tissues, and heart and lung were the most similar pair in the five tissues.

Conclusion

In conclusion, we presented a workflow combines a fine-tuned, easily adopted MS protocol and a specifically designed bioinformatics tool that are readily to be used for the glycoproteomic analysis of complex sample: 1) stepped-energy HCD-MS/MS provided abundant fragment ions for both the glycan and the peptide in one single spectrum; 2) a search engine specifically designed for glycopeptide spectra performed strict and

TO07-01

comprehensive quality control for both the glycan and peptide matches; 3) our pGlyco+ search engine is among a few software tools that support direct searching of a complete proteome and glycome database.

Keywords: glycoproteomics, intact glycopeptide, software

TO07-02

Integrated Glycoproteomics Demonstrates Fucosylated Serum Paraoxonase 1 Alterations and Functions in Lung Cancer

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Introduction and Objectives

Small cell lung cancer (SCLC) is an aggressive type of lung cancer, and the detection of SCLCs at an early stage is necessary for successful therapy and for improving cancer survival rates. We aimed to identify and validate the aberrant and selective fucosylated glycoproteins in the sera of patients with SCLC.

Methods

Fucosylated glycoproteins were enriched by the Aleuria aurantia lectin (AAL) column after serum albumin and IgG depletion. By both label-free proteomics and iTRAQ approaches, the fucosylated glycoproteins were identified as up- or down-regulated. Verification was performed by multiple reaction monitoring-mass spectrometry to select reliable markers.

Results and Discussion

Four fucosylated proteins, APCS, C9, SERPINA4, and PON1, were selected and subsequently validated by hybrid AAL ELISA and Western blotting. The PON1 protein levels were significantly reduced in the sera of patients with SCLC, whereas the fucosylation levels of PON1 were more significantly increased. The glycan structural analysis of PON1 by MS/MS identified a biantennary fucosylated glycan modification consisting of a core + 2HexNAc + 1Fuc at increased levels in the sera of patients with SCLC. In addition, the PON1 levels were decreased in the sera of the Lewis lung carcinoma lung cancer mouse model, and in lung cancer (LC) tissues of human patients and mouse orthotropic models. Intracellularly, up-regulation of PON1 supported metastatic progression of LC interjecting G1/S fraction and LC cell senescence mediated by p21Waf1/Cip1. PON1 suppressed cell death and protected LC cells from cytotoxic and genotoxic damages with maintained ATP levels, requiring p53-directed signals. PON1 promoted ROS deregulation protecting the mitochondria from dysregulation. Deletion of PON1 resulted in the blockage of antioxidant function through Akt signaling. Targeted glycolysis stimulated PON1 antioxidant activity regulating phosphorylation of AMPK-α.

TO07-02

Conclusion

Our data suggest that PON1 fucosylation levels and patterns can serve as diagnostic serological markers for SCLC, and PON1 function in LC progression and cell death machinery.

Keywords: Glycoproteome, fucosylation, lung cancer, paraoxonase 1, biomarkers

TO07-03

Decoding site-specific alteration of Sialo-glycoproteome in EGFR-subtype of non-small cell lung cancer

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Introduction and Objectives

Altered sialylation of cell surface glycoproteins has been correlated with cancer development. Due to extreme heterogeneity, variable branching and extension of glycans, decoding the complex site-specific glycan structure on glycoproteins still presents great analytical challenges in mass spectrometry-based glycoproteomics. A highly specific enrichment and quantitation approach for intact glycopeptides is critical for comprehensive sialo-glycoproteomics profiling. In non-small cell lung cancer (NSCLC), sialylation has been reported to regulate EGFR activity and enhance its sensitivity to tyrosine kinase inhibitors. Here, we report a new glycopeptide enrichment material, ZIC-cHILIC, and incorporating an isotopic labeling approach to decode the altered sialo-proteome in different EGFR subtypes of NSCLC cells.

Methods

The membrane proteins were extracted from different NSCLC cells and followed by in-solution digestion. Digested peptides from each sample were labeled by TMT6plex. After mixing all samples and desalting, the glycopeptides were enriched using ZIC-cHILIC stage-tip and eluted by stepwise fractionation. The enriched glycopeptides were analyzed by HCD using Orbitrap Fusion MS. The intact glycopeptides were identified by Byonic and MAGIC software and quantified by Proteome Discoverer 2.1.

Results and Discussion

The intact glycopeptides were enriched by our home-made ZIC-cHILIC stage-tip from NSCLC membrane fraction, separated into 5 fractions by stepwise elution and analyzed by Orbitrap Fusion MS with product-dependent stepped HCD, which targets 3 diagnostic oxonium ions (m/z 204.08 HexNAc, 138.06 Hex fragment, and 366.11 HexNAcHex) to induce the glycopeptide fragmentation. ZIC-cHILIC stage-tip afforded good enrichment specificity. By presence of sialic acid-specific diagnostic oxonium ions in MS/MS spectra, our results showed as high as 88% specificity for enrichment of sialo-glycopeptides. On the sialo-glycoproteomic scale, 2346 unique intact sialo-glycopeptides corresponding to 747 proteins were quantified by TMT6plex labeling. The comparison of the 4 NSCLC cell lines with different EGFR subtypes revealed 29 unique glycopeptides site-specifically present in wild-type NSCLC, such as integrin a3/b1 with fucosylated monosialo-biantennary glycan on N86/N363. In addition, 19 and 24 glycopeptides were uniquely identified in NSCLC cells with exon 19 deletion and L858R/T790M mutation, respectively. The proteome-scale profiling also revealed high glyco-site identification coverage of individual glycoprotein. Without immunoprecipitation, 11 of 13 N-glycosylation sites on EGFR were identified and altered site-specific sialylated glycoforms were also quantified.

TO07-03

Conclusion

The quantitative sialo-glycoproteomic approach provides high specificity to map the global site-specific glycosylation profile and its changes. On the model study of NSCLC, our results may allow better understanding how the glycosylation correlates with EGFR subtypes

Keywords: Sialylation, sialo-glycoproteome, non-small cell lung cancer

TO07-04

Identifying Antibody and Lectin Recognition to HIV N-glycans through Native Mass Spectrometry and Glycoproteomic Analysis of Viral Envelope Spikes

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Introduction and Objectives

An estimated 37 million people worldwide are HIV positive and efforts towards global eradication focus both on vaccine development as well parallel interventions to limit viral transmission, including physical barrier methods via anti-viral lectins. The surface of HIV is decorated by roughly twelve heavily glycosylated envelope glycoprotein (Env) spikes, comprised of gp120/gp41 proteins, which are 50% carbohydrate by mass. Crucially broadly neutralizing antibodies (bnAbs), which are elicited in up to 30% of infected individuals, target Env and the recent development of soluble Env mimics holds tremendous promise for an HIV vaccine. The abundance of Env glycans effectively shields the underlying peptides from innate immune responses but paradoxically many bnAbs target the viral spike via contact with conserved N-glycans. Lectins that have anti-viral properties have recently emerged as an alternative treatment to prevent HIV transmission, acting as microbiocides applied topically or even produced in situ by live engineered commensal microbes. Both bnAbs and microbiocide lectins (griffithsin and banlec) target the HIV “glycan shield” yet the precise mechanisms by which they target Env is unclear. Therefore a detailed glycoproteomic analysis of Env glycosylation is critical and will increase our knowledge of bNAb/lectin epitope recognition. Here we show that native mass spectrometry techniques, combined with molecular dynamics, NMR and HPLC reveal the complex mechanisms by which HIV glycans are targeted.

Methods

Glycan and glycopeptide site-analysis of gp120/gp41 was performed by ion mobility MS and RP LC-MS. Specific bnAb/lectin binding was studied by HPLC depletion of flourecently labelled N-glycans and binding stoichiometry and binding affinity were characterized by native MS.

Results and Discussion

Over 100 N-glycan structures were identified from HIV-1, clades A & B by ion mobility and tandem MS fragmentation. Site-specific analysis showed microclusters of high-mannose “patches” were conserved and the location of processed complex and hybrid N-glycans. Importantly these data challenge certain models of bnAb epitope recognition that were probed by native MS. We uncovered a new banlec structure and found that only a subset of high-mannose structures bind the lectin and is explained by bidentate binding to two separate carbohydrate binding sites on each monomer. Grifftithsin binding stoichiometry from native MS was 1:1 and NMR studies identified both high- and low-affinity binding sites challenging current models based on crystallography data.

Conclusion

Site-specific analysis of Env trimers is vital in HIV vaccine development and will

TO07-04

undoubtedly shed light on bnAb/lectin targeting of viral spikes. Native MS and IM-MS are also critical tools that are capable of uncovering the detailed molecular processes of these protein-carbohydrate interactions that will help shape the development of new drugs to tackle the global HIV epidemic.

Keywords: HIVglycomicsnative MSlectinsion mobility MS

TO07-05

Dynamic mapping of human frontal cortex according to the developmental stage via neuroglycomic approach

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Introduction and Objectives

Human frontal cortex (FC) is well known for its particular vulnerability to age-related deterioration. Yet, there is still weak evidence at molecular level. Investigation into the brain glycome might be the right strategy to generate new hypotheses. Glycan plays numerous roles in the nervous system during development, regeneration and synaptic plasticity, forming of a complex meshwork at the cell surface. However neuroglycomics have languished due to the lack of sensitive analytical method. As an example, gangliosides, one of our interests have been poorly characterized due to their inherent amphipathic nature. Herein, we successfully traced the changes in glycome, the most common PTM and gangliosides, major components of grey matter in human FC across the life span.

Methods

We newly established an analytical platform for exploring the two molecules- glycan attached to proteins and glycan attached to lipids- of brain using mass spectrometry. Sixty nine brain samples were obtained from the University of Cambridge. Briefly, brain tissues were sonicated and ultracentrifuged for membrane extraction and N-glycan release. Homogenized tissues mixed with chloroform/methanol/water were centrifuged to extract total lipids, followed by partitioning to collect gangliosides. After further enrichment by SPE, N-glycans and gangliosides were profiled by positive and negative ion mode nano-LC/MS respectively. Structure information was obtained by nano-LC/MS/MS.

Results and Discussion

Brain samples were divided into the 7 groups for efficient developmental mornitoring: neonates, infants, toddlers, school-ages, teenagers, young adults and adults. We successfully identified approximately 100 N-glycans and 120 gangliosides of brain with several unique “brain type” characters. For glycans, novel motifs such as bisecting GlcNAc, antennary fucose, sulfation and glucuronidation that might be associated with cellular signaling were revealed. For gangliosides, GD1 was markedly decreased whereas GT1 and GM1 were slightly increased from neonates to school-ages while all kept constant level after school-ages. Significant increases on long-chain ceramides were the key feature during development. Interestingly, hierarchical clustering derived high similarity in adults and young adults yet little correlation between neonates and adults. Furthermore, based on the structure information, we proposed biosynthetic pathway model including related enzymes such as specific glycosyltransferases.

Conclusion

This is the first systemic developmental profiling of glycome and sphingolipids in human

TO07-05

FC with observations about possible roles of related enzymes. We introduced the powerful platform with microgram-level sensitivity for comprehensive and accurate characterization of brain glycome. This study promises insights into involvement of glycan to massive flow of information that underpins brain development, providing meaningful understanding to neuropathology such as Alzheimer's and Parkinson's.

Keywords: Brain, glycome, sphingolipid, LC-MS

TP02-01

In-depth LC-MS/MS Mapping of Sulfo-sialo-glycoproteome in B-chronic lymphocytic leukemia (CLL)

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Introduction and Objectives

The sialoglycoproteome of B cells and their additional modifications such as sulfation are functionally relevant in various immuno-activation and disease states including human chronic lymphocytic leukemia (CLL) but remain mostly undefined and under-investigated. Of particular interest are the site-specific protein carriers of the cis ligands of CD22 (Siglec-2), ±6-sulfo-α2-6-sialyl lacNAc, which we aim to identify in this work via a concerted glycomic and glycoproteomic workflow.

Methods

Using 2 related single patient-derived B-CLL cell lines, MEC 1 and MEC 2, as an investigative model that reflects the clinical progression of the disease, we employed a complementary two-tier sulfo/sialo-based glycomics and glycoproteomics profiling strategy to elucidate the glycans and their attachment sites by nanoLC-MS/MS analyses. For glycomics, PNGase F-released N-glycans were subjected to permethylation prior to their fractionation into neutral-, mono- and poly-sulfated glycans. The presence of sulfated N-glycan structures were first confirmed by MALDI-MS mapping of the permethylated glycans in negative ion mode, before subjecting both unfractionated native glycan pool and the permethylated sulfated glycans to PGC and RP nanoLC-MS/MS, respectively. For sialoglycoproteomics, membrane proteins were extracted by Triton X-114 phase partitioning, trypsin-digested, and subjected to Endo H and α2,3-sialidase treatments prior to ZIC-cHILIC enrichment of α2,6 sialylated glycopeptides. Confident identification and maximal structural information of glycopeptides was derived from composite scoring of two concurrently acquired MS2 data, namely the trap-based collision-induced dissociation (CID) and higher-energy collision dissociation (HCD).

Results and Discussion

In total, we have identified over 40 sulfated N-glycans with varying degrees of sulfation, sialylation and branching in MEC 1 and MEC 2. The predominant glycotopes were determined to be α2,6 sialylated LacNAc, a fraction of which is additionally sulfated. Whilst both cell lines comprise mainly of core-fucosylated, bisecting-type bi-antennary N-glycan structures in the non-sulphated fraction, the mono-sulphated fraction in MEC 2 displayed hybrid-type mono-sialylated and non-sialylated bi-antennary N-glycans which were not identified in MEC 1. Based on these information, an in-house glycan database was constructed and utilized to

TP02-01

identify glycopeptides carrying the targeted glycotopes by complementary search engines, as well as manual assignment. Additional sulfate modifications were then sought at the MS1 level, the positive detection of which followed by a secondary, targeted MS/MS acquisition.

Conclusion

It is envisaged that the resulting systems-level acquisition of precise structural information at high MS sensitivity will shed light into the prevalence of sulfo-, sialyl glyco- epitopes and their corresponding glycoprotein carriers, which offer diagnostic and therapeutic potentials.

Keywords: Sulfo-sialo-glycoproteome, glycomics, glycoproteomics

TP02-02

Improving confidence in glycan structure characterisation using alternative CID fragmentation

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Introduction and Objectives

The structural characterisation of both N-glycans and O-glycans using ion-trap low-energy CID is a well established technique with one notable limitation, the absence of low mass range fragments. Utilising negative-mode ESI-PGC-LC-MS/MS with alternative fragmentation modes, Trap-HCD and PQD, diagnostic glycan fragments in this low mass range can be detected. Detection of low mass range diagnostic fragments can increase confidence in structural characterisation, provide a qualitative assessment of monosaccharide composition and allows further fragmentation with traditional low-energy CID.

Methods

A standard LC-PGC-ESI-MS/MS run was used to analyse both N- and O-glycans released from glycoproteins. To compare the fragmentation capabilities of Trap-HCD and PQD to the conventional low energy CID, multiple injections of the same samples were performed with optimisation of collision energy for each fragmentation technique. To analyse the acquired data, a comprehensive list of cross-ring fragments for common monosaccharides found in N-glycans and O-glycans was compiled.

Results and Discussion

Two applications of this technique are described: the structural characterisation of O-linke d glycans containing glucuronic acid found on secreted proteins of the fungus, Trichoderma reesei, that is used for recombinant protein production and the structural characterisation of N-glycans released from a standard glycoprotein, Bovine Fetuin. Negative mode ESI-LC-MS/MS using a Thermo Velos Pro PQD/Trap-HCD/CID was used to identify and characterise these glycans by annotation of monosaccharide mass and diagnostic glycosidic bond and cross-ring fragments. As an unusual O-glycan composition was identified on T. reesei secreted protein, Trap-HCD allowed targeted MS3 experiments to be performed on the glucuronic acid substituent of these structures thus validating the novel composition.

Conclusion

The undetectable low mass fragments from low energy CID can be detected using alternative fragmentation techniques of Trap-HCD and PQD. Applying these fragmentation methods to N-glycans and O-glycans allowed detection of traditionally undetected fragments and increased confidence in glycan structural characterisation using tandem MS. For both types of glycans, diagnostic ions confirmed monosaccharide components of the overall structures. To validate a novel composition of O-glycans released from T. reesei secreted protein, targeted MS3 experiments were performed on

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the glucuronic acid ion generated in MS2. The use of PQD and Trap-HCD have allowed an increase in confidence level of glycan structural assignment, allowing us to move one step closer to the automated, high confidence assignment of glycan structures in PGC-LC-ESI-MS/MS glycomics experiments.

Keywords: Glycomics, Glycoproteins, Tandem Mass Spectrometry, N- and O-linked glycans

TP02-03

Highly Selective Enrichment of Glycopeptides Based on Zwitterionically Functionalized Soluble Nanopolymers

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Introduction and Objectives

Efficient glycopeptides enrichment prior to mass spectrometry analysis is essential for glycoproteome study. ZIC-HILIC (zwitterionic hydrophilic interaction liquid chromatography)-based glycopeptides enrichment approaches have been attracting more attention for several benefits like easy operating, high enrichment specificity and intact glycopeptide retained. However, the drawbacks of ZIC-HILIC matrices like limited functional groups and inadequate interaction with glycopeptides in the heterogenous enrichment system significantly restrain the enrichment efficiency toward glycopeptides. In this work, we propose a chemical strategy for the zwitterionic functionalization (ZICF) of G5 PAMAM for highly selective and sensitive N-glycopeptide enrichment.

Methods

The ZICF-PAMAM materials were synthesized based on the commercially available G5 PAMAM via a two-step derivatization procedure. Glycopeptieds were enriched by ZICF-PAMAM through FASP-mode strategy.

Results and Discussion

The flowchart for the whole synthesis and enrichment procedure are shown in Scheme 1. The results of structural characterization show fully that the original dendrimers have successfully been zwitterionically functionalized. The multiple branched structure and good solubility of ZICF-PAMAM enables a sufficient interaction with glycopeptides. The ZICF-PAMAM combined with the FASP-mode enrichment strategy exhibits more superior performance compared with the existing methods. It has the minimum detectable concentration of femtomolar level and high recovery rate of over 90.01%, and can efficiently enrich glycopeptide from complex biological samples. A total of 395 unique glycopeptides with 417 glycosylation sites mapped to 178 glycoproteins have been successfully identified in 1 μl human serum. A total of 44 unique glycopeptides with 48 glycosylation sites mapped to 28 glycoproteins were identified from merely 0.1 μl human serum. The above results indicate that ZICF-PAMAM based enrichment strategy shows extreme efficiency and superior sensitivity.

Conclusion

TP02-03

In summary, ZICF-PAMAM combined with the FASP-mode enrichment strategy exhibits remarkable performance for highly selective and sensitive N-glycopeptide enrichment and for comprehensive glycosylation exploration in complex biological samples. Thus the proposed ZICF-PAMAM strategy highlights the potential application in in-depth glycoproteome research, which may open up new opportunities for the development of glycoproteomics.

Keywords: Glycopeptide enrichment, FASP-mode enrichment strategy, Zwitterionic hydrophilic interaction, PAMAM, Mass spectrometry

TP02-04

Glycoproteomic analysis of O-GlcNAc-modified proteins in colorectal cancer

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Introduction and Objectives

Elevated glucose uptake is an important characteristic of cancer. A few glucose enters into the hexosamine biosynthesis pathway (HBP) to generate UDP-GlcNAc, a substrate used for classical glycosylation and O-GlcNAcylation. This later modification is the attachment of a single UDP-GlcNAc to the serine and threonine residues of proteins regulated by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA). It is a dynamically reversible process similar to phosphorylation. Aberrant O-GlcNAcylation was reported to be involved in metastatic phenotypes of many cancers. However, identification of O-GlcNAcylated proteins and their role in cancer are poorly understood. Here, we aim to search and identify O-GlcNAc-modified proteins in colorectal cancer (CRC) and elucidate the interplay of this modification and phosphorylation.

Methods

CRC tissues and cell lines were used in this study. The O-GlcNAc levels was examined by O-GlcNAc immunoblotting. Two-dimensional gel electrophoresis (2-D) followed by LC-MS/MS and O-GlcNAc immunoblotting were applied to identify O-GlcNAc-modified proteins. Serine-phosphorylation was determined using phosphor serine antibody detection. RNAi against OGT was performed in CRC cell lines to observe its biological effects as well as the dynamic interplay of phosphorylation under the global O-GlcNAc reduction.

Results and Discussion

O-GlcNAcylation was increased in CRC cell lines (HT29, SW480, and SW620) compared to a normal epithelial colon cell (CCD841 Con) as well as in CRC tissues, compared to normal adjacent samples. Using 2-D O-GlcNAc immunoblotting and LC-MS/MS analysis, we successfully identified 16 O-GlcNAcylated proteins in CRC cancer tissues. Of these identified proteins, some were related to metabolic enzymes, proteins associated in stress responses, RNA metabolism, gene expression, and cytoskeleton. OGT knockdown had no effect on cell viability and led to a decrease colony formation in soft agar assay. In addition, 2-D immunoblotting revealed that several phosphoserine spots have been altered in siOGT cells compared siScramble. Among these protein spots, pyruvate kinase M2 (PKM2) was further identified as an O-GlcNAc and phosphor serine protein. Immunoprecipitated PKM2 revealed decreased O-GlcNAc and serine phosphorylation levels after siOGT knock down, but increased levels after treatment with Thiamet-G, an inhibitor of OGA. Moreover, the metastatic colorectal cancer cells, SW620, had more O-GlcNAc-PKM2 and showed lower PKM2 specific activity compared to the non-metastatic colorectal cancer cells, SW480.

TP02-04

Conclusion

The results demonstrate that aberrant protein O-GlcNAcylation is associated with CRC and O-GlcNAc-modified proteins may be a potential CRC biomarker of CRC. Moreover, O-GlcNAcylation plays a role in modulating serine phosphorylation, as well as in regulating PKM2 activity. Interfering levels of O-GlcNAcylation of PKM2 might be a novel target in controlling cancer metabolism and tumorigenesis.

Keywords: Colorectal cancer, O-GlcNAcylation, pyruvate kinase M2

TP02-05

Glycoproteomic Analysis of Serum proteins for Oral Cancer

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Introduction and Objectives

Oral cancer is the tumor grows on the lips, tongue, cheeks, floor of the mouth, hard and soft palate, sinuses, and pharynx. The high prevalence and mortality rate of oral cancer makes it important to investigate new biomarkers for the surveillance of high-risk population. We previously found that the mRNA levels of FUT8 was higher in oral cancer cell than in normal cells. High levels of core-fucosylated N-glycans were observed on the oral cancer cell surface.

Methods

In order to discover novel biomarkers, we explored the serum N-glycome and glycoproteins of normal (20, healthy volunteers) and oral cancer patients (60, obtained from tissue bank of NCKUH). Total serum proteins are lyophilized and the N-glycans are released by treating glyco-peptides with PNGase F and purified by Sep-Pak C18 cartridge. After permethylation, the purified N-glycans are analyzed using MALDI-TOF-Mass spectrometry in positive ion mode to generate a serum N-glycan profile. The lyophilized serum samples were also resuspended in buffer followed by AAL (Aleuria aurantia lectin) lectin column purification. The purified core-fucosylated serum proteins were separated by electrophoresis and stained. After compared the protein bands on gel between normal with patient serum, bands showed significant expression differences were collected, subjected for in-gel digestion, and protein identification on a Mass-spectrometry.

Results and Discussion

We find that some tri-antennary and tetra-antennary glycans with varying degrees of fucosylation and sialylation is increased in serum N-glycomes of oral cancer. We also find that the relative abundance of 7 N-glycans decrease or increase in the serum sample of oral cancer with diagnostic accuracy greater than 75%. In addition, the proportions of bi-, tri- and tetra-antennary N-glycans were significantly increased in oral cancer patients. The N-glycan structures also showed high sensitivity, high specificity, and high AUC values (>0.8) for cancer patients. Several glycoproteins were identified, and then verified by western blotting (serum) and immunohistochemical staining (tissue array).

Conclusion

In conclusion, the identified tumor markers should be considered as novel oral cancer biomarkers.

Keywords: Glycoproteomic, Oral cancer, Biomarker

TP02-06

One-fraction nanoLC-MS^2/MS^3 analysis for high throughput glycome-wide precision mapping of glycotopes

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Introduction and Objectives

Different combination and relative expression level of assorted terminal sialylated, fucosylated and/or sulfated glyco-epitopes (glycotopes) collectively define the glycomic characteristics of a cell or tissue type at a specific patho-physiological state. Since the same glycotopes can be equally or preferentially distributed over N- and O-glycans, a holistic view of their regulated expression necessitates mapping of both N- and O-glycans. Large scale glycomic studies in the era of precision medicine is beset by redundant fractionation and multiple runs in order to increase the depth of coverage. We aim in this work to develop a high throughput glycotope-centric glycomics platform that would adequately address these technical issues.

Methods

We have previously developed a nanoLC-MS^2-product dependent-MS^3 analytical approach that would separate sulfated and non-sulfated glycans into different fractions to be analyzed in negative and positive ion modes, respectively, using the same LC conditions. Taking advantage of increasing speed and performance of latest MS instruments, we now further evaluated the feasibility of one-fraction, one-shot workflow that would shorten analytical time without compromising the attainable glycomic resolution and precision. The N- and O-glycans from tissues were released sequentially into one fraction, permethylated with and without additional separation into sulfated vs non-sulfated pool, and directly subjected to RP C18 nanoLC-MS2/MS3 analysis on an Orbitrap Fusion Tribrid MS system in both positive and negative ion modes. Productive MS2 containing diagnostic ions of glycotopes measured at the high mass accuracy and resolution are then filtered out by in-house developed glycomic data mining tool, GlyPick, along with any associated MS3 for both linkage confirmation and relative quantification.

Results and Discussion

In this communication, our innovative workflow comprising one-fraction N- and O-glycan preparation along with glycosyl linkage-informative one-shot nanoLC-MS2/MS3 analysis and automated glycotope-centric data analysis tool will be fully illustrated by our recent analytical efforts on gastrointestinal tissues. Among the glycotopes identified in gastric but not in colorectal tumors or adjacent non-cancerous cells include Lewis a/x, sialyl Lewis a/x, H antigen type 1/2, as well as a wealth of sulfated fucosylated glycotopes not previously described. Many such glycotopes are recognized by a plethora of endogenous and microbial glycan-binding receptors mediating diverse immunobiological functions while others serve as potential biomarkers of disease and developmental stages.

Conclusion

TP02-06

We have developed an integrated analytical platform capable of high-throughput and high-precision glycomic mapping particularly suited for mapping glycotopes of clinical significance.

Keywords: glycomics, glycotope, precision mapping, Orbitrap Fusion Tribrid.

TP02-07

Lectin Microarray: A Powerful Tool for Glycan-Based Biomarker Discovery

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Introduction and Objectives

Cell surfaces are heavily coated with complex glycans, which play critical roles in pathogenesis, trafficking and differentiation. A slight change in the composition or structure of these glycans may lead to dramatic changes in cell phenotype, and even causing diseases, such as cancer and male subfertility. Therefore, glycans are expected to serve as important biomarkers for disease diagnosis and/or prognosis. Recent development of the lectin microarray technology has allowed researchers to profile the glycans in complex biological samples in a high throughput fashion.

Methods

We employed a microarray with 91 lectins for profiling the surface glycans of the six TNBC cell lines and human sperms with DEFB126 mutation. Candidate lectins were then verified by lectin-based flow cytometry and immunofluorescent staining assays. For TNBC cells, it was further validated by patient-derived tissue microarrays and real-time cell motility assays. Finally, LC-MS/MS was employed to identify the membrane glycoproteins recognized by candidate lectins.

Results and Discussion

Using the lectin microarray, we found the bindings of RCA-I to TNBC cells are proportional to their metastatic capacity. Tissue microarray showed the intensity of RCA-I staining is positively correlated with the TNM grades. The real-time cell motility assays demonstrated RCA-I inhibition of adhesion, migration, and invasion of TNBC cells of high metastatic capacity. Additionally, a membrane glycoprotein, POTE ankyrin domain family member F (POTEF) was identified by LC-MS/MS as a binder of RCA-I.By comparing sperm samples of three different genotypes of DEFB126, we identified six candidate lectins with lower binding affinity to sperm with del/del. As the sperm with del/del presented normal semen parameters and sometimes had the DEFB126 protein by western blotting, these lectins, especially ABA and MPL, can be employed to assess the quality of the sperm glycocalyx that may be defective because of the mutation in DEFB126. Thus, an independent set of samples was tested and lectins (ABA and MPL) verified by FACS showed high consistency with the results obtained by lectin microarray.

Conclusion

Our results indicate that RCA-I-specific cell surface glycoproteins may play a critical role in TNBC metastasis and that the extent of RCA-I cell binding could be used in diagnosis to predict the likelihood of developing metastases in TNBC patients. We also pioneered in establishing and optimizing the procedures of lectin microarray for human sperm surface glycome profiling and validated that 6 lectins, especially ABA and MPL, could have potential capability to serve as a biomarker individually or as a combination in diagnosing males with unexplained infertility due to DEFB126 mutation. Taken together, these

TP02-07

studies proved that lectin microarray, as a relatively new tool, is highly suitable for live/intact cell analyses and has the potential for rapid discovery of glycan-based biomarkers.

Keywords: Lectin Microarray, TNBC cells, Human sperm, Biomarker

TP02-08

Uncovering Target Glycoprotein Biosignatures using a One-Pot Dual Nanoprobe Mass Spectrometry Assay

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Introduction and Objectives

Aberrations in protein glycosylation are increasingly being explored for biomarker development because changes on the glycan structure, site occupancy and glycoform concentration are associated with disease development and progression. For target glycoprotein analysis, the complexity of biofluids and the low abundance of glycoproteins are recurring issues. Glycoprotein micro- and macroheterogeneity further complicate analyses. In this work, we present a nanoprobe-based strategy coupled to mass spectrometry to identify glycosylation signatures in a target protein.

Methods

We designed a one-pot enrichment strategy for intact glycopeptide analysis and targeted protein quantification using liquid chromatography MS (LC-MS/MS) after a single sample preparation. Using two nanoprobes with divergent properties—antibody-conjugated silica nanoparticles (Ab-SiO2) and lectin-conjugated magnetic Fe3O4 nanoparticles (Lectin-MNP)—we can achieve target glycoprotein isolation from biofluid and subsequent protein quantification and glycopeptide enrichment in a single container. We demonstrated the method using a clinically-relevant N-glycoprotein, alpha-fetoprotein (AFP), which is a serum marker for hepatocellular carcinoma (HCC).

Results and Discussion

Using standard AFP, 20 intact glycopeptides, composed of eight unique glycoforms, were identified. We observed a ~2-fold higher number and abundance (total peak area) of the AFP glycopeptides compared to the traditional “non-one-pot” sequential assay and 10-fold higher abundance compared to an assay that did not employ glycopeptide level enrichment. We then profiled the glycopeptides of AFP from serum HCC samples to demonstrate the clinical utility of the assay. Using a combination of higher-energy collisional dissociation (HCD) and collision-induced dissociation (CID) spectra from a LTQ-Orbitrap MS to elucidate the glycopeptide composition, we were able to identify a total of 59 AFP intact glycoforms from 6 individual HCC samples. A number of glycoforms are highly-complex, including sialylated and fucosylated types. Finally, for protein quantification, the non-glycopeptides were collected from the supernatant and analyzed by a multiple reaction monitoring (MRM-MS). A response curve from a non-glycopeptide fragment GYQELLEK/y5 was plotted to show good linearity and wide dynamic range from 0.5 ng to 500 ng (r^2=0.9999) with good precision (4.2-17.5%). The concentration of AFP in serum samples was found to correlate closely with that of the ELISA method (r^2=0.983),

demonstrating its reliability to quantify from serum samples.

Conclusion

In summary, we demonstrated that the one-pot dual-nanoprobe assay can provide a complete picture of the targeted glycoprotein, incorporating both protein concentration and glycosylation profile for “single glycoprotein biosignature”.

Keywords: Nanoprobe, mass spectrometry, multiple reaction monitoring, glycosylation

TP02-09

Integrated Proteomic and N-glycoproteomic Analyses of Alzheimer's Disease Mouse Brain Reveal the Precise Alterations in Protein N-glycosylation

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Introduction and Objectives

Alzheimer disease (AD) is a neurodegenerative disorder characterized pathologically by the accumulation of senile plaques and neurofibrillary tangles, and most of proteins associated with AD are extensively modified by glycosylation. Although glycosylation defects have been observed in AD patients, the role of protein glycosylation in AD has not been thoroughly investigated. Here, we comprehensively analyzed protein N-glycosylation changes in the progression of AD.

Methods

We integrated lectin arrays, quantitative proteomics and N-glycoproteomics as well as intact glycopeptides interpretation methods to investigate the differences between the APP/PS-1 double-transgenic mouse and wild type mouse.

Results and Discussion

Lectin arrays revealed the widespread glycosylation on mouse brain proteins, and indicated the decrease of protein sialylation in the brain of AD. Furthermore, quantitative analysis of global proteome, glycoproteome and site-specific glycoforms gave abundant information on differences in the level of glycoprotein expression, site occupancy, as well as glycan heterogeneity between the AD and the control, respectively.

Conclusion

Such detailed and deep analysis greatly increased the knowledge of protein glycosylation of AD. Furthermore, our results found that aberrant glycosylation occurred on many key proteins associated with AD progression, such as APP, BACE1. Further studies on the biological function of these important glycoproteins were ongoing in our lab.

Keywords: N-glycoproteomic Alzheimer's Disease site-specific glycoform

TP02-10

Increased confidence for the identification of N-linked glycopeptides using an optimised collision energy workflow

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Introduction and Objectives

Glycosylation regulates cell-cell interactions, recognition and diseases. The development of viable glycomarkers has been limited due to the technical challenges raised from sample preparation and analytical perspectives. Mass spectrometry (MS) is seen as an important tool for elucidating glycan structure but can prove challenging from a data acquisition and processing perspective. For example, N-glycosylated peptides often provide intense Y and B fragment ions corresponding to the carbohydrate moiety when fragmented by means of collision induced dissociation (CID). This is counteracted with inefficient fragmentation of the peptide backbone. Therefore having the ability customise the collision energy (CE) applied to glycopeptides would be advantageous. Here we describe an LC/MS method which provides efficient glycan and peptide backbone fragmentation within a single acquisition using optimised collision energies.

Methods

Results and Discussion

Acquiring 15 different m/z of N-linked glycopeptides purified from AFP proteins, we found the modest CE at 40eV provided best b and y ions fragmentation from peptide backbone and CE at 50eV possessed best fragmentation of intact glycopeptides in Trap channel using a Synapt G2-Si QTOF. Comparing with optimal CE in Transfer channel, CE at 50 eV and 50/60 eV had good peptide and glycan fragmentation, respectively. In addition, combining these spectra with varying CEs by further informatics processing also provided good decomposition of glycan structure and peptide sequencing from intact glycopeptide due to the complete information of fragmentations. Most importantly, different glycopeptides with the same m/z can be separated by ion mobility and correctly identify the glycan structure. We further applied this DDA method for identifying the N-linked glycoprotoeme of HeLa cell, totalling 193 intact glycopeptides corresponding to 112 glycoproteins, including EGFR, CD63, LAMP1/2, and integrin family proteins. For instance, high mannose type of glycan structure on EGFR and fucosylated mono-sialo-biantennary glycan on CD63 were identified. Constructing a glycoproteomic database by DDA & DIA can also provide identification of glycan structure and peptide sequencing of HeLa membrane digests.

Conclusion

Optimised CE is demonstrated to be critical for ensuring specificity when characterising and identifying N-linked glycopeptides.

Keywords: DIAIon mobilityMAGICGlycopeptides

TP02-11

GLYCOPROTEOMICS REVEALS DECORIN PEPTIDES WITH ANTI-MYOSTATIN ACTIVITY IN HUMAN ATRIAL FIBRILLATION

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Introduction and Objectives

Extracellular matrix (ECM) proteins contribute to cardiac fibrosis and hypertrophy in disease. ECM deposition facilitates the development of ectopic pacemakers and late potentials as a result of inhomogeneous stimulus conduction, and can also lead to fluctuations in membrane potential. Thus, atrial fibrotic remodeling has been implicated as a potential therapeutic target in atrial fibrillation (AF). We used proteomics to profile glycoproteins in the human cardiac ECM and explore the potential roles of decorin and anti-myostatin activity in human AF.

Methods

Atrial samples (n=65) were obtained from the atrial appendages during cardiopulmonary bypass and just after cardioplegic arrest of the heart. Atrial specimens were analyzed by mass spectrometry after extraction of ECM proteins and enrichment for glycoproteins or glycopeptides. To test the biological effects of decorin peptides, neonatal rat cardiac myocytes were used for in vitro experiments, and hearts from 10-12 week-old C57BL/6J male mice were perfused in a Langendorff system.

Results and Discussion

Out of all ECM proteins identified, the small leucine-rich proteoglycan decorin was found to be most fragmented. Within its protein core, eighteen different cleavage sites were identified. In contrast, no cleavage was observed for biglycan, the most closely related proteoglycan. Decorin processing differed between human ventricles and atria and was altered in disease. The C-terminus of decorin, important for the interaction with connective tissue growth factor, was predominantly detected in ventricles compared to atria. In contrast, atrial appendages from patients in persistent atrial fibrillation had higher levels of

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full-length decorin but also harbored a cleavage site that was not found in atrial appendages from patients in sinus rhythm. This cleavage site preceded the N-terminal domain of decorin that controls muscle growth by altering the binding capacity for myostatin. Myostatin expression was decreased in atrial appendages of patients with persistent persistent atrial fibrillation and hearts of decorin null mice. A synthetic peptide corresponding to this decorin region dose-dependently inhibited the response to myostatin in cardiac myocytes and in perfused mouse hearts.

Conclusion

This proteomics study is the first to analyse the human cardiac ECM. Novel processed forms of decorin protein core, uncovered in human atrial appendages can regulate the local bioavailability of anti-hypertrophic and pro-fibrotic growth factors.

Keywords: Extracellular matrix, Proteomics, Mass spectrometry, Atrial fibrillation, Cardiovascular disease

TP02-12

Simplified Cell Strategy for Large Scale Identification of Mucin-type O-glycoproteins

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Introduction and Objectives

Mucin-type O-glycosylation is one of the most important protein post translational modifications, and is involved in many important biological and physiological processes like protein secretion, cell adhesion, proliferation and tumor growth. However, lacking consensus sequence motif and universal enzymes capable of releasing all O-glycans make the identification of mucin-type O-glycosylation more difficult than N-glycosylation. Here we developed a simplified cell strategy using chemical inhibitors to truncate the elongation of protein O-glycans during cell culture, aiming at obtaining glycoproteins with homogenous O-glycans to facilitate further identification with mass spectrometry.

Methods

In this work, cancer cell lines were firstly cultured and treated with an optimized dose of chemical inhibitors. Cells then lysed and tryptic digested followed by filter-assisted lectin affinity enrichment. The released glycopeptides were later analyzed by LC-MS/MS using HCD/ETD and EThcD fragmentations.

Results and Discussion

Immunocytochemical fluorescence results clearly confirmed that O-glycans in chemical inhibitor treated cells was successfully truncated. Using a combination of filter-assisted lectin affinity enrichment and LC MS/MS analysis with multiple fragmentation techniques, we successfully identified 48 O-glycopeptides and 52 O-glycosylated sites mapping to 42 O-GalNAc glycosylated proteins from Hela cell lysates.

Conclusion

This simplified cell strategy is powerful and easy to handle, which may greatly benefit research on large scale identification of mucin-type O-glycosylation and O-glycoproteomics.

Keywords: Mucin-type O-glycosylation, Chemical inhibitors, Mass spectrometry, HCD/ETD, EThcD

TP02-13

INFLUENCE OF SIALYLATION ON INTRACELLULAR SIGNALLING PATHWAYS OF HeLa CELLS

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Introduction and Objectives

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase (RTK) involved in regulating cell growth and differentiation. Ligand binding to the EGFR leads to an increase in the proportion of dimerized receptor and an increase in the catalytic activity of its intracellular tyrosine kinase domain. Upon receptor dimerization and tyrosine phosphorylation, different downstream signalling cascades are activated producing the signal transduction. Although EGFR signalling is well characterized with respect to phosphorylation, little is known about the role of the sialic acids on the glycoproteins at the surface of the cell upon EGFR activation. The aim of this study was to investigate modulation of sialic acids during the recycling process of the EGFR and its activation on the modulation of sialylation on surface glycoproteins of HeLa cells.

Methods

HeLa cells were stimulated with EGF for 5 min after the inhibition of the tyrosine kinase domain of EGFR and the endocytosis with gefitinib and PitStop for 30 and 15 min, respectively. Then membrane proteins were enriched by Na2CO3 treatment and ultracentrifugation. The changes at the N-linked sialylated glycopeptides and phosphorylation upon stimulation were assessed using iTRAQ 8-plex labelling. After digestion and dephosphorylation of membrane fraction, iTRAQ labelled sialylated glycopeptides were enriched using TiO2, deglycosylated using PNGase F and fractionated using high pH reversed phase. Phosphorylated peptides from the soluble fraction were enriched by the TiSH protocol and fractionated by HILIC. All the samples were subsequently analysed by nLC?MS/MS using a Q-exactive MS.

Results and Discussion

In the experiments, we observed not only a desialylation on selected surface proteins after acute EGF stimulation, but an increase in sialylation of surface glycoproteins, not explained by de novo synthesis of the sialylated N-linked glycoproteins in the Golgi. A significant desialylation of glycans on glycoproteins involved in cell adhesion (integrins), cell-cell communication and migration, and degradation or internalization of surface receptors (ephrin B2) was observed. The present MS-based approach has allowed the study of the modulation of the sialylation pattern after the inhibition of the tyrosine kinase domain of EGFR and the endocytosis process. The results suggest that changes in the sialylation pattern are due to the activation of the EGFR and the increase in sialylation is not related with the endocytosis as similar increase is observed when this process is inhibited.

Conclusion

TP02-13

Modulation of sialylation is essential in the first events of transduction signals into HeLa cells.

Keywords: Sialylation, N-glycosylation, phosphorylation.

TP02-14

Glycome Signature for Gastric Cancer Biomarker Discovery: N-glycan Profiling of Serum Haptoglobin

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Introduction and Objectives

Gastric cancer is one of the most common malignancy and leading cause of cancer death. Classically, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) have been used for detection. However, CEA and CA19-9 are insufficient for reliable diagnosis due to low specificity and sensitivity. Glycosylation is the most common post-translational modification of protein. Glycans in blood are secreted from cells and these glycans are changed depending on health condition and diseases including cancers. Haptoglobin (Hp) containing multiple N-glycosylation sites is a major glycoprotein in human serum and a positive acute-phase protein with immunomodulatory properties. This study is aimed at analyzing and profiling the N-glycans of Hp and investigating the changes of glycosylation between gastric cancer patient and healthy control.

Methods

Cancer patient (n=30, stage IV) and healthy control (n=30) were obtained from Samsung Medical Center. Commercial sera were purchased from Sigma Aldrich for quality control. All samples were processed by established systematic protocol for clinical sample preparation. First, Hp were purified from serum using in house anti-Hp immunoaffinity column and further treated by PNGase F for N-glycan. N-glycans were enriched by solid phase extraction with graphitized carbon to remove the remaining salt and protein. Finally, glycans were analyzed by nano LC-chip Q-TOF MS and MS/MS analysis.

Results and Discussion

Samples are qualitatively similar and they have about 300 compounds including isomers and 100 compositions of glycans on average. From this results, 41 predominant glycans were selected by relative abundance and frequency. Major 41 compositions occupy quantitatively almost 99% of total glycan. Sialylated (C/H-S) glycans are the most abundant (almost 80%), on the other hand, high mannose glycans represent very low abundance (0.14 ~ 0.18%). In patient sample, C/H-S glycans are lower while fucosylated/sialylated (C/H-FS) glycans are higher (7%), and represent different quantitative results on isomer level. Interestingly, C/H-FS glycans have aberrant antenna fucosylation. For example, the largest isomer peak of Hex6HexNAc5Fuc1NeuAc3 is antenna fucosylated and we confirmed this unique structure by MS/MS analysis. We are examining quantitative and structural differences for gastric cancer biomarker discovery.

TP02-14

Conclusion

This study suggests targeted glycoprotein analysis method for gastric cancer biomarker discovery. Targeted Hp analysis can provide more specific information about Hp glycosylation than whole serum analysis. We can release the glycan that only existed in Hp and investigate glycosylation changes and differences between gastric cancer patient and healthy control. In particular, we find unusual antenna fucosylation on C/H-FS glycan through this method. In further study, we will confirm abundance of isomers and aberrant glycan structures for isomer and structural specific biomarker.

Keywords: N-glycan, Mass spectrometry, Gastric Cancer, Haptoglobin, Biomarker

TP02-15

Comparative Study of Fucosylation between Liver and Non-liver Secreted N-glycoproteins in Liver Cancer Plasma

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Introduction and Objectives

Aberrant protein glycosylation plays important roles in variety of diseases. Especially, fucosylation in N-glycoproteins has been an important biological phenomenon in Hepatocellular Carcinoma (HCC). However, few studies have been reported for the site-specific fucosylation of various liver-secreted N-glycoproteins.

Methods

In this study, we characterized the fucosylation patterns of major plasma glycoproteins using a workflow to identify and quantify site-specific N-glycoprotein using GPA system. We selected 15 major glycoproteins in plasma, including liver-secreted and non-liver-secreted glycoproteins, to analysis of differential fucosylation between control and liver cancer plasma.

Results and Discussion

As the result, a total of 215 tryptic N-glycopeptides from 15 target glycoproteins in human plasma was identified by nanoRPLC-MS/MS with HCD/CID fragmentation, where 93 fucosylated N-glycopeptides were found. The relative abundance of fucosylated N-glycopeptides from liver-secreted glycoproteins were increased 2.5-fold on average in HCC than control samples, whereas non-liver-secreted glycoproteins showed no difference.

Conclusion

We suggest that site-specific analysis of aberrantly fucosylated N-glycoproteins secreted from liver is an effective method to distinguish biomarker signatures in human plasma of liver cancer. As a next step, we will attempt to analyze aberrant fucosylation levels in liver-secreted glycoproteins using multiple reaction monitoring in various cancer plasma samples.

Keywords: N-glycoproteins, Fucosylation, Liquid chromatography, Mass Spectrometry, Human plasma, Liver-secreted glycoproteins

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In-depth N-glycoproteome Analysis of Human Metastatic Hepatocellular Carcinoma Cell Lines

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Introduction and Objectives

Glycosylation is one of the most common post-translation modification(PTM) of protein and over 50% of mammalian proteins are predicted to be glycosylated[1]. Glycoproteins, which are reported in many biological progresses such as secretion and recognition, have been considered to play important roles in tumor development and progression. Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality globally[2]. The metastasis of HCC is observed as the major reason of morality. In this study, the N-glycoproteins in 3 human hepatocellular carcinoma (HCC) cell lines with different metastatic potential (Hep 3B, MHCC97L, MHCCLM3) were investigated with in-depth strategy.

Methods

Proteins are digested to peptides by trypsin. Then peptides are separated by 2 methods strong cation-exchange(SCX) chromatography and high pH reverse phase chromatograph (RP), followed by ZIC-HILIC (zwitterionic hydrophilic interaction chromatography) glycopeptides enrichment approach.

Results and Discussion

A total of 3976 N-glycopeptides with 5166 glycosylation sites were confidently identified in these cells, which is the largest number being reported yet. 26.3% of the N-glycopeptides and 21.23% of the N-glycosylation sites are observed in both 3 cell lines. In different metastasizing conditions, these proteins kept N-glycosylated status that may be functional conserved and play important roles in tumour development.

Conclusion

The information of the N-glycosites and N-glycoproteins of the cell lines helps learn more about the occurrence and development of liver tumor, and is also valuable for discovery of glycoprotein biomarkers.

Keywords: Glycoproteome / Hepatocellular carcinoma cell lines / Mass spectrometry / In-depth identification

TP02-17

Direct mapping of additional modifications on phosphorylated O-glycans of α-dystroglycan by mass spectrometry analysis

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Introduction and Objectives

Dystroglycanopathy is a major class of congenital muscular dystrophy caused by a deficiency of functional glycans on α-dystroglycan (α-DG) with laminin-binding activity. Recent advances have led to identification of several causative gene products of dystroglycanopathy and characterization of their in vitro enzymatic activities. However, the in vivo functional roles remain equivocal for enzymes such as ISPD, FKTN, FKRP, and TMEM5 that are supposed to be involved in post-phosphoryl modifications linking the GalNAc-β3-GlcNAc-β4-Man-6-phosphate core and the outer laminin-binding glycans. Herein, by direct nanoLC-MS^2/MS^3 analysis of tryptic glycopeptides derived from a truncated recombinant αDG expressed in the wild-type and a panel of mutated cells deficient in one of these enzymes, we sought to define the full extent of variable modifications on this phosphorylated core O-glycan at the functional T317/T319 sites.

Methods

Gel bands containing αDG were subjected to in-gel digestion and the extracted peptides were analyzed by nanospray LC-MS/MS on an Orbitrap Fusion Tribrid under the HCD product ion trigger CID mode. HCD and CID MS^2 datasets were filtered for candidate glycopeptide spectra based on presence of specific MS^2 ions, and then manually interpreted. Select MS^2 oxonium ions were further targeted for CID MS^3 using the inclusion list feature.

Results and Discussion

We showed that the most abundant glycoforms carried a phosphorylated core at each of the 2 sites, with and without a single ribitol phosphate (RboP) extending from terminal HexNAc. At much lower signal intensity, a novel substituent tentatively assigned as glycerol phosphate (GroP) was additionally detected. As expected, tandem RboP extended with a GlcA-Xyl unit was only identified in wild type, whereas knocking out of either ISPD or FKTN prevented formation of RboP. In the absence of FKRP, glycoforms with single but not tandem RboP accumulated, consistent with the suggested role of this enzyme in transferring the second RboP. Our findings thus revealed additional levels of complexity associated with the core structures, only a fraction of which was fully functionalized to carry the laminin-binding glycans. The

simplified analytical workflow developed here should facilitate rapid mapping across a wider range of cell types to gain better insights into its physiological relevance.

Conclusion

We demonstrate in this work a highly sensitive analytical workflow from in-gel digestion to direct nanoLC-MS^2/MS^3 analysis without additional chemo-enzymatic treatments, for unambiguous identification of the target glycopeptides carrying further modified phosphorylated O-glycan core. Diagnostic fragment ions were afforded by complementary modes of MS^2 in positive ion mode, which can be programmed for target MS^3 and/or used for rapid filtering of large spectral dataset to allow meaningful manual data interpretation in anticipation of novel substituents.

Keywords: α-dystroglycan, glycosylation, glycopeptide analysis, phosphorylated O-glycans

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Identification and quantitation of site-specific N-glycoforms in biopharmaceutical glycoproteins using LC-MS/MS with glycoproteomic database search

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Introduction and Objectives

One of the most important post-translational modification of protein is glycosylation, which plays a key role on the stability and immunogenicity of biopharmaceutical antibodies. Alteration in N-glycoforms may significantly modify the biological activity of monoclonal antibodies (mAbs). The unexpected and heterogeneous glycoforms of monoclonal antibodies are often reported, because it is affected by the culture medium, the efficiency of protein expression, and the physiological status of the host cells. Thus, accurate identification and fast quantitation of their glycosylation forms are necessary in order to control the production processes of therapeutic monoclonal antibodies.

Methods

IgG (Human serum), two NIST (National institute of standards and technology), and three unknown therapeutic mAb samples produced from different manufacturing processes were digested by trypsin. Their glycopeptides were analyzed by nano LC-MS/MS and site-specifically characterized by GPA (GlycoProteome Analyzer) search. GPA is homemade program for automated identification and quantification of site-specific N-glycosylation.

Results and Discussion

1. The site-specific N-glycoforms from IgG (Human serum), two NIST (A and B), and three unknown therapeutic mAb samples (A, B, and C) produced from different manufacturing processes were studied. 2. The complex types of N-glycoforms were identified in IgG sample and unknown sample A, whereas different high-mannose and hybrid types of N-glycoforms were identified in two NIST samples and unknown sample B and C. 3. The quantitative patterns of N-glycoform were compared between IgG (human serum), two NIST, and three unknown therapeutic mAb samples. The quantitative patterns of N-glycoform were similar between IgG sample and unknown sample A. The hybrid type and high-mannose type of N-glycoform were quantitatively increased in two NIST samples and unknown sample B and C, respectively.

Conclusion

Our analytical protocols would be useful for verification of safety and efficacy in various biopharmaceutical products such as recombinant proteins and therapeutic monoclonal antibodies.

Keywords: Biopharmaceutical glycoproteins, Therapeutic monoclonal antibody, nano LC-MS/MS, site-specific N-glycoforms

TP02-19

Characterization of site-specific N-glycopeptides of alpha-1-acid glycoprotein from Human Plasma by an interlaboratory study using LC-MS/MS

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Introduction and Objectives

Glycoproteins is one of the most common posttranslational modifications and related to protein folding, quality control, sorting and secretion. However, glycoprotein characterization is very difficult due to microheterogeneity of glycans attached N-glycosylation sites and low sensitivity of glycopeptides. Therefore, to establish a standard analytical protocol for the characterization of the site-specific N-glycopeptides of glycoproteins with multiple N-glycosylation sites, seven laboratories were participated in this study with different type of mass spectrometers.

Methods

Alpha-1-acid glycoprotein (AGP) was used as a model compound of N-glycoprotein through interlaboratory experiments. Four samples (three AGP standards samples and one human serum sample), trypsin, HILIC enrichment kit and the analytical protocols such as protein digestion and glycopeptides enrichment by HILIC and LC-ESI/MS conditions were given to each laboratory, along with the sample information. The MS data from seven participating laboratories were automatically analyzed by using homemade GPA(GlycoProteome Analyzer) program1 and the analytical results were qualitatively and quantitatively compared to evaluate the experimental processes from sample preparation including protein digestion and enrichment of glycopeptides to LC-MS/MS analysis. Based on the protocol, N-glycopeptides of AGP were quantitatively analyzed from normal and hepatocellular carcinoma (HCC) human plasma samples.

Results and Discussion

The experimental results were reproducible in each laboratory although the number of identified N-glycopeptides from AGP is different among laboratories. The highest number of total 215 N-glycopeptides of AGP were first reported in our interlaboratory study. The quantitative patterns of ten most abundant N-glycopeptides of AGP were similar between laboratories from three AGP standard samples and one serum sample, although the different type of MS analyzers were used. Based on the results, the process was applied for the quantitative N-glycopeptide analysis between normal and HCC human plasma samples with four laboratories and the same results which the fucosylated

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N-glycopeptides of AGP were quantitatively increased in HCC samples were occurred.

Conclusion

This process could be used as the standard protocol to characterize site-specific N-glycopeptides of glycoproteins because the highest number of N-glycopeptides of AGP were identified and the relative changes of representative ten N-glycopeptides from AGP in disease samples were similar in any MS analyzers used. This quantitation for site-specific N-glycopeptides could be utilized to any glycoproteomic studies of profiling disease biomarkers from plasma samples in future.

Keywords: Keywords: Interlaboratory study, site-specific N-glycopeptide, isoforms, post translational modificationReference 1 : Scientific Reports (2016) 6, 21175

TP02-20

Smart polymer for saccharide discrimination and glycopeptide enrichment

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Introduction and Objectives

Abnormal glycosylation of proteins is highly associated with many major diseases, such as cancers and neurodegenerative diseases. However, this study is challenging owing to the difficulty to enriching trace glycopeptides (GPs) from highly complex biosamples and separate glycan isomers. The key to solving this problem relies strongly on the design of novel GP receptors to capture GPs in a specific and tunable manner. Inspired by the multiple hydrogen bonds in life systems, here we introduce saccharide-saccharide interaction, peptide-saccharide interaction based GP receptors into this study. A series of smart polymer materials were designed and prepared in order to enrich glycopeptides and distinguish glycan isomers.

Methods

Results and Discussion

Firstly, saccharide-saccharide interactions based materials were investigated. Monosaccharides display specific and pH-sensitive binding toward sialic acids (SA). Integrating monosaccharides units into a polyacrylamide chain generate saccharide-responsive smart copolymers (SRSCs). Such design significantly improves the specificity of SA binding, meanwhile, this binding can be intelligently triggered in a large extent by solution polarity and pH. As a result, SRSCs exhibit high-performance enrichment capacity toward GPs, even under 500-fold interference of bovine serum albumins digests, which is notably higher than conventional materials. In real biosamples of HeLa cell lysates, 180 sialylated glycosylation sites (GPSs) have been identified using SRSC. This is apparently superior to those obtained by SA-binding lectins including WGA (18 GPSs) and SNA (22 GPSs). Saccharide-saccharide stereoselective H-bond interaction points out a new avenue for designing artificial saccharide receptor with high specificity, just as Lectins. Secondly, peptide-saccharide interactions were utilized to design polymer materials toward glycan discrimination. Dipeptide units grafted on flexible polyethylenimine (PEI) main chains demonstrated the capability to separate chiral monosaccharides. The polymer film became substantially softer after interacting with L-ribose and became more rigid after interacting withD-ribose. This chiral effect provides a new method for determining the enantiomeric purity of an L/D-ribose mixture and facilitates the chiral separation of deoxyribose racemates as well as the separation of diverse mono-, di-, and oligosaccharides.

Conclusion

In summary, we reported several bio-inspired polymer materials. This design concept is distinct from classical chromatographic knowledge and may facilitate the construction of a new generation of materials for glycomics and glycoproteomics.

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Keywords: Smart polymers, Saccharide-saccharide interaction, Glycopeptide enrichment, Saccharide discrimination.

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Investigating the roles of glycosylation in plasticizer induced tumor migration in colon cancer cells

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Introduction and Objectives

Phthalates are environmental contaminants which are added to PVC to make it flexible. Since they are not tightly bound to the PVC molecules, phthalates may migrate from PVC to solutions. Phthalates can interfere with hormone signaling, which is especially critical to early childhood development. Recently, numerous studies also reported that plasticizers may promote tumor progression including invasion, migration and drug-resistance. However, the effects of plasticizers in tumorigenesis, cancer progression, migration, or invasion are still unclear. Glycosylation is the most common post-translation modification of proteins. Alteration of glycosylation profiles have been shown to be associated with many disease processes such as cancer progression, inflammation and virus infection. In our previous studies, we found that di-2-ethylhexyl phthalate (DEHP) or mono-2-ethyhexyl phthalate (MEHP, the active metabolite of DEHP) exposure enhances colorectal cancer cell migration by regulating glycosyltransferase activities. Thus, we aim to investigate the roles of glycosyltransferases and glycan structures after plasticizer treatment in colon cancer cells.

Methods

Cell surface N-glycans were released from glycoproteins and purified with Sep-Pak C18 cartridge. After permethylation, the N-glycome patterns were analyzed by MALDI-TOF-Mass spectrometry and annotated by GlycoWorkbench.

Results and Discussion

We found that core fucosylation and sialylation structures were significantly decreased in DEHP/MEHP treated cells. The upregulation of high mannose and simple N-glycans were also observed.

Conclusion

Finally, the relationships between the altered glycosylation with phthalates exposure and cancer behaviors will be further studied.

Keywords: Phthalates, glycosylation, MALDI-TOF-Mass

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Glycoproteome-wide identifications of novel GALNT14 substrates using tandem mass spectrometry

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Introduction and Objectives

The glycosyltransferase GALNT14 was recently shown to mediate the oncogenesis and treatment responses of multiple cancers, including hepatocellular carcinoma, cholangiocarcinoma, colon cancer, esphagial cancer, neuroblastoma and breast cancer. Particularly, the genotype of GALNT14 was tightly associated to the therapeutic response of hepatocellular carcinoma in an accumulation of more than 900 patients treated in Taiwan. However, the substrates of GALNT14 are not entirely known, except the death receptor 5 which mediates the extrinsic apoptosis signaling of cancer cells. We aim to investigate novel substrates of GALNT14 by use of a lectin enrich glycoproteome-wide screening method.

Methods

The GALNT14 genomic DNA was cloned to plasmids which were then transfected to the hepatocellular carcinoma cell line, Huh7 and J7, for generating stable GALNT14 overexpression cell lines. A control cell line was also established with only the plasmid backbone without the GALNT14 DNA. Glycoproteins of the two cell lines were captured using self-made lectin affinity column, which was packed with Peanut Agglutin (PNA) lectin, with GVS Centrex Centrifuge Filter. The captured proteins were trypsin digested and than were identified through tandem mass spectral spectrometric analyses.

Results and Discussion

A collection of 70 glycoproteins were captured and identified in the GALNT14 overexpressed cells but not the control cells, while 39 glycoproteins were found only in the control cells. Annotation of these proteins showed that the 70 candidates were particularly enriched in genes involving in the biological processes of apoptosis and immunological reactions. A scrutiny of the candidates showed that ANXA5, EIF4G1, UBE2L3, IGF2BP1, IGF2BP2, HNRNPK, which all involved in apoptosis, were only detected in the GALNT14 overexpressed cells but not the control cells.

Conclusion

In this initial proteome-wide screening of potential substrates of GALNT14, we identified six glycoproteins pertinent to the apoptosis of cancer cells. They opened new directions for elucidating the molecular mechanisms of hepatocellular carcinoma.

Keywords: GALNT14, glycoprotein, lectin affinity chromatography, tandem mass spectrometry, hepatocellular carcinoma

TP02-23

Salivary Glycomic Approach for The Distinction of Human Constitutions

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Introduction and Objectives

In oriental medicine, human organs fall into an order of strong and weak, and the groupings of these orders that are distinguishable from human constitutions. The accurate diagnosis of constitution is important because therapy can be differed depending on constitution. Human constitutions to each person are generally diagnosed by oriental medical doctor's personal opinion. Thus, modern medical studies are attempted to demonstrate the distinction of constitution based on oriental medicine.Compared to other human fluids, saliva is a readily accessible and noninvasive fluid. One of the major components of saliva is mucin, which is heavily glycosylated protein. Glycosylation is the most common post-translational modification process. Changes of glycosylation reflect health condition because glycosylation is highly sensitive to the biochemical environment. Thus, glycans may the best target for constitutional distinction.In this study, we aim to profile and to compare N-glycans from saliva to identify the difference and the potential signature depending on constitution using mass spectrometry.

Methods

Saliva was collected from 9 normal people; 7 pancreotonia, 1 pulmotonia and 1 colonotonia. To remove non-glycans, the saliva was filtered by 10K MWCO spin column. N-glycans were enzymatically released by PNGase F from saliva. Released glycans were enriched by graphitized carbon and C18 SPE. N-glycans were analyzed by MALDI-MS and nano-LC-MS. N-glycans were profiled and compared to identify the difference of glycans depending on frequency, quantitation, composition and structure.

Results and Discussion

N-glycans from saliva were enriched by SPE using both graphitized carbon and C18. Samples were examined to determine overall N-glycan profile and individual variations. Approximately 150 compositions of N-glycan were profiled. For comparison, constitution groups were compared according to glycan abundance using normalized absolute peak intensity (NAPI). We found that the most N-glycans in saliva are highly fucosylated (79-80%), while high mannose glycans (4%), complex/hybrid glycans (11-13%) and sialylated glycans (2-3%) present in low abundance.In constitution distinction, fucosylated N-glycans consisting of [Hex]5[HexNAc]4[Fuc]5 and [Hex]6[HexNAc]5[Fuc]5-7 are high in abundance in pulmotonia and colonotonia while sialylated N-glycans consisting of [Hex]5[HexNAc]4[Fuc]1-3[NeuAc]1 and [Hex]6[HexNAc]5[Fuc]3[NeuAc]1 are high in abundance in pancreotonia.

Conclusion

Salivary glycomic approach for constitutional distinction was conducted using mass

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spectrometry. We could determine that fucosylated and sialylated N-glycans are potential signatures to differentiate the human constitution. Further studies will be conducted to analyze various types of constitution and a large of samples.

Keywords: Saliva, N-Glycan, Mass spectrometry, Human constitution

TP02-24

Neural glycomics and epigenetic regulation

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Introduction and Objectives

Glycans are expressed in a cell and tissue specific manner and this results in their functional diversity. In particular, brain cells such as neurons and astrocytes are known to express unique functional glycans which play pivotal roles in high-order brain functions and involved in various neurological disorders. However, it is poorly understood how the expression of these neural glycans is established, maintained and dysregulated. Here, by using glycomic and transcriptomic approaches, we determined the N-glycan structures in each primary neural cell and examined how epigenetics contributes to the expression of neural glycans.

Methods

First, primary neurons, astrocytes and fibroblasts were isolated from mouse embryos. To identify N-glycans specific to a particular neural cell type and to clarify the underlying transcriptional basis, the structures of N-glycan on these cell membrane were analysed by LC-ESI MS, and amounts of 144 glycosyltransferase (covering nearly all glycosyltransferases) transcriptome in these cell were determined by qPCR. Next, these cells were treated with epigenetic drugs (5-aza-2´-deoxycytidine and trichostatin A) and the changes in N-glycomes and glycosyltransferase-transcriptomes were characterized.

Results and Discussion

We found that the overall patterns of the elution profiles of N-glycans were relatively similar between neurons and astrocytes, while that of non-neural fibroblasts was substantially different. We also detected several characteristic glycan epitopes predominantly in neurons such as oligo-sialic acid, type 1 LacNAc, Lewis-type fucose, bisecting GlcNAc and a mannose-6-phosphate. All of these findings are well consistent with the results of transcriptomic analysis. Furthermore, the N-glycomes in neurons were found to be highly stable and resistant to epigenetic stimulation. In contrast, astrocytes showed dynamic N-glycan changes after treatment, such as a shift in the linkages of sialic acid.

Conclusion

These results provide novel insights into how the expression of neural glycans is maintained and epigenetically regulated.

Keywords: glycomics, glycome, transcriptome, epigenetic, glycan, glycosyltransferase, LC-ESI MS, qPCR, Neuron

TP02-25

Integrated GlycoProteome Analyzer (I-GPA) for Automatic Identification and Quantitation of Site-Specific N-Glycosylation in Human Plasma

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Introduction and Objectives

Methods

All protein samples including human plasma were digested with trypsin. Then, hydrophobic interaction liquid chromatography (HILIC)-enriched site-specific N-glycopeptides were analyzed by nano-reversed-phase liquid chromatography (nRPLC) coupled to Orbitrap MS with both HCD and CID-MS/MS fragmentation. The resultant data were then computationally analyzed using specific algorithms within the I-GPA suite: glycopeptides were identified against the GPA database by id-GPA, quantified by q-GPA, and finally compared between multiple samples by c-GPA. In id-GPA, scoring entailed three steps: 1) Selection of N-glycopeptide from 15 glycan-specific oxonium ions using HCD-MS/MS spectra; (M-score); 2) Selection of candidates by matching the isotope pattern to intact N-glycopeptides against the GPA-DB (S-score); and 3) Identification of N-glycopeptide from CID and HCD-MS/MS fragment ions (Y-score) with FDR < 1%. For the automated label-free quantitation, we used the combined intensities of top three isotope peaks at three highest MS spectral points (3TIQ).

Results and Discussion

Conclusion

I-GPA, a newly developed search engine, allows direct analysis of site-specific N-glycopeptides from complex glycoprotein mixtures using the efficient glycoprotein DBs, where an analytical efficiency was similar to that currently available in proteomics with FDR <= 1% using a decoy database.

Keywords: I-GPA, Glycoprotein, N-Glycopeptide, Label-Free Quantitation, AGP, Human Plasma, Mass Spectrometry

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Glycomic Approach for Design of Humanized Mouse Model via Nano-LC/MS and LC/MS/MS

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Introduction and Objectives

Therapeutic glycoproteins are over 70% of protein therapeutics in world market. They are attatched with glycans which are expressed on the cell surface as components of glycoproteins, glycosphingolipids, and proteoglycans. These glycans contribute significantly to fundamental biological functions, such as cell-cell interaction, pathogen-host recognition, and immune responses. In previous study, we developed a highly sensitive mass spectrometry-compatible method. Herein, we have aim to design the humanized mouse model having the same type glycosylation through the analysis of cell surface glycome obtained by developed the analytical method. Mammalian expression systems such as CHO(Chinese hamster ovary) cells are preferred for the production of therapeutic glycoproteins due to their ability to synthesize glycans having similar structures with human type glycans. But, mammalian-derived cells have the limitation to produce therapeutic glycoprotein for treatment of human disease because of the glycans which are not existed in human. Therefore, we are ongoing the comparative study to target a red blood cells and white blood cells having possibility that is able to differentiate various immune cells.

Methods

Red blood cells and white blood cells were obtained from KRIBB(Korea Research Institute of Bioscience and Biotechnology). For N-glycan analysis, cell membranes were isolated by ultracentrifugation. N-glycans were enzymatically released from membrane proteins and enriched by porous graphitized carbon solid phase extraction. Enriched glycans were profiled by nano-LC/MS for isomer-specific quantification and structure information. For glycolipid analysis, membrane proteins were further extracted by organic solvent and enriched by C18 solid-phase extraction and they were profiled by negative ion mode nano-LC/MS using a C18 microfluidic chip. Extracted N-glycans and glycolipids were assigned based on the accurate mass and the structures were further elucidated using tandem MS.

Results and Discussion

We comprehensively explored cell surface-specific glycosylation on blood cells (RBCs and WBCs) using previously developed analytical platform. It is well known that sialic acid is represented as N-glyconeuraminic acid (NeuGc) in mammailian different from human (N-acetylneuraminic acid, NeuAc). We will find the different glycosylation between the species and determine the species-specific structure through the structural elucidated by tandem MS.

Conclusion

A comparative study of cell surface glycome between the species by powerful tool provide

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the features of species such as structure, linkage. These results could be targets for new signatures distinguishing the species and the beginning for design of humanized mouse model. Furthermore, species-specific glycosylated signatures may be used for possible applications of mammalian-related researchers including pharmaceutical drug development and biomarker discovery.

Keywords: Humanized mouse model, Red blood cell, White blood cell, Cell surface glycosylation

TP02-27

Building a high confidence, quantitative O-glycopeptide profile for IgA

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Introduction and Objectives

O-Glycosylation is an important PTM that play crucial roles in various biochemical processes. Comprehensive structural characterization and quantitation of all glycoforms of a protein is necessary to fully understand the different functions that these glycans confer to proteins. O-glycan distribution presents significantly different challenges for automated data processing compared to N-linked glycosylation due to the lack of static motif as well as high probability of multiple sites of modification in any peptide. The biology associated with O-linked glycopeptides requires alternative data processing strategies to balance the increased sequence options with reducing false positives. We present novel software strategies to exhaustively profile complex O-linked glycoproteins with minimal time and the results demonstrate the selectivity to differentiate true and false positives.

Methods

Recombinant IgA was reduced, alkylated, acetone precipitated then digested with trypsin. Data acquisition was performed by chromatographically separating peptides using a Thermo Scientific™ Accela™ HPLC system and Thermo Scientific™ Orbitrap Elite™ mass spectrometer (Bremen, Germany). Data dependent acquisition methods using a resolution setting of 120,000 was used for MS and tandem mass data. Electron transfer dissociation (ETD) and HCD was used for all peptide sequencing and glycan characterization using a resolution setting of 30,000. Data processing was performed using the Pinnacle software (Optys Technologies, Inc., Boston, MA) utilizing iterative spectral matching based on an O-glycan database. All data processing was done on a 12-core Xeon computing server.

Results and Discussion

The primary challenge associated with successfully processing O-linked glycoproteins is two-fold, identifying the site(s) modified as well as determining the glycan distribution in a chromatographic timescale. DDA methods were used to generate HRAM MS and product ion spectra resulting from both HCD and ETD activation. The combination of MS and tandem MS data facilitates automated data processing in the Pinnacle software to identify and quantify more than 50 O-glycoforms for IgA with high confidence, on the sequence HYTNPSQDVTVPCPVPSTPPTPSPSTPPTPSPSCCHPR. For this result, Pinnacle combines evidence from isotopic fidelity of parent ion, high resolution HCD/EThcD product ions as well as the retention time similarity between glycoforms to build a comprehensive glycopeptide lists, and score them using a multivariate scoring system. The scoring system accounts for various levels of supporting data and integrates empirical data associated with glycoforms containing high confidence to those with less such as limited tandem MS data. Pinnacle then builds a quantitative profile for all these forms showing the percentage of signal present in each glycoform.

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Conclusion

Novel software enables fast characterization and quantification of O-glycan profiles for IgA, with more than 50 glycoforms.

Keywords: glycopeptide, glycoforms, O-linked, glycoprot

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Performance Evaluation of First and Second Generation Quadrupole Dual Cell Linear Ion Trap Orbitrap Hybrid MS for Glycopeptide Analysis

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Introduction and Objectives

Large scale intact glycopeptide analysis remains challenging due to complexities associated with the glycopeptide structure. Not only must one sequence the peptide backbone, but site localization and glycan composition are also required. The challenge is further compounded by the fact that traditional fragmentations are not ideal for glycopeptide sequencing. The emergence of ETD, and by extension EThcD have alleviated a lot of these issues. Here we present a performance evaluation comparison of first and second generation quadrupole dual cell linear ion trap Orbitrap hybrid mass spectrometer for glycopeptide analysis. Parameters and workflows will be presented that highlight large scale glycoproteomics.

Methods

Glycopeptides were enriched from human serum and HeLa lysates using strong anion exchange columns. The enriched glycopeptides were analyzed using an EASY nLC 1000 with a C18 PepMap column (2um, 100A, 75umx50 cm) on an Orbitrap Fusion and Orbitrap Fusion Lumos mass spectrometer. Various ETD reaction times, AGC target values, isolation windows, supplemental activation collision energy and RF were tested to maximize glycopeptides identification. Data analysis were performed using Byonic software (Protein Metrics).

Results and Discussion

Our initial experiments focused on optimizing ETD parameters to improve glycopeptides data. Typically, longer ETD reaction times were needed for glycopeptides relative to conventional peptides. Various ETD reaction times, fixed or varied, dependent upon charge states were tested to maximize spectral quality. Similarly, we also optimized EThcD parameters, as glycopeptide fragmentation can be maximized by varying supplemental activation collision energy. The primary focus of our experiments was on ETD and EThcD, however, we observed that the quality of HCD spectra were superior to spectra acquired on other platforms for intact glycopeptides. Typically, b and y ions generated from peptide backbone of a glycopeptides are low abundant and are difficult to detect on mass spectrometers. But in these instruments we could easily detect and use them for sequencing. In total 11 parameters were tested with 21 individual runs to maximize performance. After optimization of parameters, experiments were conducted on both platforms to examine performance relative to each other.

Conclusion

Overall, Lumos identified 9% more unique glycopeptides relative Orbitrap Fusion by ETD, 43% more by EThcD and 49% by EThcD over ETD. The increase in identification came from large glycopeptides which are challenging in mass spectrometry. Glycosylation, specifically changes in glycosylation, is a hallmark of cancer. Unfortunately, proteomics

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studies tend to ignore this particular PTM. Even in unenriched samples, 15-20% of spectra are glycopeptides. Using HeLa as model we explored the possibility of sequencing these modifications in discovery proteomics experiments. The detail of data processing is on-going.

Keywords: glycopeptide Orbitrap Fusion Lumos EThcD ETD glycosylation

TP02-29

Role of CD52 glycosylation in immunosuppression

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Introduction and Objectives

CD52 is a low molecular weight glycopeptide with a single N-linked glycan structure, which is expressed on lymphocytes and in the male reproductive tract. In T cells, CD52 is immunosuppressive by binding to sialic acid-binding immunoglobulin-like lectin (Siglec-10) receptors (Bandala-Sanchez E, 2013). Lymphocyte CD52 has multiple tetra- and penta- antennary N-glycan structures with poly-acetyllactosamine repeats and variable sialylation at the N-glycosylation site (Treumann A, 1995). Whether O-glycans are also presented on CD52 has not been characterised and the most bioactive glycoform is unknown.

Methods

Results and Discussion

The profile of CD52 with the Fc N297 glycosylation site mutated indicated the presence of several bi-tri and tetra-antennary sialylated N-glycan structures, also revealed after Factor X cleavage of CD52 from the Fc. Glycan structures were determined by intact mass analysis of the glycopeptide before and after PNGase F treatment to determine N- and O-glycan site occupancy.

Conclusion

The identification of specific glycans responsible for CD52-mediated immune suppression may provide a foundation for future therapeutic targets.

Keywords: Glycosylation. Immunosuppression. Sialylation. Glycofroms. CD52.

TP02-30

Ligand-independence of the Colony Stimulating Factor 3 Receptor (CSF3R) results from loss of sialylation which leads to increased oncogenesis.

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Introduction and Objectives

Mutations in the Colony stimulating factor 3 receptor (CSF3R) have recently been found in a large percentage of patients with chronic neutrophilic leukemia (CNL) and, more rarely, in other types of leukemia. CSF3R signaling plays a seminal role in neutrophil production through activation by its ligand, granulocyte colony stimulating factor (G-CSF).

Methods

We interrogated both T618I and N610H mutants and wild-type CSF3R from blood and found that sialylation plays a role in ligand-independent signaling and demonstrate glycosite cooperation. Employing glycoproteomics and free glycan analysis of CSF3R provide vital information to signaling differences involved in the normal and malignant CSF3R signaling.

Results and Discussion

Acquired point mutations of N- and O- glycosylation sites in CSF3R lead to a ligand-independent signaling and were identified as oncogenic drivers of CNL. The most common mutation, T618I, signals without ligand and is observed 80% of CNL patients.

Conclusion

An in vitro model system was developed using FLAG-tagged CSF3R and glycoproteomic analysis revealed a loss of glycosylation drives oncogenesis.

Keywords: Proteomics, Signaling, Glycoproteins, Glycoproteomics, Chronic neutrophilic leukemia

TP02-31

Glycoproteomic Alterations in Drug-resistance Lung Cancer Cell Lines Revealed by Lectin Magnetic Nanoprobe-based Affinity Mass Spectrometry

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Introduction and Objectives

Recent studies have reported the potential involvement of glycosylation in cancer resistance. For example, sialylation influence the sensitivity to Tyrosine Kinase Inhibitors (TKI) by modulating EGFR phosphorylation in non-small cell lung cancer (NSCLC). To investigate the differential glycoprofile and glycoproteins between TKI-sensitive and resistant NSCLC cells, we developed a nanoprobe-based affinity mass spectrometry method for quantitative comparison of the glycosylation profile with high-mannose, sialylation and fucosylation. Analysis of membrane glycoproteome might allow identification of glycoproteins and their site-specific glycan structure that play significant roles associated with drug resistance in NSCLC.

Methods

Results and Discussion

Applying multi-lectin strategy for enrichment of the glycoproteome in PC9 and resistant PC9/gef, the screening of intact glycopeptides using oxonium ions (m/z 204.1 for HexNAc and m/z 366.1 for HexHexNAc) from MS/MS spectra showed that significantly differential glycoprofile was observed from MNP@AAL enrichment. Analysis using MAGIC showed that 70% of the glycopeptide spectra identified were fucosylated, wherein 5040 fucosylated glycopeptide MS/MS spectra and 4643 fucosylated glycopeptide MS/MS were present in PC9-IR and PC9 respectively. Label-free quantitation result displayed that 114 glycopeptides from 77 proteins had more than 2-fold increase in PC9-IR over PC9, including Neural cell adhesion molecule L1 (L1CAM), Hepatocyte growth factor receptor (MET), and Cation-independent mannose-6-phosphate receptor (IGF2R). In addition, analysis of intact glycopeptides using Byonic identified 208 and 386 intact glycopeptides from PC9 and PC9IR, respectively. More importantly, intact glycopeptides and fucosylated glycan structures of relevant proteins unique to PC9-IR, such as L1CAM and EGFR, have been identified.

Conclusion

We found that more fucosylated glycopeptides and glycoproteins are present in resistant cell. Verification of proteins and N-glycan alterations on glycoproteome will be analyzed and may provide new insight into the mechanism of glycan involvement in NSLC resistance.

Keywords: glycosylation, lectins, magnetic nanoprobes

TP02-32

Preference of the universal enrichment methods for N-glycopeptides with particular glycoforms

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Introduction and Objectives

Glycosylation is a one of the most common, important and complex post-translational modifications of proteins. Thus a comprehensive analysis of glycoproteins or glycopeptides is urgent. Researchers have developed many enrichment methods of glycoproteins or glycopeptides such as lectin affinity chromatography, hydrophilic interaction chromatography(HILIC), chemical technology(hydrazide capture and boronate-based capture). Unlike lectin affinity methods, HILIC and boronate-based methods are universal without bias for different glycan structures. Nevertheless there are no detailed work on investigating the preference of the universal enrichment methods for N-glycopeptides with particular glycoforms. Herein, we used four standard glycoproteins cantaining high mannose, complex type and hybrid type glycans to test the glycoforms enriched by HILIC and boronate-based method.

Methods

The glycopeptides are enriched by Hydrophilic interaction chromatography(HILIC), Boronated-based capture method followed by MALDI-TOF MS. Firstly, we investigate the impact of the factors of these methods on their enrichment efficiency, pH, salt concentration and salt type are tested to evaluate the boronate-based method for enrichment. The acid concentration, salt concentration and salt type are tested to evaluate in HILIC for enrichment.

Results and Discussion

In this study, the pH has crucial influence on different glycan structures especially the high mannose structure in boronete-based capture method. The hydrophilic interaction chromatography presents a multimodal retention mechanism. The concentration of salt and the type of the salt have a profound influence on HILIC.

Conclusion

We study the prefence and the key factors of each methods in order to offer the better and comprehensive understanding of the methods.

Keywords: Glycopeptides /Enrichment method / HILIC / Boronate-based method / MALDI-TOF MS

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Integrating Glycoproteomics in the Multi-Omics Analysis of Type II Diabetes Onset

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Introduction and Objectives

Glycosylation is one of the most ubiquitous global post-translational modifications of proteins observed in eurkaryotes. It is postulated that the heterogeneous glycosylation patterns provide an additional level of dynamic regulation that could describe disease states. The advent of omics profiling technologies across all levels of the central dogma now allows for powerful physiological analyses with relevance to predicting medical risks. This study leverages novel technologies in glycoproteomics in conjunction with other longitudinal, quantitative and high-throughput multi-omics data to elucidate the complex molecular underpinnings of weight gain in human progression to Type II Diabetes (T2DM)

Methods

Overweight individuals were recruited for this study, half of which were classified as insulin resistant and half as insulin sensitive. Each individual underwent a 30-day period of weight-gain via excessive caloric intake followed by a 60-day period of restricted dieting and return to baseline weight. Blood samples and clinical profiles were obtained at three time points per patient: baseline, peak-weight and post weight loss.DNA, RNA, and protein were then obtained from each sample. Glycoproteins were enriched using a multi-lectin affinity column, digested with trypsin, and analyzed by LC-MS/MS. The resulting data including but not limited to, RNA-seq and glycoprotein-enriched tandem mass spectrometry data, were then aligned and subjected to ANOVA-based, analyte-by-analyte analysis. Changes in a priori-defined molecular pathways were tested for using bioinformatics tools.

Results and Discussion

Primarily we have been able to produce robust data from multi-lectin affinity chromatography glycoform enrichment of protein samples before tandem mass spectrometry analysis on each of the samples. The challenge then arose to align these data with other omics profiles. This study has revealed biomolecular changes of note in various multi-omics levels. For example, on the RNA expression level, pathways that displayed the most variability between patients and across time points were in those of inflammatory response and other defense mechanisms. In addition to identifying multiple protein glycoforms from the multi-lectin affinity chromatography separation followed by LC-MS/MS analysis, the early quantitative glycoprotein alterations in these glycoforms also corresponded to analogous pathways to those found in gene expression data and epigenomic modifications.

Conclusion

These multi-omics data and subsequently discovered multi-analyte signatures suggest

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differential biomolecular landscapes in not only various timepoints of induced weight gain, but also in insulin sensitivity status of patients. In this study, we were able to both contribute to new biology that may characterize the effect of weight gain on T2DM onset, and also mature novel technologies to integrate glycoproteomics in a multi-omics analysis of complex disease progression.

Keywords: Type II Diabetes, Multiomics, Glycoproteomics, Precision Medicine

TP02-34

Ion Mobility and High-Resolution Native Mass Spectrometry Reveals Glycan-Specific Modulation of Glycoprotein Stability

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Introduction and Objectives

Glycosylation is the most prevalent protein modification and importantly modulates protein structure, activity, half-life and cellular localization. Glycoproteins are highly heterogeneous and glycan structures at any given site can vary with subtle changes profoundly altering protein function. However the ability to link these minor glycan changes (microheterogeneity) to intact protein structure-function relationships remains a key challenge in glycobiology. Bottom-up strategies are lacking in that the information does not fully represent the intact protein complex. Herein we explore glycan-dependent modulation of glycoproteins by high-resolution native mass spectrometry (MS). The high resolution Orbitrap mass spectrometer provides high accuracy of measurement (< 1Da) for glycoproteins with various glycoforms. We targeted for our study a C-type lectin receptor DC-SIGN since it plays a pivotal role in initiating the immune response and dissemination of HIV. We identified unique O-glycosylation of DC-SIGN and used ion mobility MS to determine how key glycan structures influence stability of the protein. Collectively, native MS offers a robust analysis of glycoproteins, providing complementary methodology for studying the function of glycosylation.

Methods

DC-SIGN expression constructs were transiently over-expressed in HEK293T cells and purified by affinity chromatogram. Glycosylation analysis of DC-SIGN was performed on a high resolution Orbitrap MS. The stabilization effect of distinct glycoforms was studied by ion mobility-mass spectrometry (IM-MS) to measure the change of collision cross-section (CCS) of gas-phase ions.

Results and Discussion

The native mass spectra of the DC-SIGN carbohydrate recognition domain (CRD) indicated multiple glycosylations of the protein, including the moieties of sialic acid and multiple LacNAc. Treating the protein with various glycosidases allowed further characterization of glycan structures. The data revealed the O-linked glycosylation of DC-SIGN with sialylated core 1/2 glycans. Energy-induced protein unfolding in the gas-phase showed glycan-specific modulation of DC-SIGN stability. The LacNAc structure profoundly enhanced protein stability, but sialylation produced a negative effect on stability. By uncovering this glycosylation of DC-SIGN and link this to protein stability, we propose a potential modulation mechanism involving O-glycosylation of DC-SIGN on pathogen recognition.

Conclusion

Native MS provides a powerful approach to characterize the glycosylation on intact proteins. The sensitivity and resolution of the Orbitrap offers accurate assignment of

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glycan compositions and glycan structures are further investigated by enzymatic approach. Combined with IM-MS, this method is capable of examine the structure-function relationship of glycoforms present on the protein and prompt new mechanistic insight into the mechanism of pathogen recognition.

Keywords: Native mass spectrometry, ion mobility, glycosylation, stability, DC-SIGN

TP02-35

Preparative purification of N-linked glycopeptides by using two-dimensional chromatography for the structural characterization

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Introduction and Objectives

For in-depth understanding the biofunctions of glycans attached to proteins, it is often the first priority to acquire their structural information. Comprehensive elucidation of glycan structures is usually difficult due to the complexity and heterogeneity of the glycans. Meanwhile, glycan isomers differing in topology, branching or linkage pattern further hinder the structural elucidation. Although tandem mass spectrometry (MS) has become one of the most powerful techniques for glycan structure analysis, the prior chromatographic separation is still indispensable, since the sample complexity could be significantly reduced by using LC. Due to the limited selectivity, single dimensional chromatography was insufficient for the full resolution of highly complex glycopeptides. Herein, an offline two-dimensional (2D) LC method was developed by using hydrophilic interaction chromatography (HILIC) and porous graphitized carbon (PGC) chromatography for the effective separation of glycopeptides from ribonuclease B (RNase B), prior to the MS/MS analysis.

Methods

Tryptic digestion of RNase B was first loaded onto a HILIC column packed with customized material named Click TE-Cys. The glycopeptides fractions were collected manually, evaporated, re-dissolved and transferred to the second dimensional PGC column, respectively. The purified glycopeptides from PGC were finally applied to the MALDI-TOF/TOF MS for the detailed structural analysis.

Results and Discussion

On the first dimensional HILIC, the RNase B glycopeptides were isolated from the non-glycosylated peptides and sorted into five main fractions according to the comprised mannose residues from five to nine (Man5 to Man9). Glycopeptide isomers contained in each fraction were further separated on PGC, where one component were found in Man5 and Man9, two isomers in Man6 and Man7 and three in Man8, respectively. These purified isomers were fractionated and further characterized by using tandem MS, from which the sequence and branch of the attached glycans could be identified, as shown in Figure 1.

Conclusion

A method based on 2D-HILIC-PGC coupled to tandem MS for the characterization of RNase B glycopeptides was established. The complexity and heterogeneity of the target glycopeptides were significantly reduced and the confidence for glycan structure identification was remarkably enhanced. This 2D-system also constitutes an effective approach for acquiring pure glycopeptides from natural sources.

Keywords: Two-dimensional chromatography, HILIC, tandem mass spectrometry, glycopeptide

TP02-36

New glycoproteomic tools to study N-glycosylation and to uncover novel glycophenotypes in human diseases

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Introduction and Objectives

One of the most critical post-translational modifications in proteins is N-glycosylation, influencing the folding and function of ~ 1/3rd of the cellular proteome. N-glycosylation occurs when a pre-assembled glycan is transferred en bloc to a protein acceptor at specific protein sites (sequons) by the oligosaccharyltransferase (OTase). These glycans are then further modified as proteins traverse the secretory pathway. Alterations in glycan structure and occupancy at specific sequons have significant clinical and industrial impact, leading to a growing number of diseases (Congenital Disorders of Glycosylation, cancer, etc) and to changes in secretion, half-life, and activity of proteins with biotechnological relevance. A more complete understanding of the causes and consequences of changes in N-glycosylation will lead to the design of better therapeutics, diagnostic strategies, and industrial bioprocesses. Thus, to more effectively and efficiently study the mechanism of N-glycosylation and its physiological impact, we designed several biochemical and quantitative mass spectrometry (MS) workflows. Here, we applied these tools to study the function of the OTase and to look for glycobiomarkers in clinical samples.

Methods

We developed SWATH-MS glycoproteomic workflows to measure site-specific and global changes in glycan occupancy and structure. We tested these methods in yeast and mammalian cells with normal or altered glycan biosynthetic processes, and in clinical samples.

Results and Discussion

Using Saccharomyces cerevisiae as a model, we measured glycan occupancy and structure on a range of glycoproteins in strains with mutations in the N-glycosylation pathway. We observed different degrees of hypoglycosylation in all mutants, including in mutants in the OTase subunits. The stronger hypoglycosylation phenotypes occurred in strains with defects earlier within the ER lumenal mannosyltransferase steps or glucosyltransferase steps, and only mannosyltransferase mutants showed extensive global changes in glycan structure. These results provide a quantitative and qualitative overview of the OTase ability to transfer truncated glycans, and quantitate the diverse glycosylation outcomes of defects in different parts of the glycan biosynthetic process. We also applied these workflows to the study of mammalian cells with defects in the glycan biosynthetic machinery and to clinical samples of patients with potential glycosylation disorders.

Conclusion

We have designed glycoproteomic protocols that allow for rapid relative quantitative and

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qualitative glycoproteomics of a variety of samples. Our results provide insights into regulation of site-specific glycosylation and the function of the OTase. Our methods and results have important implications in the understanding of the fundamentals of the glycosylation process and on the industrial and medical applications of glyco-biotechnology.

Keywords: glycobiology, oligosaccharyltransferase, clinical samples, yeast, glycosylation disorder, SWATH, N-glycosylation

TO03-01

Unravelling crosstalks between SUMOylation and other protein modifications in human cells using dynamic proteomics

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Introduction and Objectives

The small ubiquitin modifier (SUMO) is an ubiquitin-like (UBL) protein that is reversibly conjugated to a wide range of substrates involved in different regulatory pathways including intracellular trafficking, DNA damage response and cell cycle progression. Crosstalks between protein SUMOylation, phosphorylation and ubiquitination have recently been reported although no approach currently exists to determine the inter relationship between these modifications. Here, we report an optimized workflow that combines TiO2 media and sequential immunoaffinity purification to profile the dynamic changes in protein phosphorylation, SUMOylation and ubiquitination in human cells upon cell stimulation known to promote the formation of PML nuclear bodies.

Methods

HEK293 SUMO3 mutant cells were grown DMEM media containing light (control) or heavy amino acids (cell stimuli). Cells were treated with As2O3 and/or MG132 for periods extending to 8h. Protein extracts were enriched on Nickel NTA beads prior to tryptic digestion. SUMOylated and Ubiquitinated peptides were purified using custom monoclonal antibodies directed against their respective remnants and phosphopeptides were purified using TiO2 beads. All samples were analyzed by LC-MS/MS on an Orbitrap Fusion using HCD activation mode. All mass spectrometry data were analyzed using MaxQuant.

Results and Discussion

The optimized immunoaffinity method facilitates the enrichment of SUMOylated proteins, and enables the unprecedented identification of 10394 SUMO sites in HEK293 cells. The dynamic profiling of modified proteins in HEK293 cells treated with the proteasome inhibitor MG132 for up to 8 hours identified 676 and 114 regulated SUMOylation and ubiquitylation sites, respectively. Quantitative proteomic analyses revealed cross-talk between SUMOylated and ubiquitinated substrates that control protein degradation via the ubiquitin-proteasome system, and highlighted the unexpected regulation of deubiquitinase enzymes by UBL modifiers and the SUMOylation of proteasome subunits. Proteasome SUMOylation affects its recruitment to PML nuclear bodies, and PML lacking a SUMO interacting motif failed to colocalize with SUMOylated proteasome. PML was SUMOylated in the first 2h upon As2O3 treatment and was ubiquitinated and degraded after, while the SUMOylated form of PML was stabilized in presence of the proteasomal inhibitor MG132. We also identified other proteins such as heat shock factor protein 2, histone deacetylase 1 and Poly [ADP-ribose] polymerase 1 that exhibited similar modification profiles upon these cell treatments. Interestingly, more than 175 SUMO sites

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were also phosphorylated at neighboring residues including several proteins involved in DNA damage and cell cycle regulation.

Conclusion

The present proteomics approach provided key insights into crosstalks between UBL modifiers and protein phosphorylation that are involved in the biogenesis of PML nuclear bodies and the regulation of protein degradation.

Keywords: SUMOylation, ubiquitination, phosphorylation

TO03-02

Towards Comprehensive Analysis of Protein ADP-ribosylation

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Introduction and Objectives

Poly-ADP-ribosylation (PARylation) is a PTM that was first documented in 1963. It is catalyzed by a family of enzymes called Poly-ADP-ribose polymerases (PARPs). In particular, PARP1 is a nuclear enzyme that is involved in mediating DNA damage response (DDR). This provides the rationale for developing PARP1 inhibitors to treat human diseases. In particular, cancer cells with defects in double-strand break repair, such as BRCA1/2-mutated cells, are particularly sensitive to PARP1 inhibitors. Indeed, a PARP1 inhibitor, olaparib, has recently been approved by the FDA to treat BRCA-mutated ovarian cancer. Despite the hugely successful clinical development of PARP1 inhibitors, from a basic science perspective, we know surprisingly little about the detailed mechanism by which PARPs signal downstream. The paucity of validated PARP1 effectors and their modification sites has greatly hampered its functional study.

Methods

We used boronate affinity beads to isolate PARylated peptides. We eluted PARylated peptides using NH2OH treatment. The ester bond between the first ADP-ribose unit of PAR and the side chain carboxyl group of an Asp and Glu residue is susceptible to NH2OH attack. This generates a hydroxamic acid derivative with an addition of 15.0109 Da. This mass increment can be readily distinguished by mass spectrometers.

Results and Discussion

Recently, we were able to develop the first large-scale mass spectrometric approach towards site-specific characterization of the D/E-PARylated proteome. Using this method, we: (1) identified 1,048 unique, endogenously modified PARylation sites on 340 proteins. (2) found that the modified proteins are involved in not only DDR, but also a wide array of other nuclear functions. (3) characterized the D/E-PARylated proteome that is sensitive to clinically relevant PARP1 inhibitors, and, in so doing, identified many novel PARP1 targets. (4) found that iniparib, a compound which was previously thought to be a covalent PARP1 inhibitor, does not inhibit PARP1 in intact cells.

Conclusion

In summary, we were able to develop the first-of-its-kind approach to global and site-specific characterization of the D/E-PARylated proteome in vivo. The method can be easily performed on any mass spectrometers with conventional CID-tandem MS capabilities. We expect that this method will greatly facilitate the uncovering PARP-dependent signaling mechanisms that will lead to better strategies of therapeutic intervention for the treatment of relevant human diseases.References:1. Gibson BA, Zhang Y, Jiang H, Hussey KM, Shrimp JH, Lin H, Schwede F, Yu Y, Kraus WL, " Chemical genetic discovery of PARP targets reveals a role for PARP-1 in transcription elongation.", Science, 20162. Zhang Y, Wang J, Ding M, Yu Y, "Site-specific characterization of the Asp- and Glu-ADP-ribosylated proteome.", Nature Methods, 10,

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981, 2013

Keywords: PTM, ADP-ribosylation, BRCA, breast cancer

TO03-03

Acetylome analysis reveals carbon metabolism as a key factor enhancing thermogenesis in white adipocytes

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Introduction and Objectives

Adipocytes are essential regulators of whole-body energy homeostasis by secreting several proteins that regulate blood pressure, immune function, angiogenesis and energy balance. Recently, casein kinase 2 (CK2) was found to regulate class I histone deacetylases (HDACs) activity and compromise the cAMP-induced uncoupling and energy expenditure stimulated in white adipocyte tissues. However, how the thermogenesis in white adipocytes is promoted by CK2 or HDAC inhibitors remains unclear. In this work, we aim to elucidate the phosphorylation-engaged acetylation events promoting thermogenesis process in white adipocytes.

Methods

To explore pivotal molecular mechanism triggered by CK2-engaged acetylation events, acetyllysine-containing peptides were enriched from CK2 or deacetylase inhibitors treated adipocytes by affinity-based method. The enriched peptides were fractionated by strong cation exchange (SCX) chromatography followed by nanoLC-MS/MS. The identified acetylated peptides were quantified based on the extracted ion current of each peptide. Bioinformatics analyses, such as functional enrichment analysis, motif analysis, hierarchical clustering, pathway construction, molecular modeling were applied to interpret the influence of differentially acetylation events.

Results and Discussion

Combined with SCX fractionation, 1043 unique acetylated peptides were identified with 1887 acetylation sites from 514 proteins including histones and non-histone proteins by two multi-antibody mixtures with averaged enrichment efficiencies of 16.96% and 90.28%, respectively. We found that CK2 inhibitor caused comparative accumulation in lysine acetylation on mitochondrial proteins compared to deacetylases inhibitors, suggesting robust associations between phosphorylation and acetylation, directing thermogenesis in white adipocytes. The differential acetylated proteins were enriched in carbon metabolism of KEGG pathways, especially in TCA cycle, valine, leucine and isoleucine degradation, and fatty acid metabolism, implying versatile metabolic processes were regulated by protein acetylation. Of interests, citrate synthase and citrate lyase were the top two altered acetylated proteins which are important regulators in adipogenesis with the same trends on lysine acetylation among inhibitor treatments. Furthermore, differential acetyl modification on mitochondrial ATP synthase was on the top list of protein complex enrichment, showing that acetylation contributes to energy production. Acetylation on proteins involving in TCA cycle, fatty acid degradation and oxidative phosphorylation tips the balance to acetyl-CoA accumulation, explaining our findings in lipolysis and UCP-1 induction in inhibitor-treated white adipocytes.

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Conclusion

By integrative analysis on PTMomics data, we exhibit novel roles of protein lysine acetylation in orchestrating protein kinase activity and metabolic homeostasis contributing to the capacity of white adipocyte thermogenesis.

Keywords: Acetylome; obesity; carbon metabolism; thermogenesis

TO03-05

Efficient Enrichment of SUMOylated Peptides from Alpha-lytic Protease Digest Using K- ? -GG Remnant Immuno-affinity Purification

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Introduction and Objectives

Small Ubiquitin-like Modifier (SUMO) proteins are a family of proteins covalently attached to lysine residue of target proteins and modify their function. SUMOylation has been demonstrated being involved in various cellular processes including nuclear-cytosolic transportation, transcriptional regulation, apoptosis, protein stability, response to stress and cell cycles. Compared to ubiquitination, much fewer SUMOylation sites were identified largely due to the lack of efficient enrichment method. To this end, we have developed a straightforward and robust method by combing alpha-lytic protease (WaLP) digestion and K-?-GG remnant antibody enrichment to identify and quantify endogenous SUMOylation sites by LC-MS/MS.

Methods

Hela cells were heated shock and lysed in urea buffer. Protein digestion was performed in 2M urea by alpha-lytic protease (WaLP) that preferentially cut at the C-terminus of T, S, A and V resulting a GlyGly remnant left on the lysine residue previously carrying SUMO-1 and SUMO-2/3. Digested peptides were subject to immuno-affinity purification (IAP) using K-?-GG remnant antibody and LC-MS/MS analysis for identification and quantification of SUMOylation sites. To validate the KGG sites identified, specific sumo-proteases were used to remove endogenous SUMO tails from modified proteins, which were then digested by WaLP and trypsin, respectively. Label-free quantification was applied to determine the quantitative changes of identified KGG peptides from WaLP and trypsin digest in parallel.

Results and Discussion

There were about 1,100 non-redundant KGG peptides identified from 5mg Hela lysate digested by WaLP and enriched by KGG antibody IAP including known sumoylated proteins including TRIM28, TRIM33, SUMO1 and SUMO-2/3. By using specific SUMO-proteases, both SUMO-1 and SUMO-2/3 were efficiently removed in-vitro confirmed by western blots; while the total ubiquitination level was not affected. We compared the quantitative changes of identified KGG peptides from WaLP digest with and without SUMO-proteases treatment and found 612 out of 724 KGG sites identified in showed significant lower intensities in SUMO-proteases treated samples. While the quantitative analysis of identified KGG peptides from trypsin digest showed only 16 out of 9,031 KGG sites with significant intensity changes. The results validated the KGG sites identified by our method truly coming from SUMOylation instead of ubiquitination. Protein function classification placed proteins involved in 1) transcriptional regulator; 2) translation, 3) RNA processing and 4) DNA binding and repair as top protein groups modified by SUMOylation that indicated potential correlation between SUMOylation and epigenetics. More importantly, more than 2/3 of the KGG sites identified by our method

are novel sumoylation sites.

Conclusion

A novel and robust method for enrichment, identification, quantification and validation of endogenous SUMOylation was developed

Keywords: SUMOylation, alpha-lytic protease, K-?-GG remnant antibody enrichment

TP03-01

Apoptotic protein Bax is regulated by multiple deubiquitinating enzymes in response to DNA damage

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Introduction and Objectives

Bax is a pro-apoptotic protein included in Bcl-2 family. In healthy cells, Bax exists in the cytosol as a monomer. When the cells get stress incuding UV exposure, Bax is activated and is translocated to the mitochondria, leading to the release of cytochrome c and then trigger apoptosis. Bax has been reported to be degraded through the ubiquitin-proteasome system. And deubiquitinating enzymes (DUBs) can reverse this pathway by removing the ubiquitin from protein targets. In this study, we found that multiple DUBs bind to Bax by performing the yeast two hybrid screening and proteomic analyses. These analyses revealed that Bax-DUB1, Bax-DUB2, Bax-DUB3, and Bax-DUB4 interact with Bax, and regulate the ubiquitination of Bax. Our biochemical analysis revealed that Bax-DUB4 participates in apoptosis pathway by interacting with Bax. Taken together, our data suggest that Bax-DUB1, Bax-DUB2, Bax-DUB3 and Bax-DUB4 might be putative DUBs regulating the stability of Bax. Of these, it is confirmed that Bax-DUB4 regulates apoptosis by controlling Bax and this provides a new therapeutic target for various cancers.

Methods

Yeast two–hybrid screening Yeast strain AH109 was used for screening and was incubated in a YPD plate at 30ºC for 3-4 days. USPs which were subcloned into pGBT9 vectors and pGAD424-Bax were co-transformed and incubated in -Leu/-Tre minimal medium plates containing X-α-gal (Sigma, St. Louis, MO, USA). Immunoblotting and immunoprecipitation 293T and HeLa cells were harvested and lysed with a lysis buffer. The cell lysates were incubated with primary antibodies at 4°C overnight. And then, mixtures were incubated with protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C for 2 hours. The beads were washed 3 times and boiled in SDS sample buffer. The immunoprecipitates were analyzed by Western Blotting using α-β-actin (Santa Cruz, CA, USA), α-HA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), α-Flag (Sigma-Aldrich, St. Louis, MO, USA), and α–Bax (6A7 and 2D2) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies.

Results and Discussion

We found that Bax-DUB1, Bax-DUB2, Bax-DUB3 and Bax-DUB4 are binding proteins of Bax through yeast two-hybrid screening. And we confirmed interaction between

domain of Bax. Bax is ubiquitinated by forming Lys48- and Lys63-linked polyubiquitin chains. Also there is counter-regulation that both Lys48- and Lys63-linked ubiquitination level is decreased by Bax-DUB4. Through these data, it is suggested that Bax-DUB4 may regulate apoptosis by deubiquitinating Bax. The functional study for Bax-DUB4 at the molecular level is required to better understand an underlying mechanism for apoptosis pathway.

Conclusion

Bax-DUB4 regulates Bax through deubiquitination and may have a cellular role in the mechanism of apoptosis.

Keywords: Bax, deubiquitinating enzyme, ubiquitination, deubiquitination, and apoptosis

TP03-02

Investigating the significance of Methyltransferase-like (METTL)-family lysine methylation enzymes on Heat Shock Proteins and their role in cancer

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Introduction and Objectives

Lysine methylation is involved in the control of numerous protein properties and as a result fulfils critical roles in various biochemical pathways. Moreover the dysregulation of methylating enzymes have been shown to lead to disease. Lysine methylation has been identified as playing a role in some cancers by affecting transcription factors and chaperones. A small sub-set of lysine methyltransferases of the Methyltransferase-like (METTL) family have been shown to act on chaperones including heat shock proteins (HSPs), which in turn have been the focus of various aspects of cancer research, though mainly with respect to malignancy, progression and chemoresistance. Thus the aim of this study was to determine the substrate range of this METTL-family sub-set, and then focusing on selected proteins within the chaperone network, identify the position and function of the methylations, in order to be able to infer the role they play in cancers.

Methods

The substrates of the METTL enzymes were screened for by a Yeast-Two Hybrid set-up using a number of the METTL-enzymes including METTL20, METTL21A, METTL21B and METTL 23 as bait. A sub-set of the aquired proteins, which were considered to be of greater relevance, were re-validated and underwent further study. Among the HSPs deemed of interest are HSP27, HSP60, HSP70 and GRP78. The selected proteins were overexpressed in HEK293 cells and the position of methylated lysines was determined on the over-expressed HSPs by performing co-transfections of the methylating enzyme and HSP, followed by immunoprecipitation (IP) and identification by liquid chromatography and tandem mass spectrometry (LC-MS/MS) using an LTQ Orbitrap Velos (Thermo Fisher Scientific, USA). Furthermore, in order to try and identify the function of some of these methylations, changes in the sub-cellular localisation of the target proteins in the presence and absence of the enzyme was determined.

Results and Discussion

The first two screens by Yeast-Two Hybrid are underway and another eight baits are being prepared for the respective screens to be performed by the end of the project. Preliminary LC-MS/MS analysis of the IPed samples of over-expressed HSPs, co-transfected with their respective methylating enzyme, have yielded distinct, and previously unidentified methylations. However, so far, no changes in the sub-cellular localisation of the target proteins have been clearly identified when comparing over-expression with and without the respective enzyme. This information is essential in order to start piecing together and infer the role these enzymes and the modifications they bring about, play in cancer.

TP03-02

Conclusion

The results obtained offer a first glimpse into the full range of protein substrates this sub-set of METTL emzymes has and their possible roles. However, a lot more work is required to elucidate both the position and biochemical pathways in which they are involved.

Keywords: Lysine methylation, METTL-enzymes, heat shock proteins, signalling, localisation, activity modulation

TP03-03

Lysine propionylation is a widespread post-translational modification involved in regulation of photosynthesis and metabolism in cyanobacteria

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Introduction and Objectives

Protein lysine propionylation is a recently identified post-translational modification (PTM), in which a propinyl group (CH3-CH2-CO-) is added to the ?-amino group of lysine residue on a protein moiety. Increasing lines of evidence strongly suggest that lysine propinylation is likely a mechanism involved in the regulation of metabolism and the stress responses in organisms ranging from bacteria to mammals, but its extent and function in photosynthetic organisms remain unexplored. Cyanobacteria are the only prokaryotes capable of oxygenic photosynthesis and play crucial roles in global carbon and nitrogen cycles. we hypothesized that lysine propinylation may play regulatory roles in multiple metabolic processes in cyanobacteria, such as photosynthesis and carbon metabolism.

Methods

We carried out a proteomic survey of lysine propionylation in a model cyanobacterium Synechocystis sp. PCC 6803 (Synechocystis) using high-accuracy tandem mass spectrometry (MS/MS) analysis in combination with the enrichment of propionylated peptides and subsequent peptide identification. The functional significance of lysine propinylation on the enzymatic activity of fructose-1,6-bisphosphatase (FbpI) was determined by site-specific mutagenesis and biochemical studies.

Results and Discussion

In total, we identified 111 lysine propionylation sites on 69 proteins and a large proportion of propionylated proteins are involved in photosynthesis and metabolic pathways. Our functional results showed that propionylation could regulate FbpI activity significantly. Therefore, lysine propionylation could provide an additional layer of regulation of FbpI activity and may integrate metabolic cues such as energy or carbon status through the availability of propionyl-CoA. Further functional studies showed that the propinylation level of PsaD subunit of photosystem I (PsaD), was significantly upregulated after high light (HL) treatment, suggesting that propionylation may be a mechanism involved in acclimation to HL in Synechocystis as well as play a regulatory role in photosynthesis. Plants are also likely to possess unique mechanisms related to the control of metabolic and photosynthetic pathways via lysine propionylation because much of the energy status of plants is related to the chloroplast via photosynthesis.

Conclusion

Overall, our results indicate that lysine propionylation is widespread in cyanobacteria and that it is probably an important mechanism that regulates photosynthesis and carbon metabolism in both plants and cyanobacteria. The underlying molecular mechanisms might be crucial for fine tuning the photosynthetic efficiency and cellular metabolism in various environmental conditions.

TP03-03

Keywords: Lysine propionylation, post-translational modification, Cyanobacteria, photosynthesis, Synechocystis, metabolic pathways

TP03-04

PIAS1-mediated SUMOylation of BAF57 is a critical regulator of cell growth and drug sensitivity in ovarian cancer cells

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Introduction and Objectives

The human BAF57 protein is the mammalian SWI/SNF chromatin-remodeling complex and can alter DNA-nucleosome topology. It has been reported that BAF57 is a predictive marker of endometrial carcinoma, and that inhibition of BAF57 activity may be a target for endometrial cancer therapy. SUMOylation is a covalent and reversible modification of a target protein by small ubiquitin-like modifier (SUMO). Conjugation of SUMO to target proteins requires a single E1 enzyme to activate SUMO and a unique E2 conjugating enzyme (UBC9) that, in combination with one of the few known E3 ligases, directs conjugation and ensures target specificity. PIAS1 (Protein Inhibitor of Activated STAT, 1), is one of the well-studied SUMO E3 ligases that is known to SUMOylate PML and PML-RAR, which promotes their ubiquitin-mediated degradation. In this study, we identified PIAS1-specific substrates using a large-scale SUMO approach and studied the function of SUMOylation on one substrate, BAF57 in human ovarian cancer (HOC) cell line.

Methods

HEK293 SUMO3 mutant cells were cultured in triple SILAC medium. PIAS1 knockdown were performed in medium and heavy channels with two different siRNAs and a negative control in light channel using scrambled siRNA. Then a workflow that combines the use of Ni-NTA purification, peptide level immunoenrichment and LC-MS/MS analysis was used for PIAS1 specific-substrate identification. In vitro SUMO assay and in vivo protein overexpression in HOC A2780 to further confirm the PIAS1-mediated SUMOylation status of BAF57. Through site-directed mutagenesis combined with cycloheximide (CHX) chase assays, cell proliferation assays and cytotoxicity assays using the anticancer agents, cisplatin and paclitaxel to further investigate the function of SUMOylation on BAF57.

Results and Discussion

In total, 270 SUMO sites were quantified across 4 biological replicates using our SUMO proteomic approach. Of these, 29 SUMO sites on 29 proteins were significantly down-regulated and Lys-92 SUMOylation on BAF57 was one of the most significantly down-regulated sites. In vitro SUMO assay and in vivo overexpression of PIAS1, SUMO3 and BAF57 in HOC A2780 cells further validate the K92 SUMO site on BAF57. CHX chase assay and overexpression of BAF57 and Ubiquitin in HOX A2780 cells shows that SUMOylation on BAF57 affects its stability and promotes its degradation by the ubiquitin–proteasome system. Cell proliferation assay and cytotoxicity assay shows the BAF57 SUMOylation sensitizes cells to anticancer agents and decrease cell growth by inducing cell cycle arrest at G1 phase.

TP03-04

Conclusion

In conclusion, we established a method for high-throughput identification of SUMO E3 ligase substrates by using siRNA knockdown in tandem with quantitative proteomics. For the first time we found that BAF57 is a PIAS1 substrate. PIAS1-mediated SUMOylation status of BAF57 promotes its STUbL-dependent proteasomal degradation and further affects cell growth and drug sensitivity of HOC A2780 cells.

Keywords: SUMOylation, PIAS1, BAF57, HOC

TP03-05

A novel post-translational modification: lysine propionylation was identified in bacterial species

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Introduction and Objectives

Lysine acetylation, one of post-translational lysine acylations, is an important PTM for the regulation of cellular processes, especially in transcription. Recent studies showed this modification occurs in every cellular component including nucleus, cytoplasm, mitochondria, and is conserved not only in eukaryotes but also in bacteria. Our previous acetyl-proteome study identified hundreds of protein acetylation sites in Thermus thermophilus, an extremely thermophilic bacterium (Okanishi et al. (2013) J. Proteome Res.). During this acetyl-proteome study, we discovered that lysine propionylation, a novel type of lysine acylations, occurs in T. thermophilus. In this study, we first identified the lysine propionylation at a diverse range of proteins in the several bacterial species.

Methods

Proteins were digested by trypsin. Propionyl-peptides were enriched from the tryptic digests by immunoprecipitation using anti-propionyl-lysine antibodies which were prepared in house. The enriched propionyl-peptides were analyzed by nLC-MS/MS.

Results and Discussion

We immunopurified propionyl-peptides from T. thermophilus proteins at exponential and stationary phase, and analyzed them by nLC-MS/MS. As a result, a total of 361 propionylation sites from 183 proteins were identified (Okanishi et al. (2014) Mol. Cell. Proteomics). The proteins identified to be propionylated were involved in various cellular processes, especially in metabolism. Interestingly, only 19.5% of propionylation sites were overlapped with acetylation sites identified in our previous study. The small overlap of modification sites proposed that the regulatory mechanisms of these two types of acylations are different regardless of the similar chemical structures of propionyl- and acetyl-groups. Furthermore, the local sequence context of amino acids around propionylated and acetylated lysines exhibits different characteristics, which suggested that responsible enzymes of these two kinds of acylations are different. The frequent occurrence of lysine propionylation implies the influence on a diverse range of proteins similar to lysine acetylation. We further showed lysine propionylation occurs in other 4 kinds of bacterial species including mesophiles and thermophiles, although the number of identified propionylation sites were different. This demonstrates lysine propionylation is diversely conserved in bacterial world. In silico structural mapping of the identified propionylation sites revealed dozens of propionylation events occur at lysine residue important for protein function such as enzymatic activity, nucleic acid binding, and complex formation.

Conclusion

Our propionyl-proteome study first showed lysine propionylation occurs at a diverse range

TP03-05

of bacterial proteins. These results will expand our knowledge of proteome-wide regulation by lysine acylations.

Keywords: post-translational modification, bacteria, lysine modification, protein structure

TP03-06

Acetyl proteomics using a novel enrichment strategy for lysine acetylated peptides

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Introduction and Objectives

Protein is one the most important components that control versatile cellular phenomena, and the functions are ingeniously regulated by reversible post-translational modifications (PTMs). Lysine is the hot spot that is subjected to various PTMs including methylation, ubiquitination and acetylation. Reversible acetylation of lysine e-amino group is mediated by lysine acetyltransferases (KATs) and lysine deacetylases (KDACs), and links to basic physiological processes such as metabolism, nutrition and aging. LC-MS/MS together with immunoprecipitation of acetylated lysine peptides has been utilized to discover endogenous lysine acetylated proteins. However, this current strategy based on the use of acetyl lysine antibodies has limitations such as the antibody bias to the substrate sequence as well as the contamination of non-acetylated peptides. In addition, it is difficult to identify the responsible KATs and KDACs. In this study, we developed a novel strategy to enrich lysine acetyl peptides to solve these problems. In our method, acetyl peptides are enriched depending on KDAC selectivity so that it also enables to elucidate the substrate selectivity of KDACs.

Methods

Proteins extracted from HeLa cells or E. coli cells were digested by trypsin, V8 or chymotrypsin, and were subjected to reductive dimethylation to protect primary amine groups, in vitro deacetylation using recombinant KDACs, and the derivatization of the deacetylated amine group with hydrophobic tags. Then, the peptides were enriched by reversed phase LC, and analyzed by nanoLC-MS/MS.

Results and Discussion

In order to validate the system, tryptic peptides with chemically acetylated lysine were used as model samples. As a result, in the tagged peptide enrichment step, most of the tagged peptides (~ 99%) were included in the elution fractions and few tagged peptides were in the flow-through fractions. On the other hand, most of the non-tagged peptides (~ 95%) were identified in the flow-through fractions. Next, we examined if each KDAC had different peptides selectivity. We found that distinct tagged peptides were identified among KDACs, suggesting each KDAC have different substrate selectivity respectively, and our system was suitable to access the selectivity of KDACs. Finally, we applied this system to E. coli cell extracts. We succeeded to identify endogenous lysine acetylation sites in E. coli, while further optimization was needed to increase the sensitivity.

Conclusion

We successfully developed a system to elucidate the substrate selectivity of KDACs, and to identify the in vivo acetylation sites in E. coli.

Keywords: lysine acetylation, acetylomics, KDAC, LC-MS/MS

TP03-07

Comprehensive Analysis of Proteome and Lysine Acetylome in Human Esophageal Carcinoma Cells

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Introduction and Objectives

Esophageal cancer (EC) is the sixth leading cause of cancer deaths worldwide. However the molecular mechanism is not yet known. Protein lysine acetylation plays a key role in regulating chromatin dynamics, gene expression and metabolic pathways. Recent report indicates that dysregulation of protein acetylation may be linked with the development of cancer. Herein we used the mass spectrometry based proteomics coupled with stable isotope labeling with amino acids in cell culture (SILAC) and protein acetylation enrichment approach to characterize protein acetylation in human esophageal carcinoma cell SHEEC and esophageal epithelial cell SHEE. The differential expression of acetylated proteins was further characterized and validated to investigate the correlation of protein acetylation and SHEEC occurrence.

Methods

Human esophageal carcinoma cell SHEEC and esophageal epithelial cell SHEE were grown for more than 6 generations in RPMI-1640 medium containing 13C6-lysine and 12C6-lysine, respectively. 10 milligrams of proteins were extracted and purified, respectively and were mixed after normalizing the protein amount. The resulting protein mixture was digested and followed to separate into 10 fractions by HPLC. Lysine acetylated peptides of each fraction were enriched using anti-acetyllysine pan antibodies, and enriched peptides were finally analyzed by HPLC-MS/MS, respectively. The resulting MS/MS data were searched using Mascot search engine and Maxquant software.

Results and Discussion

We combined mass spectrometry analysis, multidimensional separations and enrichment of protein lysine acetylation and phosphorylation to quantify proteins, lysine acetylation and phosphorylation in human esophageal carcinoma cell (SHEEC) and esophageal epithelial cell (SHEE). The differential expression of proteins and PTMs were further characterized and compared in the two cell lines. For example, 1543 lysine acetylation sites in 745 protein groups were identified and 979 sites in 477 proteins were quantified. 78 lysine acetylation sites in 52 proteins were quantified as up-regulated targets and 111 lysine acetylation sites in 58 proteins were quantified as down-regulated targets. Intensive bioinformatic analysis was then carried out to annotate those quantifiable targets, including protein annotation, functional classification, and functional enrichment-based cluster analysis.

Conclusion

This work presents a significant expansion of our current understanding of acetylome in esophageal squamous cell carcinoma and will be useful to understand the function of protein acetylation in the occurrence of SHEEC cell line.

Keywords: Proteomics; lysine acetylation; mass spectrometry; cancer; biomarker

Effects of co-/post-translational modifications on protein function

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Introduction and Objectives

Co-/post-translational modifications (PTMs) are often involved in the regulation of important molecular events such as protein – protein interaction, protein – cell interaction and protein activation. Many PTM sites have been identified for the yeast 26S proteasome by proteomic techniques. In the present study, we mapped PTMs on the amino acid sequences of the proteasome according to the method used for the construction of ModProt Database for the PTMs of human proteins. Simultaneously, the effects of these modifications on the function of proteasome were investigated.

Methods

To purify efficiently the yeast 26S proteasome, a fusion gene comprising DNA encoding the Rpn11 subunit, TEV protease cleavage site-specific sequence and protein A was used. This gene was expressed in the SDL66 strain to form the 26S proteasome. The produced protein complex was purified using IgG sepharose beads. To identify effectively PTM sites by MS/MS, techniques for the enrichment of PTM peptides were used. PTM sites were determined by LTQ-Orbitrap MS and 5600 Triple TOF MS, and PTMs were mapped on the sequences of the proteasome. The function of PTM was analyzed by the comparison of the function between the normal strains and mutants with the non-modified proteasome, which were produced by techniques such as the acetyltransferase-deficient mutants constructed by homologous recombination, the PTM-deficient mutants derived by site-directed mutagenesis or enzymatic removal of PTM groups. Phosphorylation state of the subunits were analyzed by Phos-tag electrophoresis.

Results and Discussion

We identified phosphorylation, N-terminal Met processing, N-acetylation, N-myristoylation and N-methylation, and other groups ubiquitination, SUMOylation, C-terminal truncation, glutathionylation and succinylation in the yeast 26S proteasome. A total of 345 modification sites have been identified, and mapped on the sequence of proteasome subunits. By the comparison of functions between the normal strain and mutants without either phosphorylated, N-acetylated or N-myristoylated proteasome, we determined the roles of these PTMs in the 26S proteasome. We found that even one PTM site for N-acetylation, N-myristoylation, or N-methylation plays an important role in the functions of the proteasome. Although there are many phosphorylation

functional protein species with different functional phosphorylation patterns, because Phos-tag electrophoresis of the 26S proteasome subunits suggested that phosphorylation patterns were a few, suggesting that a small number of phosphorylated protein species play an important role in the proteasome.

Conclusion

A number of PTM sites have been identified and mapped on the sequences. The examples showed that even PTMs on a small number of amino acids of the subunits could affect the functions of the protein complex.

Keywords:

Co- and post-translational modifications, 26S Proteasome, Yeast, ModProt database

TP04-01

Quantification of ADP-ribosylated peptides during oxidative stress using a label-free PRM approach

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Introduction and Objectives

Protein ADP-ribosylation is a reversible post-translational modification in which the ADP-ribose (ADPr) moiety of NAD+ is transferred onto specific amino acids of acceptor proteins. Under steady-state conditions, ADPr levels are generally low and hardly detectable. However, an increase in ADPr level occurs in response to stress conditions such as oxidative stress and ionizing radiation. We hypothesize that protein ADP-ribosylation undergoes quantitative changes with an increasing degree of oxidative stress and that specific ADP-ribosylated peptides can be used as quantitative sensors of oxidative stress. Thus, the aims of the presented work are to:· Identify the cellular ADP-ribosylome upon different oxidative stress conditions (mild to severe) and to identify candidate proteins that can be used for quantitative mass spectrometric (MS) analysis. · Develop a robust assay for the quantification of defined ADP-ribosylated peptides using a targeted MS approach.

Methods

Label-free shotgun quantification. parallel reaction monitoring (PRM), immunofluerecense

Results and Discussion

By applying an enrichment strategy based on the ADP-ribose binding domain AF1521 before MS analysis, we have identified 146 ADP-ribosylated candidate peptides from HeLa cells treated with 1 mM H2O2 that can be used for the targeted analysis approach using shotgun label-free quantification. We have validated the candidate peptides and shown that the developed quantification method with prior enrichment is highly reproducible. Additionally, we have analyzed the oxidative stress-induced ADP-ribosylome of HeLa cells with the developed parallel reaction monitoring (PRM) method and succeeded to quantify ADP-ribosylated peptides even under very low oxidative stress conditions (under which the cellular ADP-ribosylation signal is undetectable by immunofluorescence analysis). Furthermore, application of the PRM method to a set of ovarian cancer cell lines has revealed quantitative differences in the level of ADP-ribosylated proteins under basal and H2O2-treated conditions.

Conclusion

The presented results provide evidence that ADP-ribosylated proteins can indeed be detected and quantified under various oxidative stress conditions.

Keywords: Oxidative stress, ADP-ribosylation, AF1521 enrichment, label free quantification, PRM.

TP04-02

Characterization of ribosomal protein modifications in response to glucose starvation

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Introduction and Objectives

Ribosomes are the machinery responsible for translating mRNA into polypeptides in all organisms. Previous studies showed that ribosomal proteins are regulated by complex mechanisms and are differentially modified under various conditions. These posttranslational modifications (PTMs) are associated with viral infection, cell differentiation, cell proliferation and other key processes, suggesting that ribosomal protein modifications play an important role on regulation of cellular function. For example, glucose starvation, as an environment stress, has been shown to change PTM profile of ribosomal proteins like rpS6.

Methods

After breaking the cell membrane of HeLa cells by NP-40, ribosomes were collected using ultracentrifugation in the presence of a sucrose cushion, which were further resolved using SDS-polyacrylamide gel electrophoreses. Protein bands in the gel were subjected to in-gel digestion and subsequent liquid chromatographic-tandem mass spectrometric analyses. In order to comprehensively characterize all of the ribosomal protein modifications, we combined MaxQuant, Sequest and segmental average MS analyses to mine the liquid chromatography-tandem mass spectrometric data acquired from ribosomes under different culture conditions.

Results and Discussion

We have identified a list of ribosomal proteins, including 46 out of 50 proteins of large subunit and all the 34 known members of small subunit. Meanwhile, multiple types of PTMs, including methylation, acetylation and phosphorylation, were indeed found on these proteins. Since most of these sites are brand new, these findings showcase the prowess of our approach in proteomic PTM studies. Several PTMs were indeed differentially expressed in glucose-starved cells.

Conclusion

Ribosome complexes are indeed differentially modified in starved cells. Our approach can be applied to comprehensive characterization of PTM in a proteome with a small number of constituents.

Keywords: Ribosome, posttranslational modifications (PTMs), glucose starvation

TP04-03

Identification of Glycosylphosphatidylinositol-anchored Proteins Profile in Organs

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Introduction and Objectives

Proteins can be localized to the cell membrane by post-translational modification with lipid moieties that are anchored in plasma membrane. Among these modifications, the glycosylphosphatidylinositol (GPI) anchor is the most complex structure and is highly conserved among eukaryotic species. GPI-anchored proteins (GPI-APs) are localized to the outer leaflet of plasma membrane microdomains, commonly referred to as lipid rafts. More than 150 GPI-APs have been identified in mammalian cells, and it is expected that additional GPI-APs will be discovered in the future. Collectively, GPI-APs have diverse functions, acting as receptors, surface antigens, adhesion molecules, and enzymes. In this study, we developed an efficient method for identifying GPI-APs and analyzing the C-terminal GPI-anchored peptide sequence by combining phosphatidylinositol-specific phospholipase C (PI-PLC) treatment, hydrogen fluoride (HF) treatment, TiO2-based affinity purification, and MS/MS analysis. Using this approach, we identified GPI-APs profiles in many organs.

Methods

Samples for this study were obtained from major organs such as kidney and liver. The GPI-AP–enriched fraction, termed detergent-resistant-membranes (DRMs), was purified by sucrose gradient fractionation. GPI-APs were enriched from DRMs by Triton X-114 phase separation followed by PI-PLC treatment. Digested GPI-anchored peptides were enriched by TiO2-based affinity purification; GPI-anchored peptides could be enriched using TiO2 due to the presence of a terminal phosphate on the GPI-anchor moiety after PI-PLC treatment. GPI-anchor moieties were removed from GPI-anchored peptides by HF treatment, which decreases the molecular weight of the GPI-anchor moiety by cleaving the phosphodiester bond. Consequently, HF-treated GPI-anchored peptides contain only the EtN moiety of the GPI anchor (+43.0422 Da modification). HF-treated GPI-anchored peptide sequences were determined by MS/MS analysis and database search.

Results and Discussion

Using our method, we identified multiple organ-specific, universally expressed, and novel GPI-APs. The organ-specific GPI-APs could provide insight into the functional properties of the respective organs. Our results suggesting that this method will be very useful for identifying the GPI-AP profile in many organs and tissues.

Conclusion

In this study, we developed a method for identifying GPI-APs by mass spectrometry. Our method may facilitate development of a comprehensive understanding of GPI-APs and the roles of GPI anchoring. Moreover, our method could be used to discover GPI-APs that could be used as biomarkers.

TP04-03

Keywords: GPI-anchor

TP04-04

A HPLC coupled mass spectrometry approach to analyze modified ribonucleosides from RNA

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Introduction and Objectives

RNA post-transcriptional modifications (PTMs) are ubiquitous among the three domains of life. Over 140 known modifications have been identified in all RNA bases and all forms of RNA such as tRNA, mRNA and rRNA via multiple pathways and over 50 types of modification reactions. However, their biological function remains elusive except those linked to a handful of PTMs such as N6-methyladenosine being involved in molecular recognition and stabilization of RNA structure. Mass spectrometry based approaches have gained popularity in recent years as an analytical tool to study RNA PTMs transcriptome-wide. Using a Q Exactive™ Plus Mass Spectrometer coupled with a UltiMate 3000 HPLC system, this study aims to develop a sensitive and robust platform to detect/quantify ribonucleosides (RNs) using standard RNs and hydrolyzed total RNA/tRNA.

Methods

A two-step 30-minute HPLC gradient with 95% acetonitrile as mobile phase was used to resolve RNs on a Hypersil Gold aQ column kept at 36°C. Q Exactive™ Plus was operated in positive ion mode with a scan range of 50-500 m/z, 70,000 resolution at full MS and MS/MS scan modes, 30% NCE and an inclusion list. Raw data were imported into Skyline 3.5.0.9319 along with a transition list for interpretation and quantification. RNs standard and E. coli tRNA were commercially available in the highest purity. Total RNA was extracted from HEK293 cells using GenElute™ according to manufacturer's protocol. RNA was hydrolyzed into mononucleosides using phosphodiesterase I, alkaline phosphatase and benzonase at 37°C for 3 hours.

Results and Discussion

Twelve modified RNs and three canonical RNs (G, U and C) were observed with reproducible retention time. The detection level was 0.02ng for Cm; 0.1ng for m3C and m5C; 0.25ng for m1A, Y and I; 0.5ng for Am, Gm, m6A and m5U; 1.75ng for Um; and 25ng for m7G. Out of these 12 modified RNs, Cm, m3C, m5C, I and Y were observed in total RNA from HEK293 cells; whereas all except Am and m7G were identified in E. coli tRNA. The three canonical RNs were found in both RNA hydrolysates with at least three order greater in quantity. Using calibration curves obtained from standard RNs, Cm, m3C, m5C and I were detected at the picogram level out of 60ng total RNA and 25ng tRNA hydrolysates. Positional isomers with same product ion mass and similar elution time were indistinguishable.

Conclusion

Using two degrees of separation (retention time of [MH+] and m/z of MS/MS product ions), natural and modified RNs were resolved and quantified at the sub-nanogram level.

TP04-04

Using this platform modified RNs were detected in nanograms of eukaryotic total RNA and bacterial tRNA hydrolysates. Further chromatographic refinements would likely improve the resolution of RNs and hence the capability of detection and quantification. Epitranscriptomics is an emerging field in its infancy and has the potential to shed light on the control of gene expression, diagnostic biomarkers and therapeutic invention.

Keywords: RNA modification; mass spectrometry; epitranscriptomics

TP04-05

Rapid identification and quantification of amino acid isomers occurring in peptides under physiological conditions: a targeted proteomics approach.

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Introduction and Objectives

Evidence show that there are optical and structural amino acid isomers in life-constituting proteins. However, state-of-the-art technique for quantitating amino acid isomers has been unsatisfactory, achieving either: (i) an isomer content in a total amino acid profile, which contains high level of false positive due to spontaneous isomerization of free amino acids in solution; or (ii) site-specific differentiation of peptide stereoisomers by using a stereo-selective protease, which requires a long and labor-intensive experimental work. To investigate further into the occurrence of isomers and its potential implication in diseases, development of an efficient workflow for site-specific quantification of isomer abundance is mandatory. Here we developed a targeted-proteomics based approach to rapidly screening for peptide isomers derived from tryptic digest of protein of interest. Peptide isomers containing Dα-Asp, Dβ-Asp and Lβ-Asp—in place of normal Lα-Asp—were chromatographically separated and detected by MRM mass spectrometry with transitions covering more than four fragment ions for identification of amino acid sequences. To demonstrate the scope of the present method, we analyzed tryptic peptides of αA-crystallin, which has been demonstrated to contain Asp isomers at high abundance, as well as synthetic peptides incorporated with Asp isomers.

Methods

Four peptides of amino acid sequence TVLDSGISEVR, corresponding to 55-65th residues of αA-crystallin, were synthesized with Dα-Asp, Dβ-Asp, Lα-Asp and Lβ-Asp. Chromatographic separation was achieved using L-column Metal Free (3 μm, 2.1 x 150 mm) by 30-minute gradient of acetonitrile increased from 5% to 20% at 0.5% per minute. Mass spectrometry was performed with Shimadzu LCMS-8060, wherein the MRM transitions for monitoring the peptides were built and optimized using the Skyline software.

Results and Discussion

By the targeted MS/MS analysis, we demonstrated that synthetic peptides of Asp isomers could be baseline separated and correctly identified, by co-detection of all related fragment ions, from as low as 1 fmol peptide injection. Moreover, we showed that this method also provided highly reproducible fragment ion intensity ratio that

reflected the structural conformation of the isomer. For example, in the case of peptide TVLDSGISEVR, intensity ratio of y8 and y7 ions was the most prominent feature for differentiating between α- and β-Asp containing peptides; although further investigation is needed to generalize such information for identification of isomers without the aid of synthetic peptide standard.

Conclusion

The targeted MS/MS analysis was demonstrated to be useful for screening for isomeric amino acids occurring in peptides. The data provided was easy to interpret and, due to wide quantification range of the triple quadrupole mass spectrometer, showed potential to detect low-abundance isomers of less than 0.1% at high accuracy.

Keywords: D/L amino acid, targeted proteomics, triple-quadrupole mass spectrometer

Global identification of free protein N-termini and protease substrates by chemical modification of N-termini

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Introduction and Objectives

Understanding where a protein begins, i.e. assigning its amino terminus, constitutes essential and highly informative biological data. It indicates the extent of the protein coding region of a gene, provides information on the half-lives of proteins, and helps to elucidate their functional states. Such information can also be used to identify the protein targets of proteases, which generate novel N-termini, termed neo-N-termini, upon digestion of their protein substrates. In principle, neo-N-termini can be used to identify protein substrates for a given protease. However, the isolation of such peptides (N-termini and neo-N-termini) from a complex mixture remains challenging, despite the development of several useful biochemical strategies. This study focuses on the use of a specific chemical modification to convert the alpha-amino groups of N-termini and neo-N-termini to carbonyl groups, thereby allowing the isolation and identification N-terminal regions by mass spectrometry (MS).

Methods

Three model proteins were used initially to develop a chemical approach that specifically modifies protein N-termini to carbonyl groups (transamination). Various carbonyl-specific biotin tags were attached to the modified proteins, which were then affinity isolated with streptavidin agarose, and digested with specific proteases (trypsin or Glu-C). The resulting peptides were analysed by nanoLC-MS/MS (Orbitrap Elite). Immunoblotting and SDS-PAGE were used to assess the efficiency of derivatization and affinity isolation, respectively. Following refinement, this workflow was applied to whole-cell protein extracts from human Jurkat T-lymphocytes to identify proteins with free N-termini.

Results and Discussion

Immunoblot and SDS-PAGE analyses confirmed the successful derivatization of model proteins after transamination and biotin tagging, as well as the isolation of the derivatized proteins through streptavidin-biotin interaction. Several carbonyl-specific tags were tested to optimise labelling. LC-MS/MS detected the biotin tags at the N-termini of the proteins after tryptic or Glu-C digestion. Scale-up to Jurkat T-cell proteins demonstrated biotin tagging of a range of proteins after transamination.

Conclusion

The work describes the use of a chemical modification approach to identify protein N-termini and neo-N-termini (after proteolytic processing). It may complement the existing “positive” and “negative” N-terminal proteomic approaches to define protein N-terminal modifications and to identify protease substrates.

Keywords: poteomics, N-termini, protease substrates, transamination, mass spectrometry

TO08-01

Copy number alteration programmed protein turnover quantified by pSILAC and SWATH mass spectrometry

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Introduction and Objectives

Gene copy number alterations (CNAs) are frequent genomic alterations and causal for many diseases. Previous studies suggested that gene copy number changes only weakly impact on protein expression. Herein we show by proteome-wide turnover analysis that CNV's substantially remodel the proteome. In the presentation we will discuss two specific instances of CNV's and their associated disease phenotypes: trisomy 21 (T21) in human Down Syndrome, caused by a third, extra copy of chromosome 21 (Chr21), and Williams-Beuren Syndrome (WBS) caused by 7q11.23 hemideletion.

Methods

We utilized SWATH mass spectrometry (SWATH-MS) to accurately quantify protein degradation through a pulse SILAC experiment (pSILAC) up to 24 hours. For T21, we used fetal skin fibroblasts derived from a pair of monozygotic twins discordant for T21. To validate the results, we also analyzed fibroblasts from 11 unrelated T21 individuals bearing Down Syndrome and 11 controls. For WBS, patient-derived induced pluripotent stem cells were analyzed. Data were processed by OpenSWATH workflow. The absolute copies per cell of proteins were estimated by absolute label-free quantification (aLFQ) strategy with spike-in peptides. The combination of protein degradation and absolute quantities further provided a proxy of protein synthesis rate in steady states.

Results and Discussion

In the case of Down Syndrome, we investigated the effect of T21 at the levels of transcript quantity, proteome quantity and protein turnover rate. We quantified 4056 unique proteins for expression and ~2200 proteins by pSILAC experiment for turnover analysis in both normal and T21 twins. The T21/normal fold-change correlation between transcript and protein levels was extremely low, indicating substantial post-transcriptional regulation and buffering effects in T21. Overall, the protein degradation was faster in trisomy cells than the controls. Remarkably, those Chr21 encoded proteins that are members of heteromeric protein complexes were largely exempt from responding to CNAs, primarily through accelerated protein degradation. Moreover, we found that both mitochondrial and cytosolic ribosomal proteomes were degraded heavily in T21, but different degree of translational regulation shaped their final, divergent expression levels. In the case of WBS, briefly, we found that proteins with significant differences in turnover at the

TO08-01

pluripotent state are components of pathways involved in translation, ubiquitination, splicing and basal metabolism.

Conclusion

Our data suggests that protein specific degradation presents a primary mechanism of proteome remodeling in response to CNAs and thus reinforces the understanding of "gene dosage imbalance" in genetic disorders.

Keywords: SWATH, Protein degradation, pSILAC, Copy number alteration, Down Syndrome, Williams-Beuren Syndrome, Gene dosage effect.

TO08-02

High Resolution Mass Spectrometry Cellular Thermal Shift Assay (HR-MS-CETSA) - post-translational modifications impact on protein stability

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Introduction and Objectives

Observation of the proteins thermal stability towards increasing temperature forms the foundation for methods that explore thermally induced protein unfolding. Heat treatment induced protein unfolding and aggregation can be graphically presented as a sigmoidal melting curve which allows estimation of melting temperatures (Tm). It was shown that ligand engagement changes the functional state of the protein and induces thermal shift (Tm). This is the principle of thermal shift assays (TSA) and recent extension of this method - Cellular Thermal Shift Assay (CETSA). CETSA principle takes into account the fact that biophysical thermal stability (thermal induced unfolding) of individual proteins can be monitored and quantified in lysates/intact cells/tissue samples. MS based CETSA tackles thermal shifts in whole proteome using quantitative mass spectrometry. To further extend CETSA application in drug target deconvolution, here we proposed high resolution (HR) MS-CETSA to increase a target specificity. Accordingly, we investigated further impact of post-translational modifications on the protein stability.

Methods

Human K562 cells were treated with phosphorylation inducing agent (perVanadate). Subsequently samples are subjected to temperature induced denaturation which triggers proteins aggregation. Soluble protein from each temperature point is recovered and processed for mass spectrometry. Samples were digested with trypsin followed by differential labelling with TMT 10plex and TiO2 enrichment. Peptides were separated using Easy LC 1000 coupled to Orbitrap Fusion Instrument (Thermo) followed by identification and quantitation with PD 2.1 (Thermo). Melting temperature is estimated after fitting the melting curve to modified peptide pools which reflects modified proteins population. Thermal shift is calculated from the difference in the protein melting properties with and without compound induced modifications, using in house developed R base analysis pipeline.

Results and Discussion

Following the data analysis using our established strategy we were able to detect from the sample with limited input (< 2mg) more than 5000 distinct phosphorylation residues in the single experiment. It corresponds to more than 5000 unique melting profiles.We have confirmed our hypothesis that phosphorylated proteins exhibit different stability towards increasing temperature. Overall the data points towards destabilizing global effect of the

phosphorylation, although detailed mechanism has to be further determined. Along the way we have developed new approach to handle High Resolution MS-CETSA data. High complexity of the melting profiles prompted us to generate reference system which enabled us to look into modification induced changes in protein stability.

Conclusion

The Cellular PTM resolved MS-CETSA has potential to add a very valuable dimension to current pre-clinical and clinical drug development defining drug target engagement and off target effects in relevant cell and tissue systems

Keywords: Thermal Shift Assay, MS-CETSA, PTM, quantitative mass spectrometry

TO08-03

A dynamic picture of the proteome and ubiquitinome upon proteasome inactivation.

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Introduction and Objectives

The 26S proteasome is a large protein complex, which degrades unneeded and damaged proteins that are tagged with the small protein ubiquitin. As such, it controls key regulator abundance levels and is critical in regulating proteostasis. Malfunctioning of the ubiquitin–proteasome system (UPS) has been implicated in diseases such as cancer and neurodegenerative disorders. Here, we dissect the molecular mechanisms of the proteasome by monitoring the dynamic global proteome and ubiquitinome (all ubiquitinated proteins). A central question is why the proteasome needs three different proteins that can clip off ubiquitin chains (DUBs) and how they function. In addition, we develop innovative tools to significantly improve the coverage of the ubiquitinome.

Methods

The proteome and ubiquitinome are analyzed upon proteasome inactivation by selective RNAi knockdown of subunits using a SILAC and diGly peptide enrichment approach in mammalian and Drosophila cells. Both the diGly peptide enrichment protocol and the decision tree on the Orbitrap Fusion are modified to achieve a deeper coverage of the ubiquitinome.

Results and Discussion

The proteome is hugely affected when the proteasome is dysfunctional. After inactivation by chemical inhibitors, the abundances of several 100s of proteins are upregulated, including proteins involved in stress response, cell cycle regulation, apoptosis and the UPS itself. Similar effects are observed after inactivation of the proteasome by selective RNAi knockdown of various selected subunits. In addition, the ubiquitinome is dramatically remodeled upon proteasome inactivation. Although the far majority of proteins become increasingly ubiquitinated, several proteins are surprisingly identified with simultaneous increased and decreased ubiquitination on different lysine residues. Proteomic analysis of cells in which one of the three proteasome associated DUBs is depleted reveals major differences and suggests different functions and/or specificities for these enzymes. Finally, using a powerful combination of extensive peptide fractionation, a more economical use of diGly antibody beads and a highly efficient Orbitrap decision tree in which least intense peptides are fragmented first, we can now identify >23,000 diGly peptides (with MaxQuant score >40) in a single sample routinely.

Conclusion

Analysis of the dynamic proteome and deep ubiquitinome after perturbation of proteasome function by depletion of specific subunits gives detailed insight into the regulatory mechanisms of this cellular machinery. The mass spectrometric detection of diGly peptides is optimized to achieve a deeper coverage of the ubiquitinome.

TO08-03

Keywords: Proteasome, proteome dynamics, ubiquitin, ubiquitinome, SILAC, PTMs, diGly peptide enrichment

TO08-04

Proteome Turnover analysis reveals substrates and physiological role of membrane proteases

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Introduction and Objectives

Due to technical challenges, substrates and physiological role of membrane proteases remain largely elusive. We reasoned that a recently developed proteome turnover approach, employing metabolic labeling for the dissection of protein synthesis and degradation, could adequately address this challenge. Two prokaryotic/microbial membrane proteases, LonB from the archaeon Haloferax volcanii and FtsH from the bacterium Corynebacterium glutamicum were subjected to proteome turnover analysis in strains to discover substrates based on changing proteolysis kinetics.

Methods

For H. volcanii, a conditional lonB mutant producing suboptimal or physiological protease levels were used. The mutant strain was first cultivated in Hv-Min medium with $^{14}NH4Cl$ in absence of trp to mid-exponential phase and then shifted to fresh medium containing ^{15}N. Cultures were supplemented with 1 mM trp to induce LonB synthesis. For C. glutamicum, the WT and ftsH deletion mutant were grown on MME-SN medium containing $^{14}NH4Cl$ as nitrogen source till the end of the exponential growth phase and then switched to ^{15}N to monitor dynamic label incorporation. Different time points were compared via an internal standard grown on ^{13}C-glucose. An LTQ-Orbitrap Elite was used in DDA mode. Proteins were identified with Sequest embedded in Proteome Discoverer 1.4 and protein turnover, as well as statistical analysis, was achieved with QuPE (Albaum et al., MCP 2012).

Results and Discussion

Of the 1100 quantified proteins in the H. volcanii membrane fraction, 204 proteins displayed significantly changed degradation rates. Most noteworthy was phytoene synthase (PS), a key enzyme in carotenoid biosynthesis which showed a degradation pattern consistent with a LonB target. This makes sense considering that Lon deficiency causes hyperpigmentation. Interestingly, the concomitant increase of PS synthesis suggests a feedback regulation mechanism. Furthermore, several ABC transporters (among others) could be LonB substrates.Deletion of the ftsH gene did not result in a C. glutamicum growth phenotype in agreement with previous works (Luedke et al. BMC Microbiology 2007). Of the 1200 quantified proteins in the membrane fraction, 211 differed in degradation. A striking decrease of degradation in the ftsH deletion strain was observed for the succinate dehydrogenase subunit A., though other proteins of energy metabolism were affected too.

Conclusion

This study demonstrates the great potential of proteome turnover analysis for a sensitive and comprehensive analysis of membrane proteases and substrate discovery. Of note, the two opposing strategies, i.e. increasing the protease amount in H. volcanii and

decreasing the protease amount in C. glutamicum, were found equally suitable and successful to uncover substrates. We anticipate that proteome turnover analysis will substantially facilitate the discovery of substrate candidates not only of AAA-type, but also other substrate-degrading (membrane) proteases.

Keywords: AAA Protease, membrane proteolysis, proteome turnover

TO08-05

A Novel Function of CRL2 Ubiquitin Ligase in Protein Quality Control

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Introduction and Objectives

The integrity of proteome is essential in all aspects of cellular processes. To maintain a functional proteome, cells take advantage of protein quality control (PQC) systems to either re-fold or remove abnormal proteins by proteolysis. Malfunction of PQC systems has been linked to human diseases such as Alzheimer's disease, but how PQC systems recognize abnormal proteins for degradation is largely unknown. The ubiquitin-proteasome system (UPS) is the major pathway for cellular protein degradation and the substrate-specificity of this system is largely controlled by E3 ubiquitin ligases. CRL2 is a modular ubiquitin ligase that utilizes an interchangeable set of BC-box proteins as substrate receptors, forming ~40 unique CRL2 complexes with different substrate specificities. We are interested in finding substrates of CRL2 ubiquitin ligases, whose function is largely unexamined.

Methods

We applied global protein stability (GPS) profiling to look for CRL2 substrates. GPS profiling is a fluorescent- and FACS-based system whose reporter cassette enables translation of two fluorescent proteins from a single transcript via an internal ribosome entry site. The first fluorescent protein RFP serves as a non-degradable internal control, while the second fluorescent protein GFP is fused to the N-terminus of the protein of interest. The GFP/RFP ratio is a read-out for protein stability. Multiplexed GPS profiling allows 15,438 protein stability measurements at once. Coupling with genetic ablation of ubiquitin ligase function, GPS profiling can serve as a generic platform for the identification of ubiquitin ligase substrates.

Results and Discussion

We identified 102 putative CRL2 substrates and found 75% of substrates to be abnormal resulting from frameshift mutations or premature termination, including selenoproteins. The synthesis of selenoprotein has known to be failure prone, but how PQC system eliminates truncated selenoproteins is unclear. We found that CRL2 selectively removed truncated selenoproteins resulting from failed UGA redefinition but spared the full-length ones. CRL2 used distinct substrate receptors to recognize the C-terminal tails of various truncated selenoproteins. Beyond targeting truncated selenoproteins, CRL2 eliminated other aberrant substrates using a similar recognition mechanism.

Conclusion

Our findings suggest a general function of CRL2 in proteolysis-mediated PQC and push forward current understanding of PQC substrate recognition mechanisms.

Keywords: GPS Profiling, protein quality control, CRL2, selenoproteins

TP05-01

Identification of HTRA1 Substrates in the Context of Age-Related Macular Degeneration

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Introduction and Objectives

Age-related macular degeneration (AMD) is the major cause of irreversible blindness in elderly population. The pathogenesis of AMD is associated with the retina pigment epithelial (RPE), a monolayer of cells between light-sensitive photoreceptor and blood vessels. Several studies including genome-wide association studies and in vivo transgenic mice model have shown that elevated expression of HTRA1, a secreted multi-functional serine protease, might induce AMD. However, the precise role of HTRA1 in AMD remains elusive.

Methods

Results and Discussion

Here we investigated how HTRA1 shapes the secreted proteome (''secretome'') and proteolytic profile (''degradome'') of human RPE cells by using transwell culture system, which enables us to distinguish apical and basolateral targets. > 470 apical proteins and > 270 basolateral proteins have been robustly identified and quantified in multiple replicates by liquid chromatography – tandem mass spectrometry. HTRA1 overexpression has a strong impact on the RPE secretome, including a pro-angiogenic and pro-proliferative signature.Moreover, we characterized the biochemical specificity of HTRA1 by using proteome-derived peptide libraries. Our results corroborate that HTRA1 has a preference for aliphatic residues (such as Leu, Ile and Val) in the P1 site and for aromatic/aliphatic residues in the P2' position. To elucidate HTRA1-dependent cleavage events, we performed multiplexed Terminal Amine Isotopic Labeling of Substrates (TAILS). A total of 3593 and 2513 non-redundant protein N-termini were identified in the RPE apical and basolateral extracellular space, respectively. In combination with the HTRA1 specificity profile, a list of putative direct substrates was assembled with several candidate substrates being implicated in angiogenesis mechanism.

Conclusion

Taken together, our data provides molecular insight into the role of HTRA1 in AMD pathology.

Keywords: Age-related macular degenerationSerine protease HTRA1SecretomeDegradome

TP05-02

Old-age proteins asymmetrically inherited in mother cells of budding yeast

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Introduction and Objectives

Budding yeast cells divide asymmetrically into mother and daughter cells. After 20 to 30 times of daughter cell production, mother cells no longer divide because of their ageing, called as replicative life-span. In contrast, daughter cells emerging from mother cells are rejuvenated to replenish ageing stage. During such an asymmetrical cell division, some groups of old-age proteins originally present in mother cells are known to be also asymmetrically inherited in mother and daughter cells. So far, global view of asymmetrically inherited old-age proteins has been still unveiled. In this study, we carried out proteome-wide analysis of old-age proteins differentially inherited between mother and daughter cells, to reveal whether old-age proteins retained in mother cells can affect processes of yeast ageing.

Methods

In order to separate mother and daughter cells just after the completion of a cell division, cell cycle was synchronized in G1 phase and cell wall of mother cells was labeled with biotin. During one round of the cell cycle, newly synthesized proteins were labeled with stable isotope to discriminate old-age proteins originally localized in mother cells. After separation of mother and daughter cells using streptavidin-immobilized magnetic beads, the ratio of newly synthesized and old-age molecular species were quantified for each protein by quantitative mass spectrometric analysis and compared between mother and daughter cells.

Results and Discussion

Several dozen of proteins were successfully identified as old-age proteins asymmetrically retained in mother cells. In consistency with previous studies, some groups of old-age proteins were involved in cell wall biogenesis and localized at plasma membrane, demonstrating a proof-of-principle of our method. Transporters for proton, metal ion and chemical compounds were also asymmetrically inherited during cell division, suggesting that disturbance of cellular homeostasis due to deterioration of these types of transporters could lead to progression of cellular ageing. In addition, identification of old-age proteins related to endoplasmic reticulum (ER) organization raise an question whether quality control of proteins in ER would be associated to ageing process of budding yeast.

Conclusion

Here we present the global view for old-age proteins asymmetrically retained in mother cells. It will be examined whether this set of proteins could have causal effect on ageing processes of budding yeast, using a method by which protein age can be rejuvenated by promotion of turnover of target proteins.

Keywords: Ageing, Budding yeast, Old-age proteins, Asymmetric cell division, Asymmetric protein inheritance

TP05-03

A novel ubiquitin ligase complex regulates gastric cancer proliferation by modulating biosynthesis of guanine nucleotides

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Introduction and Objectives

Gastric cancer is one of the leading causes of cancer-related deaths worldwide. With almost one million cases diagnosed each year, gastric cancer is also one of the most common cancers particularly in East Asia. Mortality rate of gastric cancer remains higher than that of other malignancies, mainly due to the lack of noninvasive early gastric cancer diagnostics. Moreover, the pathogenic mechanism underlying gastric tumorigenesis is still ambiguous and the only curative treatment for gastric cancer remains to be surgery. Aimed at full understanding of the molecular determinants that drive gastric cancer, we sought to identify and elucidate the functions of the genes dysregulated in gastric cancer.

Methods

Microarray analysis of gastric cancer and normal tissues was carried out in order to identify the genes that are differentially expressed. A candidate gene was subjected to quantitative interactome analysis using SILAC-based immune-precipitation followed by mass spectrometry (IP-MS). Interactions found by mass spectrometry were confirmed using in vitro reconstitution and Western blotting. Further functional assessment of the candidate gene was performed with various techniques such as application of chemical inhibitors, gene depletion and over-expression, cell proliferation assay, and ubiquitylation assay.

Results and Discussion

We found an ankyrin repeat domain-containing gene (ANK), a gene with no previously known functions, to be down-regulated in gastric cancer patients. In line with this observation, depletion of ANK in HEK-293 cells promoted cellular proliferation. Quantitative IP-MS and IP-Western of ANK suggested its role in protein degradation, as prominent ANK interactors included Cul5, elongin B, elongin C, and Rbx2, all of which are the established subunits of a cullin-RING ubiquitin ligase machinery. Interestingly, ANK contains a sequence that distantly resembles SOCS box found in Cul5 substrate receptors. We found that inosine monophosphate dehydrogenase (IMPDH), another strong ANK interactor, is down-regulated by the ubiquitin-proteasome machinery in Cul5- and ANK-dependent manner. IMPDH is a key metabolic enzyme involved in guanine biosynthesis essential for cellular proliferation and cancer progression.

Conclusion

Taken together, we propose a novel model for gastric cancer proliferation and progression— dysfunction of ANK leads to the accumulation of IMPDH, a substrate normally recruited by ANK for Cul5-catalyzed ubiquitylation and proteasomal degradation. Consequently, increased IMPDH activity in the cell promotes uncontrolled cellular growth by building up available guanine nucleotides.

TP05-03

Keywords: Gastric cancer; ubiquitin-proteasome system; cullin-RING ubiquitin ligase; substrate receptor; inosine monophosphate dehydrogenase

TP05-04

Proteome-wide analysis of protein stability in *E. coli* using pulse proteolysis

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Introduction and Objectives

The native structures of most proteins represent a compromise between thermodynamic stability and the conformational flexibility required for function and regulation. Consequently, proteins are only marginally stable in their physiological environment and thus susceptible to misfolding and aggregation upon cellular stresses, such as aging and heat stress. A complex network of factors, including the machineries of protein biogenesis, protein folding and degradation, has evolved to maintain protein homeostasis. In particular, molecular chaperones have important roles in preventing aggregation, assisting folding and mediating degradation of misfolded proteins. Here, we demonstrate the detection of the in-vivo folding states of proteins on a proteome-wide scale and show that the Hsp70 chaperone machinery (DnaK-DnaJ-Grpe) affects protein folding in E. coli.

Methods

Pulse proteolysis was used to degrade unstructured proteins in E.coli under normal growth and heat stress conditions. Quantitative changes of degraded proteins were detected by metabolic SILAC labeling and mass spectrometry.

Results and Discussion

Out of more than 1700 quantified proteins in wild-type E. coli cells, 501 proteins were found to be protease-sensitive. Bioinformatic comparison with the whole detected proteome revealed that these protease-sensitive proteins tend to be more hydrophilic, charged and enriched in unstructured regions, and less aggregation-prone. Furthermore, under heat stress, 219 proteins were substantially more degraded compared to normal growth conditions, thus defining the thermosensitive proteome. About 60% of these thermosensitive proteins overlap with the set of protease-sensitive proteins, demonstrating that a large set of proteins with flexible regions becomes further destabilized under heat stress. Moreover, these thermosensitive proteins are enriched in large, abundant, and hetero-oligomeric proteins as well as proteins with complex α/β topologies. Interestingly, most of these large proteins are efficiently protected against destabilization in a strain overexpressing the Hsp70 chaperone machinery (DnaK-DnaJ-Grpe), but are increasingly structurally destabilized in a DnaK-DnaJ depletion strain.

TP05-04

Conclusion

The analytical strategy described here allows the proteome-scale detection of protein structural changes under various conditions of conformational stress. Our study shows that mainly large proteins with complex and flexible domain topologies are susceptible to heat stress, and demonstrates the pervasive role of molecular chaperones in assisting protein folding and maintaining proteome integrity.

Keywords: Pulse proteolysis; molecular chaperone; proteomics; protein folding.

TO01-01

The Regulatory Role of Methylation and Methylation–Phosphorylation Crosstalk in Protein Interaction Networks

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Introduction and Objectives

Methylation of proteins occurs predominantly on arginine and lysine residues in the eukaryotic cell. Until recently, its predominance was unknown and its role obscure. Here we describe the construction of the first 'methylproteome network' for a eukaryotic cell and present evidence that methylation modulates protein-protein interactions in this network. We also report that arginine methylation can exist in crosstalk with phosphorylation in an SRGG motif found on many proteins. Strikingly, this can modulate the interaction specificity of proteins with many interaction partners.

Methods

The yeast protein was analysed for methylated proteins and to determine precise sites. Targeted data acquisition - electron transfer dissociation LC-MS/MS was used, as were yeast proteome arrays (containing 4,400 chips spotted on to microscope slides). To build the intracellular methylation network, all known and putative methyltransferases in yeast were knocked out and the methylproteome re-analysed to determine which enzymes were responsible for which methylation events on which sites. Enzyme-substrate links were further investigated by the LC-MS/MS analysis of recombinant substrate proteins methylated by recombinant enzymes, by in vivo methylation assays and/or the incubation of proteome arrays with recombinant enzymes. Validated enzyme-substrate links were integrated with the yeast interactome to generate the 'methylproteome network'. Kinase-substrate interactions were also co-mapped onto this network, highlighting proteins that are potentially subject to modification crosstalk. A new 'conditional two-hybrid' (C2H) system was constructed to test whether modifications can modulate protein-protein interactions.

Results and Discussion

Our analyses, together, showed that protein methylation is widespread in the eukaryotic cell. We discovered three new eukaryotic protein methyltransferases, elongation factor methyltransferases 2, 3 and 7. Mammalian orthologs were also discovered for a number of these. Our integrated methyltransferase-substrate protein and protein-protein interaction network suggested that methylation might modulate protein-protein interactions. This was proven by or new 'conditional two hybrid' system, in that half of the protein pairs involving arginine methylation showed a significant increase in interaction on methylation. Where phosphorylation was found adjacent to methylation, in a conserved but repeatedly used SRGG motif, we found that this can interfere with methylation and decrease certain protein-protein interactions. In this manner, we found that modification crosstalk involving methylation and phosphorylation can regulate protein interaction specificity.

Conclusion

TO01-01

The 'methylproteome network', built here for the first time, has revealed novel means of regulating protein-protein interactions and thus biological function in the eukaryotic cell.

Keywords: protein interaction networks protein post-translational modifications protein methylation protein phosphorylation methyltransferases

TO01-02

Network analysis reveals a dominant role of protein-level regulation in coordinating gene functions

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Introduction and Objectives

Cellular functions require coordinated expression of genes involved in the same biological pathways or protein complexes. Gene expression is regulated at both the transcript and protein levels, but the relative contributions of these two levels of regulation in coordinating gene functions have been largely unknown. Here, we address this fundamental question in cancer using matched mRNA and protein profiling data from The Cancer Genome Atlas (TCGA) and the Clinical Proteomic Tumor Analysis Consortium (CPTAC).

Methods

We constructed gene co-expression networks based on mRNA and protein profiling data sets, respectively, for each of the three cancer types and then performed comprehensive comparisons between the mRNA and protein co-expression networks.

Results and Discussion

There was very limited overlap in edges between the mRNA and protein co-expression networks for all three cancer types, suggesting that mRNA and protein co-expression networks are wired very differently. Whereas protein co-expression was driven primarily by functional similarity between co-expressed genes, mRNA co-expression was driven by both co-function and chromosomal co-localization of the genes. Functionally coherent mRNA modules were more likely to have their edges preserved in corresponding protein networks than functionally incoherent mRNA modules. Proteomic data strengthened the link between gene expression and function for at least 75% of Gene Ontology (GO) biological processes and 90% of KEGG pathways. Our analyses also identified specific biological processes and pathways whose assessment would benefit most from direct protein measurements. These processes and pathways covered a wide range of biological phenomena including cell cycle, tricarboxylic acid cycle, focal adhesion, mRNA surveillance pathway, spliceosome, antigen processing and presentation, gluconeogenesis, regulation of ligase activity, lipid oxidation, mitotic DNA integrity checkpoint, mitochondrial transport, among others. To make the highly functionally relevant breast, colorectal, and ovarian cancer protein co-expression networks available and useful to the broad scientific community, we developed a web application Gene2Net

(http://cptac.gene2net.org), which allows retrieval of known functions for a gene, prediction of new functions, and identification of new genes for a biological process of interest.

Conclusion

Our network approach revealed a dominant role of protein-level regulation in coordinating gene functions. Gene function and disease studies would benefit immensely from broad adoption of global proteome profiling technologies.

Keywords: co-expression network, gene expression regulation, systems biology, proteomics, RNA-Seq, cancer, gene function prediction

TO01-03

Profiling the phosphotyrosine interactome of receptor tyrosine kinases

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Introduction and Objectives

The human proteome contains 58 Receptor Tyrosine Kinases (RTKs) which play crucial roles in cell signaling both in health and disease. Activation of RTKs by growth factors or other ligands induces receptors homo- or hetero-dimerization followed by the phosphorylation of tyrosine residues. This in turn leads to the recruitment of adaptor proteins containing SH2 or PTB domains that convey the signal into downstream pathways. To systematically understand phosphorylation mediated RTK signaling, we are profiling phosphotyrosine interactome of RTKs using affinity proteomics.

Methods

We synthesized ~900 phosphotyrosine peptides representing all intracellular tyrosine residues of the 58 RTKs. Peptides were coupled to sepharose beads and used as baits to purify interacting proteins from cell lysates in a 96-well format. Bound proteins were eluted and digested with trypsin followed by nLC-MS/MS analysis on an Orbitrap mass spectrometer. Maxquant was used for protein identification and quantification.

Results and Discussion

The resulting interactome highlighted that some RTKs preferably interact with certain proteins but there was also substantial overlap. This indicates unique and shared signaling capacity of RTKs. For instance, the discoidin domain receptor tyrosine kinase 1 and 2 (DDR1 and DDR2) shared several interactors such as Growth factor receptor-bound protein 2 (Grb2) and 1-phosphatidylinositol 4, 5-bisphosphate phosphodiesterase gamma-1 (PLCG1), illustrating their potential similarity for transmitting the extracellular signals into the cell when activated by their respective ligands. However, Lymphocyte cytosolic protein 2 (LCP2) was found as a specific binding partner of DDR2, demonstrating the potential for alternative signaling of the two receptors. In addition, the work discovered new players in the interactome of DDR1 by identifying Docking protein 4 (DOK4) and Cytokine-inducible SH2-containing protein (CISH) that are recruited to Y513 and Y796, respectively.

Conclusion

The systematic analysis of the proximal interactome of the RTK family aids in the understanding of redundant and specific signaling pathways mediated by receptor activation.

Keywords: receptor tyrosine kinase; protein-protein interaction; cell signaling; SH2 domain; PTB domain; post-translational modification

TO01-04

Phylointeractomics reconstructs functional evolution of protein binding

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Introduction and Objectives

Molecular phylogenomics investigates evolutionary relationships based on genomic data and has been substantially advanced in recent years by the increasing availability of high-throughput DNA sequencing. Despite genomic sequence conservation, changes in protein interactions can advance rapidly and with strong functional diversification. To help investigate functional evolution, we here combine the power of phylogenomics with that of interaction proteomics.

Methods

In 'phylointeractomics', a bait is interrogated with the proteome of evolutionary diverse organisms using a DNA pull-down assay combined with label-free quantitative proteomics. We demonstrate the concept by investigating the molecular evolution of the shelterin complex, which protects telomeres from being recognized as DNA double strand breaks, across 16 vertebrate species from zebrafish to humans covering 450 million years of divergent evolution, but still all sharing the same TTAGGG telomeric repeat motif.

Results and Discussion

Our phylointeractomics screen discovers previously unknown telomere-associated proteins and reveals how homologous proteins undergo functional evolution. For instance, TRF1, a well-characterized member of the shelterin complex, evolved as a telomere-binding protein in the common stem lineage of marsupial and placental mammals. Despite being present in the entire vertebrate lineage, TRF1 does not show TTAGGG-binding capacity in non-mammalian vertebrates or monotremata, whereas it consistently binds to telomeric repeats in all marsupials and placental mammals tested. By selected single amino acid exchanges, we identify the necessary gain-of-function mutations for this evolutionary switch and thus approximate key changes enabling TRF1 to directly bind telomeres.

Conclusion

Our findings establish that simple presence of gene homologs in different species does not necessarily equate to functional conservation and recapitulate how TRF1 became a telomere-binding protein. Beyond the specific example, our phylointeractomics screen unveils previously unknown telomere-associated proteins that are consistently identified in several vertebrate species. This cross-species comparison represents a valuable resource for novel factors with putative roles in telomere homeostasis. Overall, phylointeractomics is a versatile and scalable approach to investigate evolutionary

TO01-04

changes in protein function and interactions, e.g. protein complexes, RNA folds and DNA elements. It can provide experimental evidence for phylogenomic relationships and helps to extrapolate the results obtained in model organisms.

Keywords: Phylointeractomics, Molecular Evolution, DNA-protein interactions, Quantitative Proteomics, Telomeres

TP06-01

Systematic protein interactome analysis of glycosaminoglycans revealed YcbS as a novel bacterial virulence factor

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Introduction and Objectives

Microbial pathogens have evolved several strategies for interacting with host cell components, such as glycosaminoglycans (GAGs). Some microbial proteins involved in host–GAG binding have been described; however, a systematic study on microbial proteome–mammalian GAG interactions has not been conducted. Here, we used Escherichia coli proteome chips to probe four typical mammalian GAGs, heparin, heparan sulphate (HS), chondroitin sulphate B (CSB), and chondroitin sulphate C (CSC), and identified 185 heparin-, 62 HS-, 98 CSB-, and 101 CSC-interacting proteins. Bioinformatics analyses revealed the unique functions of heparin- and HS-specific interacting proteins in glycine, serine, and threonine metabolism. Among all the GAG-interacting proteins, three were outer membrane proteins (MbhA, YcbS, and YmgH). Invasion assays confirmed that mutant E. coli lacking ycbS could not invade the epithelial cells. Introducing plasmid carrying ycbS complemented the invading defects at ycbS lacking E. coli mutant, that can be further improved by overexpressing ycbS. Preblocking epithelial cells with YcbS reduced the percentage of E. coli invasions. Moreover, we observed that whole components of the ycb operon were crucial for invasion. The displacement assay revealed that YcbS binds to the laminin-binding site of heparin and might affect the host extracellular matrix structure by displacing heparin from laminin.

Methods

Results and Discussion

Conclusion

Keywords:

TP06-02

Metastatic hepatocellular carcinoma cells enrich translation regulatory proteins in exosomes

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Introduction and Objectives

It is known that exosomes (endosome derived vesicles) play important roles in the formation of the tumor microenvironment. Hepatocellular carcinoma (HCC) is a highly malignant cancer, whose metastasis is largely influenced by the tumor microenvironment. The possible role and the specific content of the HCC derived exosomes are however largely unknown.

Methods

We performed super-SILAC-based mass spectrometry (MS) analyses to interrogate the differential proteins in the exosome of three human HCC cell lines, MHCC97H, MHCCLM3 and Hep3B cells, with varied metastatic potential. Exosomal proteins were systematically compared with multi-omics strategies, considering both proteomics and translatomics.

Results and Discussion

With stringent data quality control (quantified unique peptides >= 2, FDR <= 0.01 at both protein and peptide level), 1907 exosomal proteins were confidently identified from the three HCC cell lines, out of which 469 and 443 exosomal proteins significantly altered in the metastatic cell lines (MHCC97H/Hep3B and MHCCLM3/Hep3B), respectively. ClueGo and IPA analyses on the differentially expressed proteins (DEPs) revealed that translation and ubiquitination biological processes and pathways were significantly more encapsulated in the exosome of metastatic cell lines. We further observed significantly negative correlation of exosomal protein to translating mRNA in terms of relative abundances comparing metastatic and non-metastatic cells. The negatively correlated genes are also translation regulation-centric.

Conclusion

In conclusion, we demonstrated that the exosomal enrichment of translation regulatory proteins is related to the metastatic ability of HCC cells.

Keywords: Exosome, Super SILAC, Proteomics, Translatomics, signaling pathway

TP06-03

Unraveling the dynamic signaling network capacity of HBx in HBV host infection

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Introduction and Objectives

Viruses are obligatory intracellular organisms. Their survival and propagation rely on the interplay with host proteins and the hijacking of host signaling machineries. A detailed knowledge of viral-host protein-protein interactions, and virus perturbed host signaling protein network nodes is informative for the understanding of key signaling mechanisms of viral infection and propagation as well as for the identification of novel cellular targets for potential antiviral therapies.

Methods

To gain a comprehensive and quantitative understanding of viral-host interaction networks we used two orthogonal quantitative mass spectrometry-based proteomic approaches: affinity purification mass spectrometry (APMS) and proximity-dependent biotinylation coupled to affinity purification and mass spectrometry (BioID).

Results and Discussion

The combination of these two complementary strategies coupled to functional genetic approaches enabled the elucidation of the viral-host protein interaction map of the hepatitis B virus (HBV), in particular of the pleiotropic HBV X protein (HBx) which plays an essential role in HBV replication. The obtained high confident HBx interaction network confirm and extend current knowledge on the pleiotropic activities of HBx, pointing towards an involvement in multiple distinct cellular signaling processes. Identified interactors show HBx involvement and potentially modulation of cellular proliferation, mRNA processing and proteasome-dependent degradation.We further dissected the dynamic protein expression changes induced by HBx signaling at the total proteome level. Among the differentially expressed proteins we observed specifically nuclear import/export proteins, mitochondrial proteins and splicing factors. These results indicate that HBx achieve its pleiotropic effects not only through direct interactions with a set of distinct proteins but also indirectly through the regulation of expression and/or turnover of proteins involved in distinct secondary signaling processes.

Conclusion

Together, probing signaling network structure of HBx-host interactions by the combination of APMS, BioID and whole proteome analysis revealed a defined subnetwork structure moonlighting the pleiotropic signaling capacities of HBx, which provides in turn new leads for pharmacological intervention.

Keywords: Interactomics, APMS, BioID, dynamic protein-protein interaction, signaling network, host-pathogen interactions

TP06-04

Tête-à-TET: Elucidating the Interactome of Tet1 and Tet3

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Introduction and Objectives

Tet enzymes are α-ketoglutarate depending dioxygenases which oxidise the DNA base 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxycytosine. In vertebrates, three different Tet proteins were identified (1-3) that are differentially expressed during development, raising the question about their functional roles.

Methods

Here, we report a novel affinity proteomics workflow using Tet loaded beads as baits.

Results and Discussion

The thus obtained large scale proteomics data reveal distinct interaction profiles for all Tet family members in mouse embryonic stem cells, neural progenitors and brain. As Tet1 and Tet3 are found to significantly differ in the interaction with chromatin remodelling complexes, we hypothesize that Tet1 maintains 5-hydroxymethylcytosine levels in open chromatin structures whereas Tet3 is active at loci undergoing chromatin remodelling. In brain, we observe strong interactions between Tet3 and α-ketoglutarate biosynthesis enzymes indicating that the metabolic Tet co-substrate α-ketoglutarate is produced directly in the nucleus.

Conclusion

Hence, our modified co-immunoprecipitation approach using Tet fishing baits reveals diverse interactomes for Tet1 and Tet3, suggesting different functional contexts for the enzymes. Moreover, we propose an α-ketoglutarate supply route, which is vital for the Tet activity.

Keywords: Tet1, Tet3, 5-hydroxymethylcytosine, α-ketoglutarate, affinity proteomics, chromatin remodelling

TP06-05

Identification of HAX1 interacting proteins by BioID system

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Introduction and Objectives

HCLS1-associated protein X-1 (HAX1) was originally identified as a 35 kDa protein that interacts with Src kinase substrate, HS-1, actin-regulatory adaptor protein. HAX1 has been known as an anti-apoptotic protein and its expression is correlated with malignancy of tumors. For example, the high expression levels of HAX1 were observed in cancerous tissue from patients with colorectal cancers. However, molecular mechanism how HAX1 is regulated remains elusive.

Methods

To identify novel regulators of HAX1, we utilized BioID system which features biotinylation of neighboring proteins. Biotinylated proteins isolated by streptavidin affinity capture were analyzed by mass-spectrometry.

Results and Discussion

We found several HAX1 interacting proteins including Cofilin-1, HSP90 already discovered as a HAX1 interacting protein, reflecting that BioID system was working properly.

Conclusion

Our result showed a list of HAX1 interacting proteins including Cofilin-1, HSP90 already discovered as a HAX1 interacting protein, reflecting that BioID system was working properly.

Keywords: HAX1, BioID system, Cofilin-1, HSP90

TP06-06

Discovery of Parasite-Host Interaction Contributing Changing of Host-Lipid Metabolism for Malaria Liver Stage Development

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Introduction and Objectives

Malaria causes from Plasmodium spp. pathology to vertebrates and they have many forms corresponding its life cycle. Liver stage is the first form of malaria after human have been bitten by mosquitoes. Plasmodium sporozoites move straightly forward to hepatocyte and start settle itself for multiplication. Protein-protein interaction between host-parasite facilitates transportation of essential nutrients across parasitovacuole membrane in order to support merozoite development in hepatocytes. UIS3-FABP interaction is the most well-known host-parasite interaction in malaria liver stage so that this study attempts interrogation of changing overall protein-protein interaction of host FABP in the presence of malaria UIS3 contributing changing host lipid metabolism.

Methods

HepG2 was transfection with pcDNA3.1 containing sequence of Plasmodium vivax UIS3 which tag with V5 epitope and human FABP tagged with V5 epitope. Coimmunoprecipitation was performed and subject to LC MS/MS orbitrap for protein identification. Prior to LC MS/MS orbitrap, protein samples were digested with trypsin, the digested peptides were labelled with dimethyl labeling to perform quantitative proteomics. The mass spectrometry results were analyzed with Thermo Proteome Discoverer 2.1.

Results and Discussion

Apparently, the most of proteins that bind to FABP and UIS3 were found that they are relevant to, in term of molecular function, protein and nucleotide binding. However, most of identified proteins are also involved in metabolic process such as glycolytic and lipid metabolism. Some of the interesting proteins were documented that it could support merozoite development. Therefore, this could be the drug target for eradicate malaria furthermore.

Conclusion

Dimethyl labeling is a technique that provides more reliable proteomic results for interrogation of host-parasite interaction. This study demonstrated some proteins that could be a clue for further study in term of drug target to eradicate malaria.

Keywords: Interactome, malaria, host-parasite interaction, Liver, Protein interaction, Parasite

TP06-07

Characterization of the EGFR Interactome in Non-Small Cell Lung Cancer Cells

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Introduction and Objectives

Non-small cell lung cancer (NSCLC) often harbor activating mutation within epidermal growth factor receptor (EGFR) and develop resistance to tyrosine kinase inhibitor (TKI). The most frequent occurrence of acquired resistance is associated with second site EGFR mutation, like T790M, which consequently causes limited TKI effectiveness. Although several pathways responsible for TKI resistance have been reported, the mechanism how EGFR drive resistance remain to be elucidated. Affinity purification coupled with MS was utilized to characterize the EGFR interactors from two TKI-sensitive/resistant NSCLC models carrying different EGFR mutation (L858R/Del19). The EGFR signaling-associated protein complexes may provide information delineating the altered downstream pathways in resistant cell and therapeutic opportunities to prevent/overcome resistance relapse.

Methods

Two groups of drug sensitive/resistance NSCLC cell lines, H3255/H1975 and PC9/CL68, were used to pull-down EGFR complex with immunoprecipitation. The affinity purified proteins were enzymatically digested and profiled by LC-MS/MS. Database search was carried out via X!Tandem on Trans-Proteomic Pipeline and label-free quantitation was processed with spectral counts by ABACUS. Nonspecific binding elimination (EGFR/IgG ratio>2) and protein-protein interaction scoring (observation frequency<10) were performed on CRAPome. The associated interaction networks were constructed and revisualized by STRING/Cytoscape. The functions of identified protein were classified on DAVID, enriched biological processes annotation on GO and mapped signaling pathway to KEGG.

Results and Discussion

NSCLC cell lines with primary-secondary EGFR mutation were used as gefitinib sensitive/resistant model. A total of 325 and 623 interaction proteins were identified from two groups, respectively. After nonspecific binding elimination and interaction scoring, 55 proteins were obtained to represent potential EGFR-binding in H3255/H1975 and 99 proteins in PC9/CL68. 14 proteins were commonly present in both groups, such as ERBB2, SHC1, CDC37, which are related to EGFR signaling, cell adhesion, miRNA regulation. Bioinformatics analysis reveals enriched pathway potentially crucial for resistant occurrence caused by T790M mutation. For example, glycolysis/gluconeogenesis related proteins had higher abundance in H1975, suggesting that central carbon metabolism is more dominant in resistant cell. In PC9/CL68, EPHA2 and catenin members which have been reported to associate with

drug-resistance/tumor-development induced by EGFR mutation, had increased abundance in CL68. The selected proteins still need further validation to confirm their role in EGFR-TKI resistant mechanism.

Conclusion

The interactome demonstrate a systematic approach to identify protein/pathway associated with EGFR-dependent TKI resistance, and the knowledge may provide treatment strategies to overcome drug resistance in lung cancer therapy.

Keywords: EGFR interactome, protein-protein interaction, drug resistance, NSCLC

TP06-08

Proteomic Analysis Reveals a Role for PKM2 in modulating DNA damage response

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Introduction and Objectives

Pyruvate kinase catalyzes the conversion of PEP and ADP to pyruvate and ATP, a final and rate-limiting reaction in glycolysis. It is noted that PKM2 is predominantly expressed in tumor cells, but the exact role of PKM2 in tumor initiation, growth and maintenance is not yet fully understood. In this study, using affinity-proteomics, we aim to unravel non-metabolic activities of PKM2 in DNA damage signaling pathway.

Methods

Results and Discussion

To investigate whether PKM2 modulates tumor growth under DNA damage stimuli, luciferase-labeled MCF7 cells were used in xenograft experiments. Compared with no significant difference of tumors generated from the NC and shPKM2-infected MCF7 cells without etoposide treatment, the average volumes of tumors from NC-infected MCF7 cells were much bigger than that of shPKM2-infected ones with etoposide.To explore the potential functions of nuclear PKM2 in response to DNA damage, the nuclear extracts of etoposide-treated and untreated MCF7 cells were fractionated by size-exclusion chromatography and detected by anti-PKM2 antibody. Higher molecular PKM2-complex was shifted upon etoposide treatment. Hence, we extrapolated formation of different nuclear PKM2 complexes in response to DNA damage. LC-MS/MS followed by immunoprecipitation using anti-PKM2 antibody identified 133 and 91 non-redundant proteins respectively in etoposide-untreated and treated MCF7 cells. Gene Ontology analysis showed that these PKM2-interacting proteins were involved in various nuclear bioprocesses and some proteins induced by etoposide were enriched in DNA damage signaling. String database highlight the core proteins among nuclear PKM2-interacting DNA-damaged-related proteins.

Conclusion

PKM2 expression acquire growth advantage in the presence of DNA damage stimuli, which can be extrapolated that PKM2 acts as a new critical modulator of DNA damage response. As a result, our finding reveals an important link between metabolite kinase to DDR.

Keywords: PKM2, DDR, affinity-proteomics

Interactome analysis reveals a critical role of DDX3-hnRNPK interaction in the hnRNPK-mediated apoptosis

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Introduction and Objectives

Heterogeneous nuclear ribonucleoprotein K (hnRNPK) is an RNA/DNA-binding protein involved in multiple molecular processes such as chromatin remodeling, RNA processing and DNA damage response through its interaction with diverse DNA, RNA and proteins. In addition, elevated expression of hnRNPK in various cancers has been reported as well as its positive association with aggressive metastasis, poor prognosis and cancer stem cell phenotype. On the other hand, hnRNPK serves as a p53 co-activator for the transcription regulation of cell cycle arrest and apoptosis. It remains largely unknown how hnRNPK plays such distinct roles in cancer progression.

Methods

LC-MS/MS GST pull-down assay Immunoprecipitation Split YFP assay Gene ontology analysis Clusters of Orthologous Groups analysis STRING network analysis Ingenuity Pathway Analysis TUNEL assay

Results and Discussion

In the present proteomics study, we demonstrated that arginine methylation of hnRNPK affects its interactome. Among the hnRNPK-interacting proteins, DDX/DHX proteins were recognized by STRING and IPA analysis. Moreover, the in vitro and in vivo interaction of hnRNPK with several DDX proteins was suppressed by hnRNPK arginine methylation. Notably, we demonstrated that hnRNPK-DDX3 interaction is necessary and sufficient for the hnRNPK-mediated regulation of apoptosis.

Conclusion

Our findings suggest that hnRNPK-DDX3 interaction is responsible for the role switch of hnRNPK in apoptotic regulation, providing a new insight for hnRNPK-mediated apoptosis as well as a putative target for therapeutic strategy.

Keywords: Interactome Apoptosis Arginine methylation HnRNPK DDX3

TP06-10

PTPLAD1 suppresses colorectal cancer metastasis through interacting with PHB

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Introduction and Objectives

Colorectal cancer is the third most common cause of cancer-related deaths worldwide with a poor prognosis due to metastasis. Identification of important proteins and signaling pathways involved in this lethal disease is important for the development of effective treatment strategy, which is urgently needed in clinic. PHB is a highly conserved and widespreadly expressed protein with pleiotropic functions in cell-cycle control, apoptosis and stabilization of mitochondrial proteins. PHB also regulates epithelial cell adhesion and migration through several signaling pathways. PTPLAD1 belongs to PTPLA superfamily and according to the former report it involves in HCV replication, but the role of PTPLAD1 in human cancer cell is unclear. In this study, we showed that the level of PTPLAD1 has an important impact on cancer epithelial-mesenchymal transition and metastasis through interacting with PHB.

Methods

To identify the molecular mechanisms involved in colorectal cancer metastasis, we used SILAC proteomics and high throughput RNC sequencing to compare gene and protein profiles between two human colorectal cancer cell lines, SW480 and SW620, which were derived from primary colorectal tumor and metastatic tumors of the same patient, respectively. With SILAC-immunoprecipitation (SILAC-IP) and co-immunoprecipitation (co-IP) assay, we identified dozens of proteins that may interact with or being regulated through PTPLAD1. We further verified the protein-protein interaction through bimolecular fluorescence complementation assay (BiFC) and immunofluorescence technique.

Results and Discussion

Our results demonstrated that overexpression of PTPLAD1 in colon cancer cell line inhibited invasion and conversely, transfection with PTPLAD1 siRNA significantly promoted metastatic behaviors. With SILAC-immunoprecipitation (SILAC-IP) and co-immunoprecipitation (co-IP) assay, we identified dozens of proteins that may interact with or being regulated through PTPLAD1. With proteomics analysis, we finally focused PHB, a highly conserved and widespreadly expressed protein with pleiotropic functions in cancer cell. In order to indentify whether PHB interacts with PTPLAD1, we used PTPLAD1 antibody immunoprecipitation assay and fortunately obtained PHB. Conversely, through PHB antibody immunoprecipitation assay could also obtain PTPLAD1. We further verified the interaction between PTPLAD1 and PHB through bimolecular fluorescence complementation assay (BiFC) and immunofluorescence technique. Taken together, we demonstrated that PTPLAD1 suppresses colorectal cancer metastasis via the interaction with PHB.

TP06-10

Conclusion

1, PTPLAD1 is a potential cancer suppressor gene with significant ability to suppress cancer cell metastasis. 2, Some phosphorylated proteins are regulated by PTPLAD1. 3, PTPLAD1 interacts with PHB in colorectal cancer cell.

Keywords: colorectal cancer / proteomics / PTPLAD1 / metastasis / protein-protein interaction / phosphorylation

TP06-11

Proteomic exploration of invasiveness-associated KPNA2 (importin α1) interactome and its upstream signaling in lung cancer

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Introduction and Objectives

KPNA2 (importin α1) is overexpressed in various human cancers and is associated with cancer invasiveness. However, the functional complexes and regulation mechanisms of KPNA2 in cancer cells are unclear. This study aims to understand the essential role and regulation of KPNA2 protein complexes in lung cancer.

Methods

We applied the SILAC-based quantitative proteomic strategy combined with immunoprecipitation (IP) to investigate the differential KPNA2 protein complexes in lung adenocarcinoma cell lines with different invasiveness potentials (CL1-5 *vs.* CL1-0). The abundance, protein-protein interaction and subcellular localization of these invasiveness-associated KPNA2 protein complexes were verified by IP, immunofluorescence staining and subcellular fractionation analyses. The transwell migration assay and MTT assay were used to determine the cell migration ability and cell viability, respectively. The effects of EGFR-related kinase inhibitors on KPNA2 expressed level and complex integrity were determined by quantitative PCR and Western blot.

Results and Discussion

We identified 64 differentially abundant KPNA2-interaction proteins between CL1-5 and CL1-0 cells. The complexes formation of KPNA2 and cytoskeleton remodeling-related proteins was associated with cancer invasiveness. The IP demonstrated that the levels of KPNA2-vimentin-pErk complexes were significantly higher in CL1-5 cells than CL1-0 cells and up-regulated in the advanced stage compared with the early-stage lung adenocarcinoma tissues. Importantly, we observed that the dysregulated EGFR pathway may enhance KPNA2-vimentin-pErk complex formation. DUSP inhibitor treatment revealed that KPNA2 prevents pErk from phosphatase. We also found that the protein levels of KPNA2 were decreased in EGFR-related tyrosine kinase inhibitor-treated cells. Specifically, rapamycin treatment

induced proteasome-mediated KPNA2 protein decay and attenuated the transcriptional activation of KPNA2 by decreasing Dp1/E2F1 level.

Conclusion

Our results demonstrate that KPNA2 plays a role in the maintenance of vimentin-pErk complex levels *in vitro* and *in vivo.* Our results also provide a new insight into the mTOR-mediated modulation of KPNA2 level and complex integrity in lung cancer cells.

Keywords: KPNA2, lung cancer, invasiveness, vimentin, interactome, mTOR

TP06-12

Unfolded protein and preeclampsia: a proteomics investigation

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Introduction and Objectives

Preeclampsia (PE) is a pregnancy-specific syndrome with hypertension and proteinuria. We have shown that unfolded protein response (UPR) is significantly activating in PE placentas, while at the same time NO-relevant inflammation is enhanced. We hypothesize that the onset of PE-associated inflammation is mechanistically-relevant to the unfolded proteome.

Methods

We previously realized the first proteomic characterization of unfolded proteome of human cell lines. In this study, we extended and optimized this technology to human tissue sample, and performed the deep coverage mass spectrometry (MS) analysis comparing the unfolded proteome of 5 normal and 5 PE placentas. The relevance of unfolded protein and PE was analyzed in the view of systems biology.

Results and Discussion

We identified 807 unfolded proteins (FDR < 1% at protein level, unique peptide counts > 1, unique peptide length > 8) across all samples. Among them, 579 proteins were quantified. We found 31 significantly up-regulated proteins and 33 significantly down-regulated proteins in PE placentas. Pathway analyses suggested that the differentially expressed unfolded proteins were focusing on the positive regulation of vasoconstriction in PE placentas, and thus have the potential to increase blood pressure. With the correlation analysis of E3 ligase and differential unfolded proteins in PE, we found potentially specific ubiquitin E3 ligase CUL7 from protein-protein interaction network analyses.

Conclusion

In conclusion, we report here the first unfolded proteome of human PE placentas that links the specific UPR directly to the increased blood pressure. In addition, dysfunction of the CUL7-specific degradation in PE is potentially relevant to the PE progression.

Keywords: Preeclampsia/inflammation/unfolded protein proteomics/mechanism

TO01-05

Determining network topology, distance restraints and activation markers from endogenous protein complexes

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Introduction and Objectives

Protein complexes are essential players and regulators of cellular processes. Studying their assembly and structure in a close to native manner is fundamental to understanding their function. Complex interactions have been studied by affinity purification mass spectrometry (AP-MS) and cross-linking mass spectrometry (XL-MS). AP-MS can identify the interactors of a tagged bait protein but requires multiple bait and control purifications to find high confidence interactors and determine protein complexes. XL-MS can instead pinpoint physical interactions, domain interactions and structural restraints between single amino-acid residues but requires large sample amounts and high purity.We wanted to combine the ease and throughput of AP-MS from mammalian cells with the capability of XL-MS to obtain topological and structural restraints. We aimed at a simple protocol that could afford reproducibility and quantification of individual cross-linked peptides.

Methods

We developed qAXL-MS, combining affinity purification from human cell lines with an efficient on-beads cross-linking reaction. We enriched cross-linked peptides and identified them on a high-resolution MS. We then employed the xTract software to measure the relative abundance of individual cross-linked sites between conditions. We can thus consistently purify native complexes from as little as tens of million cells and reliably quantify cross-linked peptides within and between protein complex subunits.

Results and Discussion

First, we applied qAXL-MS to a scaffold protein involved in the signal transduction of cell proliferation. With a single bait purification we could dissect the transduction interaction network. By integrating inter-protein cross-links across the network we determined what proteins physically interact and their interaction degree. We could also narrow the interaction site to specific protein domains and determine mutually exclusive interactors at the domain level. Next, we differentially stimulated the cells and observed the interaction network rearrange; we could then reconstruct integrative structural models of the signalling complexes at different time points after stimulation. Finally, we applied qAXL-MS to the osmotic stress response in HEK293 cells. We purified signalling kinase complexes and determined changes in the abundance of cross-links, defining conformational markers for active and inactive kinase states upon cell stimulation.

Conclusion

In summary we developed a quantitative method to define, for a given complex, interaction topologies, physical interactions, distance restraints and structural activation markers from different cellular states.

Keywords: Structural proteomics, protein-protein interactions, network topology, affinity

TO01-05

purification mass spectrometry, cross-linking mass spectrometry, quantitative cross-linking mass spectrometry

TP07-01

Optimization of crosslinked peptide analysis on an Orbitrap Fusion Lumos mass spectrometer

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Introduction and Objectives

Chemical crosslinking in combination with mass spectrometry is a powerful method to determine protein-protein interactions. In this study, we evaluated traditional and MS-cleavable crosslinkers for crosslinked peptide analysis using an Orbitrap Fusion Lumos mass spectrometer. For MS-cleavable crosslinkers, we also compared different types of fragmentation (CID, ETD) and levels of tandem mass spectrometry (MS2 vs MS3). Our data provided insight to the relative performance of different crosslinking compounds and acquisition parameters relevant for improving identification of protein-protein interaction sites.

Methods

Different amine-reactive, homobifuctional crosslinkers including disuccinimidyl suberate (DSS), disuccinimidyl sulfoxide (DSSO) and disuccinimidyl dibutyric urea (NHS-BuUrBu-NHS) were used to crosslink BSA and E. coli whole cell lysates. Crosslinked samples were reduced, alkylated and digested with trypsin for MS analysis. Protein and peptide concentrations were determined using the Pierce™ BCA Protein Assay Kit and the Pierce™ Quantitative Colorimetric Peptide Assay, respectively. BSA samples were separated using a 50cm Thermo Scientific Easy-Spray™ column and an EASY-nLC™ 1000 UPLC system in 90min gradient, followed by the detection on the Thermo Scientific™ Orbitrap™ Lumos™ mass spectrometer. E. coli samples were fractionated using SCX to enrich for crosslinked peptides before LC-MS/MS analysis. Data were analyzed using Thermo Scientific™ Proteome Discoverer™2.2 software with an XlinkX node (xlinkx.hecklab.com).

Results and Discussion

The complexity of crosslinked MS spectra and subsequent data analysis has limited the wide adoption of crosslinking MS as a routine application. Crosslinkers which contain MS-cleavable linkers for fragmentation during MS/MS enable peptide identification using traditional database searching. In addition, some MS-cleavable crosslinkers also provide fragmentation ion patterns that can be used during data analysis for identification of crosslinked precursors. We compared two MS-cleavable crosslinkers, DSSO and BuUrBu to DSS for protein crosslinking labeling efficiency and crosslinked peptide identification using MS2 and MS3. For both DSSO and BuUrBu, we identified over 30 BSA crosslinked inter peptides using MS2-MS3 approach compared to less than 20 using MS2 CID using a novel XlinkX search engine integrated into Proteome Discoverer 2.2. We also compared these crosslinkers using an E. coli whole cell lysate that was fractionated after crosslinking using strong cation exchange chromatography. Our preliminary results show an increase of ~2-fold of identified peptides after crosslinking

using the MS2-MS3 method compared to CID/EThcD MS2 method.

Conclusion

Overall, the combination of MS-cleavable crosslinking, MS3 acquisition methods and novel crosslinked peptide search software enabled the analysis of protein-protein interactions in complex samples.

Keywords: DSS, DSSO, crosslinking, protein structure, protein interactions

TP07-02

Characterization of EV71 replicative mechanisms targeted by anti-EV71 cocktail therapy

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Introduction and Objectives

Enterovirus type 71 (EV71) is a member of the enterovirus A species within the genus Enterovirus of the family Picornaviridae. Enteroviral infections, which are mainly caused by EV71 or Coxsackie virus (CV) infection, are mostly asymptomatic or usually result in mild illness, such as hand, foot and mouth disease (HFMD). EV71 has become one of the most important clinical issues in Taiwan and other Asia-Pacific countries due to high numbers of annual case reports and several outbreaks in the past. Although there are several types of EV71 vaccines currently under investigation, there are no approval of effective antiviral drugs available so far.

Methods

Five compounds, coded as A, B, C, D and E, were screened using a proprietary screening method. The various combinations of these five compounds, notably below their respective therapeutic levels, were exhaustively tested for their synergistic inhibition using a cell-based assay. The inhibitory effects on viral protein synthesis and RNA replication were examined using western blot analysis and qRT-PCR, respectively. Finally, viral replication machinery targeted by these antiviral regimens was investigated by implementation of a new method that includes gel filtration chromatography (GFC), two-dimensional differential electrophoreses and mass spectrometry.

Results and Discussion

The antiviral effects of these regimens were not limited to particular strains or types of enteroviruses. These antiviral regimens exhibited similar inhibitory effects on viral protein synthesis and RNA replication, suggesting that the actions of these anti-EV71 regimens on viral protein synthesis are most likely due to suppression of RNA replication. We finally have identified a group of proteins, which have lower expression in high-molecular mass GFC fractions from treated cells. These proteins are likely parts of viral replication complex.

Conclusion

These results suggest that protein complexes are involved in viral replication and disruption of these complexes by cocktail therapy may suppress the progression of viral replication. This new therapeutic strategy has the potential to suppress the infection caused by various enterovirus, including EV71 and CV.

Keywords: Enterovirus type 71, viral replication complex, cocktail therapy

TP07-03

Examination of Aptamer-Protein Complex Structure by Mass Spectrometry

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Introduction and Objectives

The oligonucleotide aptamers can specifically recognize target molecules such as proteins, drugs, and small molecules due to their special oligonucleotide sequences with the formation of unique spatial structures. Aptamers have good chemical stability and can be easily prepared by chemical synthesis; therefore, they have high potential to be the substituent for antibody in biotechnology application, clinical diagnosis, and therapeutic usage. The systematic evolution of ligands by exponential enrichment (SELEX) method was developed for screening proper aptamers to a variety of target ligands, including small molecules, proteins and tumor cells. However, to determine the spatial interaction between aptamer and its target ligand still depends on time-consuming methods such as x-ray crystallography or NMR techniques. We hereby developed a mass spectrometry based method to investigate the aptamer-protein complex structure. The spatial correlation of aptamer-protein complex was fixed by chemical cross-linking reaction and further investigated by MS analysis. This method can identify target proteins for SELEX selected aptamer as well as to determine their interaction region on molecule.

Methods

A model aptamer-protein complex with structure information available on Protein Data Bank is utilized in this study. Cytochrome C and the corresponding aptamer are incubated at room temperature for complex formation followed by the initiation of chemical cross-linking reaction. After the removal of excess chemical cross-linking reagents, cytochrome C-aptamer complex is purified by SDS-PAGE separation followed by the typical in-gel trypsin digestion process. Peptides with cross-linked aptamer are enriched by anion exchange column followed by the DNase treatment to resect aptamer. Peptides containing partial sequence of aptamer are then analyzed by LC-MS/MS.

Results and Discussion

When the cytochrome C and the corresponding aptamer are mixed and cross liked with chemical reagent, we could observe the band of cytochrome C on SDS-PAGE become light, so we cut off the band of the molecular weight from 20kD to 37kD in order to obtain the possible protein-aptamer complexes. After in-gel digestion, samples are analyze by mass spectrometry. We find out high sequences coverage after database search, the result show that molecular weight shift on SDS-PAGE probably due to the cytochrome C with attached aptamer.

Conclusion

MS data will be analyzed by either in-house program or RNPxl. After the data analysis, the amino acids sequence of peptide-oligonucleotide can be determined and the amino acid residue which has oligonucleotide attached can be identified. The method is validated through comparing with known data, for example, thrombin binding aptamer and

thrombin. Finally, this procedure can be used to characterize target proteins for aptamers prepared by SELEX.

Keywords: aptamer; mass spectrometry; chemical cross-linking

TP07-04

Interactome analysis identified the specific interaction of TCTP and EF1A2 in Neurofibromatosis type 1 (NF1)-associated tumors

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Introduction and Objectives

Neurofibromatosis type 1 (NF1) is an autosomal dominant disease that predisposes individuals to developing benign neurofibromas and malignant peripheral nerve sheath tumors (MPNSTs). Owing to the lack of information on the molecular mechanism of NF1-associated tumor pathogenesis, biomarkers, and therapeutic targets, a radical treatment for NF1 tumors has not been established. By a unique integrated proteomics, comprising iTRAQ, 2D-DIGE, and DNA array, using MANGO/iPEACH, we identified a novel NF1-related abnormal network, “Translationally controlled tumor protein (TCTP)-mediated oncogenic signaling”. To further analyze the function of TCTP in detail in NF1-associated tumors, we identified the TCTP-interacting proteins by proteomic approach.

Methods

We constructed the plasmid expressing Flag-tagged TCTP, and transfected it into MPNST cells. Using antibodies for FLAG-tag, TCTP complexes in the cells were purified, digested with trypsin/LysC, and analyzed by nanoLC-ESI-MS/MS and SWATH, and in-silico protein-protein network analysis. The interactions of TCTP and the binding partners were further validated by the immunoprecipitation using anti-FLAG antibody followed by Western blot and mass spectrometric (LC-MS/MS, MRM) analyses.

Results and Discussion

TCTP-interacting proteins include the molecules related to protein translation machinery and stress response. Especially, TCTP most dominantly binds to the translation elongation factor complex composed of EF1A, EF1B, EF1D, EF1G and valyl-tRNA synthetase. We observed that TCTP preferentially interacts with the tissue specific and tumor-associated elongation factor, EF1A2, rather than ubiquitously expressed elongation factor, EF1A1, despite the 92% identity and 98% homology of EF1A1 and EF1A2 amino acid sequences. Our results also suggest that TCTP enhances the EF1A2 function on translation elongation via the inhibition of EF1A2 self-dimerization by the binding of TCTP to EF1A2.

Conclusion

These findings indicate that the interaction between TCTP and EF1A2 may contribute to the formation of NF1-tumor specific translational machinery, and demonstrate that TCTP is functionally implicated in the genesis and progression of NF1-associated tumors with EF1A2 via the activation of protein translation machinery.

Keywords: NF1, TCTP, Interactome analysis, EF1A2, translation elongation factors

TP07-05

Establishment of an APEX-based procedure that effectively helps analyze protein quaternary structures

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Introduction and Objectives

Protein quaternary structure defines the number and arrangement of multiple components in a protein complex assembly. Since proteins perform their biological functions at this level of protein structure, it is important to expand the repertoire of methods for analyzing such protein structures. Here, we incorporate the newly developed ascorbate peroxidase(APEX) chimera technology to establish a general approach.

Methods

To validate its utility, we designed a vector that encodes ribosomal protein S6 (rpS6)-APEX chimera, which was expressed in HEK-293T cell through DNA transfection. The transfected cells were subjected to APEX-mediated biotinylation and the cell lysates were prepared and fractionated using native gel filtration chromatography (GFC). Proteins in GFC fractions were resolved using SDS polyacrylamide gel electrophoreses, transferred onto nitrocellulose membranes and stained with streptavidin-peroxidase method. Streptavidin affinity purification was also used to isolated rpS6-APEX-containing ribosome complexes as well as those assemblies juxtaposed to rpS6-APEX chimera. Nanoflow HPLC-MS/MS with Orbitrap mass spectrometry and data interpretation with Maxquant was used for protein identification and quantification.

Results and Discussion

We confirmed that rpS6 is primarily present in high-molecular mass protein complexes, i. e. the ribosomes and 40S ribosomal subunits. We found that several highly biotinylated proteins are indeed those neighboring rpS6 in ribosomal complexes. In parallel, affinity purification successfully isolated ribosomal complexes with high purity, whose protein elements could be documented and characterized. A group of rpS6-neighboring complexes were also characterized

Conclusion

While validating the utility of this approach in protein quaternary structure analyses, our results also help understand the interplay between the ribosomes and their associated protein complexes.

Keywords: APEX, ribosomal protein S6, ribosome complexes, methodology

TP07-06

Effect of Different Lipid Membranes on the Structural Dynamics of Aquoporin Z

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Introduction and Objectives

Aquaporins are integral membrane proteins performing water transport in most living cells. They have attracted much interest in water purification. Despite the engineering success, much crucial information regarding the fabrication of an optimally functioning biomimetic membrane is lacking. A common observation is that aquaporin Z (AQPZ) biomimetic membrane shows lipid dependent functional differences. Thus, to understand the mechanistic cause of this observation, we aim to identify the effects of different lipid membranes on the structure and function of AQPZ.

Methods

We embedded AQPZ in different lipid nanodiscs. We then employed amide hydrogen/deuterium exchange mass spectrometry (HDXMS) to distinguish the causative effects of finely different membranes on the structure of AQPZ as a read out of changes in deuterium labelling of amide hydrogens. Quenching of reaction followed by online pepsin digestion and subsequent peptide separation by LC-MS identifies peptides that show lipid dependent changes.

Results and Discussion

AQPZ solubilized in DDM exhibits exceptional stability as low overall relative deuterium uptake of less than 10 %. In the presence of lipid membrane, relative deuterium uptake increases ranging from 0 – 50% giving a unique deuterium labelling profile. Due to favorable interactions between the membrane and AQPZ, there is less compaction. Interestingly, all three systems: DDM (peptides 52-70, 180-192), DOPC lipid (peptides 60-79, 178-187), E.coli lipid (peptides 52-66, 180-192) had near 0 % relative deuterium uptake along peptides containing NPA and NPAR motifs, indicating their inherent stability. Effect of different lipids is highly pronounce from differences in deuterium labelling kinetics of all peptides along the six transmembrane α helices which are in contact with lipid membrane. Peptides exhibit higher deuterium exchange in DOPC membrane as maximal labelling is achieved at the earliest time point of 0.5 m, while in E.coli lipids slow increase in deuterium exchange with time was observed. Our results indicate that functional differences in different lipids may attribute to the stability of peptide 190-208 which showed lower deuterium uptake thus higher stability in E.coli lipid across all labelling time compared to higher deuterium uptake and

maximal deuterium labelling at 0.5 m in DOPC membrane. Stability around peptide 190-208 confers higher water permeability presented by the microenvironment of E.coli lipid membrane, while destabilization of this region leads to compromised water permeability.

Conclusion

Overall, we showed that while key structural elements for water transport is unaffected by environment and channel remains water permeable in different lipids, protein-lipid interactions along peptide 190-208 affects its stability thus controls overall permeability.

Keywords: Aquaporin; structural dynamics; hydrogen/deuterium exchange mass spectrometry; nanodisk; lipid environment

TP07-07

Characterizing folding and misfolding properties of human serum amyloid A1

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Introduction and Objectives

Serum amyloid A (SAA) is an acute-phase protein that circulates mainly in plasma. In acute infection, the plasma concentration of SAA can elevate more than 1,000-fold reaching up to 1 mg/ml in 1~2 days and thus plasma levels of SAA provide a useful biomarker to assess the severity of the inflammation and the response to anti-inflammatory therapies. Persistently high levels of SAA can lead to life-threatening chronic inflammation in human named amyloid A (AA) amyloidosis which results from misfolding and deposition of the N-terminal fragments of SAA, termed AA, in organs and tissues. Although SAA isoforms (SAA1~4) are differentially expressed during inflammation, SAA1 is a major precursor of AA in human and thus was chosen to be the research target. The objective of this study focuses on the characterization of folding and misfolding properties of SAA1. This research may have a potential to provide a new insight into the biophysical properties of SAA1 and to reveal more detail about its pathogenic misfolding mechanism.

Methods

Recombinant SAA1 was firstly purified using affinity and size exclusion chromatography (SEC). Further, this study focuses on the characterization of SAA1 in terms of its secondary and tertiary structure using spectroscopic techniques such circular dichroism (CD) and fluorescence spectrometer. Oligomeric states of SAA1 were assessed by western blotting, SEC and analytical ultracentrifugation (AUC). The determination of SAA1 conformational stability at equilibrium was conducted using CD and intrinsic/extrinsic fluorescence as probes under conditions of varying pH and temperature.

Results and Discussion

The CD spectrum of SAA1 in the native state shows a characteristic of an α-helical protein while the spectrum of denatured SAA1 shows a reduction in secondary structure. When excited at 280 nm, fluorescence emission of SAA1 peaks at ~335 nm in the native state and displays a red shift to ~360 nm upon denaturation. In the conformational stability studies, denaturation curves show a two-state transition without observable sign indicating the existence of intermediates. Moreover, changes in protein concentration (2 μM versus 10 μM) does not affect equilibrium unfolding

process. More hydrophobic surface detecting dye ANS binds to native SAA1 than denatured SAA1.

Conclusion

From the equilibrium unfolding results, unfolding mechanism of SAA1 is likely to be a two-state process. Furthermore, increase in protein concentration among a certain range may not significantly influence the unfolding mechanism of SAA1. On the other hand, the native state of SAA1 has more hydrophobic surface when compared to the denatured state. Also, the hydrophobic surface of SAA1 decreases upon denaturation.

Keywords: Serum amyloid A, conformational stability, folding, misfolding

TO06-01

Status of the Affinity Binder Knockdown Initiative

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Introduction and Objectives

During the past year, there have been numerous discussions about antibody quality and validation procedures, and questions have been raised about reproducibility of scientific findings. The number of affinity binders available on the market is overwhelming, and redundancy is not uncommon; in the open-access database Antibodypedia (www.antibodypedia.com) more than 2.4 million antibodies are listed. Finding the right antibody for your research is not an easy task. Considering the amount of money spent on research antibodies, estimated to be around $2 billion in 2014, and rising to about $3 billion by 2019, there is a great incentive to profile antibodies as well validated and of high quality. A quality assurance program in proteomics research would potentially help researcher save a lot of material, time, and money. The Affinity Binder Knockdown Initiative[1] was initiated at HUPO2015 under the aegis of Antibodypedia in order to collect data for antibodies that have been target validate using genetic methods.

Methods

The Affinity Binder Knockdown Initiative relies on crowdsourcing and the engagement from the research community to upload their knockdown/knockout results from their research. The validation submission system is simple and only takes a few minutes to complete at www.antibodypedia.com/validate.php.Rewards are given for positive results submitted to the initiative for antibodies where companies have agreed on supporting the initiative. Information about the participating companies and selected antibodies is found on Antibodypedia together with the terms and conditions that apply.

Results and Discussion

Currently, data that verify the target binding of the antibody have been uploaded for 244 antibodies, and 57 of these antibodies have been validated for both western blot and immunocytochemistry. In total 301 validations have been accepted and are now publicly available on Antibodypedia.

Conclusion

Identifying good affinity reagents is key to reproducibility and advancement of science. Most research is in one way or another relying on other scientific findings and a systematic exploration of affinity reagents to verify the quality will improve research outcome in all areas of proteomics. The initiative is grateful for the submitted data, and we would like to see many more researchers contributing in building a user community where data is shared among scientist. We encourage all researchers to submit validation data to Antibodypedia in order to build a comprehensive knowledgebase of validated antibodies.

(1) Alm et al, Introducing the Affinity Binder Knockdown Initiative—A public–private partnership for validation of affinity reagents, EuPA Open Proteomics, 2016

Keywords: Antibody quality; Validation; Reproducibility; Crowdsourcing; siRNA; CRISPR

TO06-02

Heterogeneous Ribonucleoprotein K (hnRNP K) binds the 5' terminal sequence of the hepatitis C virus RNA and mature miR-122

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Introduction and Objectives

Stem-loop I (SL1) located in the 5' untranslated region of the hepatitis C virus (HCV) genome initiates binding to miR-122, a microRNA required for hepatitis HCV replication. However, proteins that bind SL1 remain elusive.

Methods

In this study, we employed a human proteome microarray, comprised of ~17,000 individually purified human proteins in full-length, and identified 313 proteins that recognize HCV SL1.

Results and Discussion

Eighty-three of the identified proteins were annotated as liver-expressing proteins, and twelve of which were known to be associated with hepatitis virus. siRNA-induced silencing of eight out of 12 candidate genes led to at least 25% decrease in HCV replication efficiency. In particular, knockdown of heterogeneous nuclear ribonucleoprotein K (hnRNP K) reduced HCV replication in a concentration-dependent manner. Ultra-violet-crosslinking assay also showed that hnRNP K, which functions in pre-mRNA processing and transport, showed the strongest binding to the HCV SL1. We observed that hnRNP K, a nuclear protein, is relocated in the cytoplasm in HCV-expressing cells. Immunoprecipitation of the hnRNP K from Huh7.5 cells stably expressing HCV replicon resulted in the co-immunoprecipitation of SL1. Since the hnRNP K binding site on HCV RNA overlaps with the sequence recognized by the liver-specific microRNA, miR-122. We also probed the proteome chip with miR-122 and also identified hnRNP K as one of the strong binding proteins. In vitro kinetic study showed hnRNP K binds miR-122 with a nanomolar dissociation constant, in which the short pyrimidine-rich residues in the central and 3' portion of the miR-122 were required for hnRNP K binding. In liver hepatocytes, miR-122 formed a coprecipitable complex with hnRNP K. High throughput Illumina DNA sequencing of the RNAs precipitated with hnRNP K was enriched for mature miR-122. SiRNA knockdown of hnRNP K in human hepatocytes reduced the levels of miR-122. These results show that hnRNP K is a cellular protein that binds and affects the accumulation of miR-122.

Conclusion

Its ability to also bind HCV RNA near the miR-122 binding site suggests a role for miR-122 recognition of HCV RNA.

Keywords: Human proteome microarrayHCVmicroRNA5'-UTR

TO06-03

A High-Content Functional Mycobacterium Tuberculosis Proteome Microarray and Its Applications

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Introduction and Objectives

Better proteomic tools with which to investigate the basic biology of MTB and its interactions with the host at the systems level are urgently needed. Proteome microarrays are powerful platforms for globally profiling thousands of molecular interactions simultaneously. We built a MTB Proteome microarray based on our high-throughput protein expression/purification technology and high-content microarray platform.

Methods

We first shuttled more than 4,700 open reading frames (ORFs) of MTB in yeast expression vectors, and then constructed the protein library using our high-throughput protein expression and purification platform. We determined the protein quality using GST immunoblotting and capillary electrophoresis. To identify PknG-interacting proteins, we purified PknG and used it to probe the MTB proteome microarray. To evaluate the reliability of the microarray results, we further test the PPIs using BLI and yeast-two hybrid analysis.

Results and Discussion

We built a functional MTB proteome microarray, composed of 4,262 proteins, covering most of the proteome and an ORFome library. For the MTB protein library, many of the MTB proteins (222/249) showed a clear band of the expected size (±10 kDa) and good purity. For the PPI application, we identified 59 candidate PknG-interacting proteins. BLI analysis were obtained for 47 of the 59 putative interactors, and 72.3% (34/47) of these showed strong interactions with PknG. We focused one binfer, RmlA and found that PknG sharply inhibits the activity of RmlA by phosphorylation.

Conclusion

We built a high-content MTB protein library and fabricated a functional MTB proteome microarray. We also demonstrated the utility and reliability by investigating the global protein interactions of PknG. As a proteomic tool, this microarray can be used in broad applications at system level: 1) It could facilitate identification of the host processes and interactions. 2) It provides a versatile platform for investigating global protein interactions with proteins, DNAs, RNAs, lipids. 3) It is a powerful platform for the direct global identification of drug-targeted proteins.

Keywords: Mycobacterium tuberculosis, Proteome Microarray, PknG, Protein-Protein Interactions

TO06-04

Utilizing protein microarray to monitor blood-brain barrier disruption and active inflammation in plasma samples from multiple sclerosis patients

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Introduction and Objectives

Extracellular vesicles (EVs) are readily available in the blood stream and hereby constitute potential biomarkers of human diseases. Studies have identified plasma EVs as useful markers in several diseases. In the present study, we have refined and used the novel protein microarray technology termed EV Array to detect and phenotype the EVs present in plasma from patients with multiple sclerosis. Multiple sclerosis is an inflammatory demyelinating disease of the central nervous system (CNS). Disruption of the blood-brain barrier (BBB) plays a major role in disease activity and damage of the BBB may be initiated by systemic or CNS inflammation and contribute to escalation of pro-inflammatory responses within the CNS. We hypothesized that damage of the BBB is reflected in the appearance of endothelium-derived EVs in the plasma, and correlate with soluble biomarkers of endothelial stress and regulators of systemic inflammation.

Methods

Five patients with untreated multiple sclerosis were followed by weekly blood sampling for 7 weeks. Plasma samples were analyzed for 41 soluble biomarkers by Mesoscale V-PLEX whereas EVs were analyzed by the EV Array (Joergensen et al., 2013, JEV). The EV Array was customized to include known general EV markers and endothelial stress induced EV markers as determined by quantitative proteomics to include 33 different antibodies against EV markers involved in inflammation and endothelial stress. For detection of EVs, a cocktail of biotinylated antibodies against the EV surface markers CD9, CD63 and CD81 was used, prior to Cy5 labelling and scanning.

Results and Discussion

The EVs were extensively phenotyped with the use of the EV Array technology. Analyzing for 33 specific EV markers in the plasma samples it was possible to generate specific EVs profile signatures for the time lines of the patients. The plasma contents of EVs was compared to soluble biomarkers indicating systemic inflammation and endothelial stress. Each patient revealed an individual phenotype of plasma EVs, which changed over time. Pearson correlations between the EV phenotypes, and soluble biomarkers were made. Hierarchical clustering of biomarkers and patients, and time-series clustering were

generated.

Conclusion

EV markers from stressed endothelial cells were identified by quantitative proteomics and afterwards found to be present on EVs in plasma from multiple sclerosis patients. By including these EV markers on a customized EV Array and relating the findings with soluble biomarkers and their individual changes over time several correlations were identified.

Keywords: EV Array, Extracellular vesicles, Protein microarray, multiple sclerosis, active inflammation

TO06-05

Mapping Transcription Factor Interactome Networks using HaloTag Protein Arrays

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Introduction and Objectives

Employing a newly developed technology, HaloTag-NAPPA arrays, we increase the capacity of in situ protein microarray technology several-fold, such that proteome-scale screening becomes feasible. Many examples of novel protein-protein interactions (PPIs) among plant signaling pathways were observed. With few exceptions, nearly all of these newly discovered connections are undocumented in the existing literature. This study has resulted in an important new resource for the plant biology community — a plant transcription factor-anchored protein-protein interaction network map. Such TF-based PPI networks may help in the identification of novel genes for use in the improvement of agronomic traits such as grain quality, disease resistance, and stress tolerance.

Methods

Results and Discussion

Conclusion

Protein microarrays enable investigation of diverse biochemical properties for thousands of proteins in a single experiment, an unparalleled capacity. Using a high-density system called HaloTag nucleic acid programmable protein array (HaloTag-NAPPA), we created high-density protein arrays comprising 12,000 Arabidopsis ORFs. We used these arrays to query protein-protein interactions for a set of 38 TFs that function in diverse plant hormone regulatory pathways. The resulting transcription factor interactome network, TF-NAPPA, contains thousands of novel interactions. Validation in a benchmarked in vitro pull-down assay revealed that a random subset of TF-NAPPA validated at the same rate of 64% as a positive reference set (PRS) of literature curated interactions. Moreover, using a bimolecular fluorescence complementation (BiFC) assay, we confirmed in planta several interactions of biological interest and detrmined the interaction localizations for seven pairs. The application of HaloTag-NAPPA technology to plant hormone signaling pathways allowed the identification of many novel transcription factor protein interactions, and led to the development of a proteome-wide plant hormone TF interactome network.

Keywords: Protein arrays, Interactome, Hormone, Systems biology, Network mapping

TP08-01

Molecular design of scFv antibodies for site-specific photochemistry-based applications in affinity proteomics

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Introduction and Objectives

The ability to perform site-specific photochemistry-based immobilization and/or functionalization of antibodies could pave the way for novel approaches in affinity proteomics. Here, we have evaluated the Dock'n'flash approach, where the unnatural amino acid pBpa (p-benzoyl-L-phenylalanine) is site-specifically incorporated into scFv antibodies and covalently photo-crosslinked to its affinity binding partner β-CD (β-cyclodextrin). This enables modified scFvs to be either immobilized in an orientated manner on a β-CD functionalized surface or site-specifically functionalized with e.g. a short β-CD-oligonucleotide-tag. The aims were to design and characterize pBba mutated scFvs and generate proof-of-concept for the approach.

Methods

Site-directed mutagenesis was used generate pBpa mutated scFv antibodies. Thirteen different mutation sites, located opposite to the antigen binding site in the framework or in the C-terminal affinity tag, were evaluated. Several different scFv were used to investigate the impact of the point mutation on their functionality. The scFvs were expressed in E. coli and the mutation was verified by sequencing, SDS-PAGE and MALDI-TOF MS. The specificity and affinity of the pBpa mutants were validated using in-house antibody microarrays. Dock'n'Flash coupling to β-CD was performed, both in solution and onto β-CD functionalized surfaces at 365 nm. The coupling was verified using MALDI-TOF MS (in solution) and antibody microarrays (on solid support).

Results and Discussion

Based on molecular performance and location of the mutation in the antibody structure, a top molecular antibody design, denoted Tag 7 (mutation located in the affinity tag), was selected. The pBpa mutated scFv Tag 7 could be produced with an adequate yield and retained function. Further, coupling to β-CD in solution was confirmed by MALDI-TOF MS. However, further optimization and/or purification will be needed as the degree of coupling was not yet complete. Preliminary experiments also confirmed immobilization of the pBpa mutated scFv to a β-CD functionalized slide surfaces. While some of the other mutants showed similar properties, the location of

the mutation (affinity tag vs. framework) was considered a key advantage, minimizing any potential impact on the overall structure of the scFv.

Conclusion

We have demonstrated proof-of-concept for a molecular design of scFv antibodies that enables site-specific photocoupling for applications in affinity proteomics. Using Dock'n'Flash it was possible to site-specifically couple pBpa mutated scFv to β-CD. A top candidate mutant was defined and selected for further studies. Ongoing and future work involves expanding this mutation to several scFv clones and to optimize the protocols for coupling both in solution and to β-CD functionalized slides.

Keywords: Antibody microarray, ScFv, Protein engineering, Dock'n'Flash, Photochemistry, Site-specific coupling, Unnatural amino acids

TP08-02

Deciphering systemic lupus erythematosus associated serum biomarkers reflecting apoptosis and disease activity

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Introduction and Objectives

Systemic lupus erythematosus (SLE) is a severe chronic inflammatory autoimmune disease. Despite major efforts, SLE remains a poorly understood disease with unpredictable course, unknown etiology, and complex pathogenesis. Apoptosis combined with deficiency in clearing apoptotic cells is an important etiopathogenic event in SLE, which could contribute to the increased load of potential autoantigen(s). The lack of disease-specific protein signatures reflecting SLE and the underlying biological processes is, however, striking and represents a key limitation. The aim of this retrospective pilot study was to identify differential immunoprofiles, modulated by the disease, reflecting apoptosis, a key process in the etiology of SLE, and disease activity, using affinity proteomics.

Methods

We have employed an in-house designed recombinant antibody microarray technology platform for miniaturized, multiplexed serum protein profiling of SLE, targeting mainly immunoregulatory proteins. A cohort of healthy controls, and two cohorts of well-characterized SLE serum samples have been analyzed, and the array data was correlated to various clinical states.

Results and Discussion

The results showed that multiplexed panels of SLE-associated serum biomarker candidates could be deciphered, in particular reflecting disease activity, but potentially also the apoptosis process. While the former biomarkers could display a potential future use for prognosis, the latter biomarkers might help shed further light on the apoptosis process taking place in SLE.

Conclusion

Hence, we have shown that antibody microarrays could be used to de-convolute crude serum immunoproteomes, extracting molecular fingerprints of SLE, in particular reflecting the apoptosis process and disease activity. This could help to advance and further foster our understanding of SLE and the underlying biology. In addition, it will also enhance our fundamental understanding of this complex autoimmune condition.

Keywords: SLE, Antibody microarrays, immunoproteomes

TP08-03

Quantitative Analysis of Acetylated Protein and Its Modification Level based on UCNPs

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Introduction and Objectives

Protein acetylation plays an important role in various aspects like epigenetics of beings, but the weak affinity of acetyl-antibody and no efficient enrichment technique specific for lysine-acetylated proteins has limited the study of lysine acetylation. Protein microarray is an emerging biotechnology. It has been widely studied and applied in recent years. Based on the attractive optical properties of upconversion nanophosphors (UCNPs), therefore, a new type of antibody microarray was designed to quantitative detection of proteins and its acetylated level in this study.

Methods

The microarray was designed as follows: Firstly, a specific kind of capture antibodies was spotted and fixed on a solid glass support to form a microarray. Secondly, the microarray was incubated with proteins solution, blocking buffer (5% goat serum in TBST) and detection antibody in proper sequence. Lastly, the microarray was incubated with UCNPs@SiO2 suspension. In order to quantify the specific protein content and its modification levels on the microarray, the fluorescence intensity of UCNPs@SiO2 combined with the detection antibody was measured.

Results and Discussion

Acetylated BSA (BSA-ac), histone H3 and acetyl-histone H3 were successfully quantified by optimizing the detect condition with this kind of protein micriarray. The limit of detection (LOD) of the new protein microarray for BSA-ac was 0.1ng/mL, which is highly sensitive than that of protein microarray detected by Cy3 (50ng/mL). Besides, we applied the protein microarray to quantify histone (H3) and the variation of lysine acetylation on histone H3 in hepatocyte L-02 and hepatoma cell HepG2 of human beings. The result shows that acetylation level of histone H3 was lower in HepG2 than in L-02. The research provides a new method to study lysine acetylation in the future.

Conclusion

This kind of chip could detect BSA-ac at the concentration as low as 0.1ng/mL with a good linear relationship. Compared to protein chip labeled by Cy3 in previous researches, the designed protein chip increases the LOD by two or three orders of magnitudes, which has great significance for clinical research. In addition, we used this chip to detect lysine acetylation variation on H3 in serum between hepatocyte L-02 and hepatoma cell HepG2 successfully. And by comparing the ratio of lysine acetylation level to protein level, the chip proved the correlation between histone H3 lysine acetylation and Hepatocellular carcinoma. As a conclusion, the research provides experimental basis for quantitative studies on the variation of protein acetylation modification in disease samples and control samples in the future.

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Keywords: protein microarray, UCNPs@SiO2, lysine acetylation, histone H3, quantitative detection

TP08-04

Development of lung cancer diagnostic method using exosomal protein DDOST.

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Introduction and Objectives

Lung cancer is fatal disease of high mortality. Because Lung cancer is hard to early diagnosis and treat. Thus, there are many trials to develop a new method for early diagnosis. In this study, we investigated exosome that expressed many type of cell. However lung cancer cell release more exosomes than normal cells to vessel. Therefore we can diagnosis with less blood easily. There are many markers for detecting exosome, we found DDOST for effective marker of membrane protein. By sandwich ELISA using anti-CD63 antibody and anti-DDOST antibody, we confirmed that early diagnosis can be possible through detection of Exosome derived from blood.

Methods

Sandwich ELISA BD OptEIA Reagent Set A (BD bioscience, US) was used for sandwich ELISA. First, anti-CD63 antibody (abcam, UK) was coated on 96well plate using coating buffer for overnight at 4℃. Next day, after 3 times wash with washing buffer, blocking buffer was treated for 2h at 25℃. Then, plasma from cancer xenograft mice or human was diluted for one-fiftieth with blocking buffer and added on wells for 2h at 25℃. While reacting, anti-DDOST antibody was binding with anti-mouse Ig G(HRP) for 30min at 25℃. After 2h, wells were washed 5 times and add antibody for 1h at 25℃. Finally 7 times washed, substrate A and B mixed at 1:1 ratio, added for 30min and stop solution treated for 10min. Optical density at 450nm was immediately measured.

Results and Discussion

To confirm existing of exosome in lung cancer, we analyzed zeta-sizer, and SEM image. Also, to examine the expression of DDOST on the surface of lung cancer cell, western blotting analysis with anti-DDOST antibody was conducted. As a result, DDOST is more expressed on the surface of cancer cells. At last, using Sandwich ELISA, we validate that DDOST is good biomarker for diagnosis of lung cancer. In this study, we identified that exosome is more derived from lung cancer cell than normal cells. Sandwich ELISA is useful method to diagnosis of lung cancer by targeting DDOST that specially expressed on surface of exosomes. DDOST can be used for diagnosis of lung cancer or targeting protein for treatment, because it is overexpressed on lung cancer tissues or cells, in addition, exosome has similarity to

cell membrane. Furthermore, it is possible that invasive cancer diagnosis. Our results suggest that DDOST protein is valuable target molecule for early cancer diagnosis.

Conclusion

According to these result, we found that DDOST is effective biomarker for diagnosis lung cancer. However, we can't confirm DDOST expression on the surface of other cancers, so that additional experiments are needed by using other type of cancer plasma. To specific detecting lung cancer, it is useful for combined DDOST with other protein known as lung cancer biomarkers already.

Keywords: Exosome, Diagnostic, CD63, DDOST, Lung cancer, Biomarker

TP08-05

Development of humanized antibody against EpCAM for cancer and cancer stem cell theranostics

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Introduction and Objectives

Epithelial cell adhesion molecule (EpCAM) is highly expressed in epithelial cancer and tumor initiation cells (TICs), which is associated with enhanced malignant potential, particularly in colorectal carcinoma (CRC) and head and neck squamous cell carcinoma (HNSCC). EpCAM overexpression associates with enhanced cancer cell proliferation, invasion, metastasis, malignant potential, chemo-/radioresistance, and decreased overall survival of cancer patients. Recent literature showed a more multipotent role of EpCAM in cell-cell adhesion, cell signaling, migration, and differentiation. As it is frequently highly expressed in tumor tissues and metastatic cancer cells in transit via blood or lymphatic vessels, EpCAM has gained attention as a potential target or diagnostic and antibody-based immunotherapies for a spectrum of malignancies.

Methods

The mouse hybridoma technology was employed to to develop pro-apoptotic monoclonal antibodies against EpCAM. From more than 3,000 hybridoma clones, we identified 49 anti-EpCAM mAbs, which possessed high binding activity to several human cancer cell lines, but not normal cell lines. Apoptosis, MTT assay and animal studies were used to determine the anti-cancer function of anti-EpCAM antibodies. We further used domain mapping and phage-displayed random peptide library methods to identify binding epitope of EpAb2-6.

Results and Discussion

Here we show that EpCAM enhances tumor initiation and tumorigenesis via activating reprogramming factors (c-Myc, Oct4, Nanog, and Sox2) and epithelial-mesenchymal transition gene expression. To further evaluate whether progression of malignance can be directly inhibited by targeting EpCAM, we have generated five novel monoclonal antibodies (mAbs) against EpCAM. One of these anti-EpCAM mAbs, EpAb2-6, was found to induce cancer cell apoptosis in vitro, inhibit EpICD cleavage, suppress tumor growth, and prolong the overall survival of both a pancreatic cancer metastatic mouse model and mice with human colon carcinoma xenografts. EpAb2-6 also increases the therapeutic efficacy of irinotecan, fluorouracil, and leucovorin (IFL) therapy in a colon cancer animal models and gemcitabine therapy in a pancreatic cancer animal models. Furthermore, EpAb2-6, which binds to positions Y95 and D96 of the EGF-II/TY domain of EpCAM, inhibits production of EpICD, thereby decreasing its translocation and subsequent signal activation. The molecular mechanisms of tumor inhibition by EpAb2-6 have been elucidated.

Conclusion

Collectively, our results indicate that EpCAM may be a promising target antigen for the development of cancer therapy. The novel anti-EpCAM mAbEpAb2-6, which recognizes a

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particular epitope on EpCAM, can directly induce cancer cells death and may be a suitable basis for devising treatments for colon and pancreatic cancer.

Keywords: EpCAM, therapeutic antibody, cancer stem cell, tumor imaging, cancer therapy

TP08-06

Subpopulations of Hepatocellular Carcinoma with High MAPK and mTOR Signaling Activity Revealed by Reverse-Phase Protein Array Analysis

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Introduction and Objectives

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related deaths worldwide. A multi-kinase inhibitor, sorafenib, is currently the only systemic therapeutic agent proven effective for HCC treatment. Only a small percentage of patients receiving sorafenib, however, experience the anticipated therapeutic benefits. The precise mechanisms underlying the anti-tumor activity of sorafenib remain elusive because of its broad inhibitory spectrum. The known targets of sorafenib are RAF kinases, which are primary regulators of the MAPK signaling pathway, VEGFR-2, PDGFR, FLT3, Ret and c-kit. We decided, therefore, to profile the signaling pathways activated in 23 HCC cell lines and then investigated the correlation between these signaling profiles and their sensitivity to sorafenib.

Methods

We examined the activation status of 180 key signaling nodes across a panel of 95 cancer cell lines derived from eight different types of cancer using reverse-phase protein microarrays (RPPAs). The sensitivity of the 23 HCC cell lines to sorafenib was assayed with the CellTiter-Glo Luminescent Cell Viability Assay kit and we then calculated their respective IC50 values. Unsupervised hierarchical clustering analyses were subsequently performed on the HCC cell lines based on the phosphorylation status of the signaling components.

Results and Discussion

Conclusion

The RPPA platform has broad utility in precision medicine including the identification of cancer subtypes most likely to respond to a specific treatment thereby facilitating the cancer therapy decision-making process.

Keywords: Reverse-phase protein array, mTOR pathway, MAPK pathway, hepatocellular carcinoma

TP08-07

Autoantibody profiling in healthy heavy smokers at risk for COPD

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Introduction and Objectives

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease characterized by an accelerated decline in lung function. With the aim to explore the role of autoimmunity as disease driver in COPD, we performed autoantibody profiling in healthy heavy smokers with an increased risk of COPD by utilizing antigens from the Human Protein Atlas in planar and suspension bead array formats.

Methods

The study sample set consisted of 364 healthy heavy smokers from a lung cancer screening trial, covering the first plasma collection visit (visit 3). All smokers were at an increased risk for COPD and classified as GOLD stage 0 at this time point. In addition, subjects were categorized in four quartile groups of annual lung function decline (slow, second, third, rapid) based on the ratio of forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC).First, in an untargeted screening phase, plasma from 80 subjects with either slow or rapid lung function decline were analysed for their IgG profiles on planar antigen microarrays. This created reactivity profiles for in total 1536 unique antigens. Four subjects were then selected for an even deeper screen of 42,100 unique antigens on planar microarrays (over 19,000 proteins). Last, in a targeted screening, the full set of 364 samples was profiled on a selected set of 380 antigens corresponding to 398 proteins on suspension bead arrays. These antigens were selected from the two screening steps as well as from the literature including antigens and proteins mentioned in relation to COPD, inflammation and lung disease. Fisher's exact test was used to compare reactivity frequencies between different groups.

Results and Discussion

From the screening phases, reactivity towards 229 antigens were identified according to certain sample specific cut off thresholds (20-120 times the sample median absolute deviation + the sample median) as well show a difference between slow and rapid lung function decline. In the targeted phase, 13 antigens showed reactivity in at least 21% of the subjects (range 21-67%). Contrasting the extreme groups of slow and rapid decline, four antigens showed a significant difference in recognition frequency ($p<0.05$). Two of these were unique to this comparison. Interestingly, when stratifying the subjects into their follow-up GOLD stage, reactivity towards one of these antigens was also observed with increased frequency in those who had developed moderate COPD (GOLD II) two years later compared to those that were still healthy (GOLD 0).

Conclusion

In summary, autoimmunity profiling in three phases were performed on various microarray formats. We have here identified autoantibody profiles that indicate a potential association to healthy heavy smokers at risk for COPD and a relation to lung function decline and future COPD status.

TP08-07

Keywords: Autoantibody profiling, autoimmunity, microarrays

TP08-08

Generation and purification of highly phosphorylated site specific antibodies for human N1ICD

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Introduction and Objectives

Abnormalities of Notch1 protein may be associated with a variety of diseases, such as tumor, blood system, cardiovascular system, stem cell and other diseases. Notch1 intracellular domain (N1ICD), a form of activation of protein Notch1 with phosphorylation, is important for the dynamic control of Notch1 signaling, and it would be degraded in a short time by ubiquitylation and proteasome. Generate and purify highly phosphorylated site specific antibodies for human N1ICD for the further study of Ntoch1 signaling pathway.

Methods

To detect the activated N1ICD, some phosphopeptides of specific phosphorylated sites in human N1ICD including Thr1861, Tyr2145 and Ser2162 were synthesized, as well as their C-terminus carrying protein KLH. And then these phosphorylated peptides of N1ICD were injected into three rabbits respectively for specific polyclonal antisera. After one priming injection, three biweekly injections and one booster injection followed by the production bleed, the antibodies responded to the phosphorpeptides were assayed via ELISA. The polyclonal antisera were purified by a subtraction step to remove antibodies that cross-reacted with total protein including the non-phosphorylated form, and an affinity purification step to remove antibodies that are against phosphorylation sites with low sequence complexity. The Dot-blot assay detected the reactivity of the eluted antibody with phosphorpeptide and non-phosphopeptide. The specificity of antibody preparation to the phosphoprotein was demonstrated by western blot with cells or organisms which undergoes the Notch signaling with phosphorylated Notch1.

Results and Discussion

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Conclusion

Three specific polyclonal antibodies for N1ICD sites Thr1861, Tyr2145 and Ser2162 were successfully generated, which will provide a tool to study the role of NOTCH protein phosphorylation in disease developmen.

Keywords: Notch1 signaling pathway/ N1ICD/ phosphorylation / polyclonal antibodies/ purification

TP08-09

Autoimmunity screening and antibody validation using high-density protein microarrays

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Introduction and Objectives

There is value and information to gain within affinity proteomics using high-density protein arrays for the analysis of autoantibodies in diseases. The Protein and Peptide Arrays facility at the Science for Life Laboratory provides technologies and resources for autoimmunity profiling, epitope mapping and antibody validation, as well as an infrastructure for commercial protein array analysis of different array density, content and sample capacities. Two recent large-scale projects will also be described. Firstly, a large-scale sample analysis for validation of Anoctamin 2 as a Multiple Sclerosis (MS) associated autoimmunity target [B. Ayoglu et al, PNAS 2016]. Secondly, the large-scale production of a unique 42K high-density protein microarray using protein fragments generated and validated within the Human Protein Atlas [R. Sjöberg et al, New Biotechnol 2016 and Uhlen et al, Science, 2015].

Methods

Planar protein microarrays, in the format of 384 unique antigens in 21 arrays per slide, are routinely produced by the Human Protein Atlas, for the validation of corresponding antibodies' on and off target binding (www.proteinatlas.org). The antigens are recombinantly produced protein fragments with unique representations of their corresponding proteins. The arrays are produced at different antigen densities with different antigen selections. The protein arrays are applied for autoantibody screening in samples such as plasma and CSF, from patients with a known or potential autoimmune component. Identified reactivities are subsequently verified in larger patient sample material using a selection of reactive antigens on suspension bead arrays.

Results and Discussion

ANO2 was recently identified as an autoimmune target in MS. The autoantigen ANO2 together with 383 other antigens, selected from an initial screening with 11 520 antigens on planar arrays, were used on a bead array to profile 2 169 plasma samples from population based controls and cases. The sample profiling effort revealed increased autoantibody reactivity against ANO2 in cases. Together with corroborative independent assays, a potential new subphenotype in MS was demonstrated. A high-density protein array was recently produced using 42 100 Human Protein Atlas antigens, corresponding to 19 055 unique Ensemble Gene IDs. The array is thereby qualified to be denoted the world's largest protein microarray. These arrays provide confirmation of on-target recognition as well as detection of off-target interactions for an antibody. This enables a previously unprecedented broad exploration of human autoimmune profiles both in diseases with known and unknown autoimmune components.

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Conclusion

Exploration of both autoantibody repertoires and antibody-antigen interactions were performed using high-density protein microarrays and with large-scale sample analysis.

Keywords: Autoimmunity profiling, high-density protein microarrays, Human Protein Atlas and antibody validation.

TP08-10

PEAKS AB – A software tool for monoclonal antibody sequencing and characterization with LC-MS

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Introduction and Objectives

Monoclonal antibodies (mAbs) have been more and more prevalently used in both diagnostic and medical applications. It stimulates the increasing demand of antibody sequencing and the comprehensive characterization It has been proved that current LC-MS technologies can provide an accurate and sensitive solution for both mAb sequencing and characterization. We propose a software tool for antibody sequencing and characterization with LC-MS.

Methods

The algorithm of the tool includes three steps below:1. The reduced mAb sample is analyzed by LC-MS to measure the intact mass of mAb chain. Our top-down MS de-convolution algorithm analyzes the top-down MS data to calculate the intact mass values;2. De novo sequencing tags are generated from the MS/MS data with multiple enzymes and then constructed the mAb sequence candidates. Thereafter, the intact mass measured above and the fragment ions are used to validate the constructed mAb sequences;3. PTMs and sequence variants, including glycosylation and disulfide bridges are qualitatively and quantitatively analyzed.

Results and Discussion

We used a standardized procedure to prepare mAb samples for LC-MS/MS. The heavy and light chains of a mAb sample were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, the sample was reduced with dithiothreitol (DTT) and free cysteine residues were then alkylated using iodoacetamide. The heavy chain bands were deglycosylated with PNGase-F and three enzymeswere used to digest both chains overnight. The digested peptides were extracted and subjected to LC-MS/MS analysis on a Thermo-Fisher Scientific Q-Exactive (Q-E) orbitrap mass spectrometer. Three resulting raw data files for each chain were collected for further mAb sequencing and characterization.With the tool, we got the full sequences of both the heavy and light chains, of which each amino acid is confidently supported by more than 20 peptides on average. Comparing to the true sequences, the constructed sequences of both chains are 100% correct. This sequencing technology was also successfully tested to mAb samples from our customers and collaborators.The light chain sample was reduced and analyzed by LC-MS to generate top-down MS data for intact mass validation. The calculated intact mass of the light chain has a 0.97 Da (41 ppm, introduced by deamidation) difference from the one measured by LC-MS and this difference is smaller than the commonly used 1.2 Da range. The heavy chain sample without disulfide bond breakage was digested using chymotrypsin and then analyzed by LC-MS/MS. From the data we successfully identified four pairs of cysteines connected by disulfide bonds with high confidence.

Conclusion

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PEAKS AB provides a tool for automated antibody sequencing and characterization from LC-MS data.

Keywords: Antibody sequencing, LC-MS, antibody characterization, antibody sequence validation

TP08-11

Harnessing Translational Research with Protein Microarrays

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Introduction and Objectives

With the rapid development of omics technologies such as the next-sequencing and mass spectrometry, researchers have the capability to systematically explore the molecular changes of human disease and discover the molecular biomarkers that are used for accurate diagnosis and treatment. Life science begins to enter into the era of precision medicine. Development of high-throughput and high-sensitive technologies would be valuable to protein function study, vaccine development, drug screening and biomarker discovery, etc.

Methods

In LaBaer lab, we developed a high-throughput proteome microarray technology based on Nucleic Acid Programamble Protein arrays (NAPPA), with which thousands of human genes can be transcribed & translated into proteins and displayed on the microarray surface in situ through a human HeLa lysates based cell-free protein expression system.

Results and Discussion

With NAPPA, we built human, tuberculosis and virus proteome microarrays which have been demonstrated in the researches of protein-protein interactions, post-translational modification, antibody mapping and (auto) antibody biomarkers, etc.

Conclusion

All these results demonstrate NAPPA technology will be a powerful proteomics approach for the translational research.References: 1. Xiaobo Yu and Joshua LaBaer (2015) High-throughput identification of proteins with AMPylation using self-assembled human protein (NAPPA) microarrays. Nature Protocols, 10(5):756-767.2. Xiaobo Yu, Brianne Petritis and Joshua LaBaer. Advancing Translational Research with Next Generation Protein Microarrays, Proteomics, 16(8):1238-50.3. Xiaofang Bian, Garrick Wallstrom, Amy Davis, et al. Immunoproteomic Profiling of Anti-Viral Antibodies in New-Onset Type 1 Diabetes Using Protein Arrays, Diabetes, 65(1):285-96.4. Xiaobo Yu, Kimberly B. Decker, et al. (2015) Host-Pathogen Interaction Profiling Using Self-Assembling Human Protein Arrays. Journal of Proteome Research, 14(4):1920-36. 5. Rafael Prados-Rosales, Leandro J. Carreo, et al. (2014) Immunization with Mycobacterial membrane vesicles protect against Mycobacterium tuberculosis in mice. mBio, 5(5): e01921-14. 6. Xiaobo Yu, Andrew R. Woolery, Phi Luong, et al. (2014) Click chemistry-based detection of global pathogen-host AMPylation on self-assembled human protein microarrays. Mol. Cell. Proteomics, 13(11):3164-76.7. Xiaobo Yu, Xiaofang Bian, Andrea Throop, et al. (2014) Exploration of Panviral Proteome: High-Throughput Cloning and Functional Implications in Virus-Host Interactions. Theranostics, 4(8): 808-822.

Keywords: Nucleic Acid Programmable Protein Arrays, Proteomics, Translational Medicine, Biomarker

TP08-12

Autoantibody Profiling Using Ultra-Dense Peptide Microarrays

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Introduction and Objectives

During the last decades there has been a lot of focus on the pathogenic roles of the B-cells, which have lead to drugs such as Rituximab, available for treating the autoimmune disorders: SLE, RA and multiple sclerosis (MS) by depleting circulating B-cells, which eventually leads to a dampening of the autoantibody production. However a more selective way of treatment, than depleting all B-cells, could involve a depletion of the autoantibodies. That kind of treatment would need an increased understanding of autoimmunity and specifically the autoantibodies and the autoantigen. Characterization of the autoantibody signatures present in an autoimmune disorder could also lead to increased understanding of disease mechanisms. Given that immunoglobulins are highly abundant in blood and rather stable proteins, they could also serve as excellent biomarkers. Here we present an approach to identify novel autoantigens and explore autoantibody repertoires in serum in the context of narcolepsy and multiple sclerosis by utilizing ultra-dense peptide microarrays.

Methods

Whole proteome peptide arrays were made with an in situ photolithographic synthesis of a total of 2.1 million overlapping peptides, each approximately 12 amino acid residues long with a six amino acid lateral shift. Then, based on peptide reactivity of the initial experiments, a set of proteins was selected to be included in a targeted design array with 175,000 peptides. The targeted design allows for higher resolution with 11 amino acids peptide overlap and the smaller size enabled more samples to be analyzed since each microarray slide fits 12 identical subarrays. Finally, verification of interesting autoantibody epitopes was performed using peptide suspension bead arrays, a format which enables hundreds of samples to be analyzed.

Results and Discussion

Initially autoantibody reactivity against over 2 million peptides was analyzed on the whole-proteome arrays with serum samples from multiple sclerosis and narcoleptic patients. Large biological variation was observed between the analyzed sample, and it could be seen that peptides that were reactive in many samples, were rich in glycine and alanine; suggesting high sequence identity to microbial peptides, especially Epstein-Barr nuclear antigens. We also found apparently disease-associated peptides and proteins from the ultra-dense peptide microarrays, such as NRXN1 with higher reactivity found in narcolepsy patients. This particularly protein was later verified using peptides on suspension bead arrays, in two cohorts from narcolepsy cases in Sweden (n=112) and Finland (n=57), showing significant differences ($p<0.05$).

Conclusion

This peptide array approach allowed us to resolve the epitopes of human autoantibodies with relevance in two different disease contexts and gave us insights into what the

TP08-12

common antibody repertoire reactivity looks like at peptide level.

Keywords: Peptide microarrays, autoantibodies, narcolepsy, multiple sclerosis

TO05-01

Personalized proteomic characterization of hepatitis B virus-associated hepatocellular carcinomas

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Introduction and Objectives

Hepatocellular carcinoma (HCC) is a highly lethal cancer, ranking as the ?fth most prevalent tumor and the second leading cause of cancer-related deaths worldwide. In China, HCC has been ranked as the second most frequent fatal cancer since the 1990s, and chronic infection with hepatitis B virus is one of its major risk factors, most patients are diagnosed at advanced stages of HCC with poor survival. A comprehensive molecular view of cancer is necessary for understanding the underlying mechanisms of disease, improving prognosis, and ultimately guiding treatment.

Methods

To provide a detailed analysis of the molecular components and underlying mechanisms associated with HBV-related HCC, we performed a comprehensive mass-spectrometry-based proteomic characterization of 126 HCC tumors and paired no tumor tissues.

Results and Discussion

A total of 11,099 proteins including 947 transcription factors and 34,191 phosphosites were confidently identified. Five pathway categories were characterized for HCC including some rarely reported but might be important in HCC such as RHO GTPase, p38 MK2 signaling, ubiquitin mediated proteolysis et al.To build an unbiased molecular taxonomy of HCC, we used protein abundance data from these tumors to identify subtypes. Three of the proteomic clusters showed a clear correspondence with clinical characteristics and subtype specific signatures.

Conclusion

It enabling a deeper understanding of HCC with the potential to more-effective clinical implementation of emerging targeted agents.

Keywords: HBV-related HCC, protein expression profile, phosphoproteome, proteomic subtyping

TO05-02

In vitro investigation of an adverse outcome pathway of cholestatic liver injury using quantitative phosphoproteomics

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Introduction and Objectives

Cholestasis is a liver disorder caused by the abnormal accumulation of bile in the liver. It accounts for about half of the cases of drug-induced liver injury and is characterized by bilirubinuria and hyperbilirubinemia that subsequently lead to icterus and pruritus. An adverse outcome pathway (AOP) construct has been introduced to pinpoint the mechanisms involved in the development of cholestasis. Hereby, the inhibition of the bile salt export pump (BSEP) is considered as the main molecular initiating event. The present study evaluates the reliability and predictive capacity of the established AOP for cholestatic liver injury.

Methods

Human hepatoma-derived HepaRG cells were exposed to sub cytotoxic concentration of bosentan [IC10 - 250 µM], a potent BSEP inhibitor and a clinically relevant cholestasis inducer. The cellular response to the inflicted toxicity was evaluated by proteomics and transcriptomics. Cell lysates were prepared for proteomic studies at the end exposure with bosentan for 1 h, 24 h and 24 h followed by a washout period of 72 h. Each experimental time point was prepared in triplicate with respective controls. Samples were processed with FASP and the resulting tryptic peptides were subjected to iTRAQ labeling and subsequent TiO2 based phosphopeptide enrichment (325 µg starting material).

Results and Discussion

We could relatively quantify ~3,600 proteins (>= 2 unique peptides; 1% FDR) at each of the three time points and notably ~4% proteins were significantly (p-value <= 0.01) differentially regulated in HepaRG cells after 24 h exposure to bosentan. Further, phosphoproteome analysis led to the quantification of 4,006 phosphorylation sites (pRS probability >= 90%; 1% FDR) at the same time point (i.e. 24 h) of which ~8% were differentially altered. Some of these proteins and phosphoproteins could be mechanistically linked to cholestasis and may represent unidentified key events and thus toxicity biomarkers. In parallel, transcriptomics analysis showed that half of the predicted gene changes related to the activation of nuclear receptors was correctly modulated. Pathway analysis further identified cholestasis as a major toxicological event induced by bosentan. Furthermore, 37 genes could be selected as potential novel transcriptional biomarkers of bosentan-induced cholestasis based on the discrepancy in expression between treated cells and respective controls.

Conclusion

Overall, the results of this study underscore the scientific soundness of the established AOP and demonstrate that AOPs are flexible tools that may be continuously optimized by

TO05-02

feeding in new experimental data.

Keywords: Adverse outcome pathway, cholestasis, phosphoproteomics, transcriptomics

TO05-03

Differential proteomic analysis of cholangiocarcinoma cells and cell-derived extracellular vesicles by label free mass spectrometry

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Introduction and Objectives

Cholangiocarcinomas (CCA) are the second most common primary liver tumor, accounting for around 3% of all gastrointestinal cancers. CCA is very deadly due to its aggressiveness, late diagnosis and high chemoresistance. But no specific CCA markers have been found to date. Therefore, new therapeutic and diagnostic strategies are needed. Extracellular vesicles (EVs) are small membranous vesicles (30-1000 nm) that contain a specific subset of cellular biomolecules including proteins, RNA species and metabolites. EVs seem to be important mediators of cell-to cell communication by presenting proteins of their surface or by fusion with the target cell, delivering their cargo. Recently some studies pointed out a relevant role for EVs in cancer progression. The goal this study was to characterize by label free nLC MS/MS the whole cell extract (WCE) proteomes and cell-derived extracellular vesicles (EVs) proteomes of: NHC2, H69, extrahepatic bile duct carcinoma cell line (TFK), and human biliary tract cancer cell line (EGI).

Methods

WCE and EVs protein extracts were digested by FASP method. Resulting peptides were analyzed by nano liquid chromatography (nano Acquity, Waters) coupled to either Synapt G2S (Waters) or Orbitrap XL (Thermo-Electron). Protein identification was performed by Mascot search engine (MatrixScience) and label free method was conducted for relative protein quantification by Progenesis QI (Nonlinear Dynamics, Waters).

Results and Discussion

Common differences between tumoral cells (TFK and EGI) and controls (NHC2 and H69) were selected following an overlapping analysis. Proteins consistently and coherently deregulated in both NHC2 vs TFK and NHC2 vs EGI comparisons were considered as reliable NHC2 vs TUMOR differences. The same was performed using H69 as control. Finally, the set of proteins that better discerns between tumor and control cells was selected by analyzing the overlap between NHC2 vs TUMOR and H69 vs TUMOR. The PCA analysis provided by Progenesis software reveals clear differences between tumor and control cells, but it also pinpoints that despite they are both used as control, NHC2 and H69 are very different.

Conclusion

TO05-03

We have observed a relatively large number of differences between control and tumor cells both at WCE and EVs proteome side. These differences are currently being validated by orthogonal methods. Further analysis of these proteins may help understand the molecular mechanism of CCA and eventually lead to the discovery of diagnosis/prognosis-related proteins that should be further validated in patients. Moreover, this proteomic analysis reveals that the commonly used as a control H69 cell line differs in a very significative manner from normal cholangiocytes.

Keywords: Cholangiocarcinoma, CCA, NHC2, H69, TFK, EGI, liver tumor, Label Free, Quantification, Extracellular Vesicles, Exosomes, cancer, tumor.

TP09-01

Relative quantitation for expression evaluation of drug metabolizing enzymes using surrogate peptide approach by Quadrupole Time of Fight

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Introduction and Objectives

Xenobiotic clearance is an important area in pharmaceutical drug development. The in vitro-in vivo extrapolation (IVIVE) studies for Drug clearance requires easy and accurate quantification of drug metabolizing enzymes in the studied in vitro system. Therefore, to quantitate drug metabolizing enzymes (DME) in human liver S9, Liquid Chromatography Mass Spectrometry (LCMS) method was applied. Quadrupole Time of Fight mass spectrometry (QTOF) is an alternative to MRM based quantitative analysis where both qualitative and quantitative information can be obtained in a single run. In the present study, QTOF mass spectrometry was used as a single platform (LC/Q-TOF) to identify and quantify the enzymes. In this presentation estimation of protein expression of the Human DMEs having no literature reports: Aldehyde Oxidases, Human esterases (hCE-1) and Sulfotransferase (SULT1A1, 1A2 and 1E1) will be discussed.

Methods

Surrogate peptides for the enzymes Aldehyde Oxidases, Human esterases (hCE-1), Sulfotransferase (SULT1A1, 1A2 and 1E1) were selected using NCBI BLAST database and following the peptide selection criteria. The unique peptides were solid phase synthesized and spiked into microsomal matrix and quantified using LC_QTOF to construct the calibration curves. The solvent systems used were 0.1 % formic acid in water and 90 % acetonitrile in water with 0.1 % formic acid. The HPLC system was run at 0.3mL/min flow rate with C18 - column. The column was operated at 60°C. Mass spectrum was acquired in positive ion mode. All Ions approach was used for quantitative and qualitative analysis. The data was analyzed with qualitative and quantitative Software tool of the instrument. For quantification of enzymes in liver microsomes, the samples were trypsin digested and analyzed using the calibration curves.

Results and Discussion

LC method was optimized to get good separation between the peptides of interest. The peptides of interest were spread out evenly across the chromatogram with good peak shape. In addition, MS parameters were also optimized to get good ionization signals for the peptides in this study. Three different ms/ms acquisition strategies such as auto, targeted and All Ions approach were tested for getting good b and y ion signals for confirmation of the desired peptide, while maintaining good MS signal (EIC) for quantification purpose. All Ions approach was found to be optimum for our study and hence was adopted for quantification of the peptides of DMEs. The results of quantification for calibration curve were excellent with good linear responses. Using these

calibrations curve the amount of DMEs were estimated in the samples.

Conclusion

• All Ions approach was used for both quantitation and for confirmation of the peptides in the samples• The study shows that Q-TOF can be used as a single platform to identify and quantify endogenous proteins in complex matrix.

Keywords: Quadrupole Time of flight, surrogate peptide, All Ions, drug metabolizing enzymes

TP09-02

Hepatic proteome analysis of dioxin-sensitive and -resistant mice exposed to 2,3,7,8-tetrabromodibenzo-*p*-dioxin

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Introduction and Objectives

Dioxins cause various toxic effects through the aryl hydrocarbon receptor (AhR) in vertebrates with inter-species and strain differences in susceptibility. C3H/*lpr* and MRL/*lpr* mice are inbred dioxin-sensitive and -resistant strains, respectively. However, the molecular mechanism underlying this different susceptibility still remains unclear. We adopted a proteomic approach to clarify the difference in effects of 2,3,7,8-tetrabromodibenzo-p-dioxin (TBDD) exposure on the hepatic proteome between C3H/*lpr* and MRL/*lpr* mice.

Methods

Female C3H/*lpr* and MRL/*lpr* mice (10–11 weeks old) were intraperitoneally injected with 10 µg/kg body weight of TBDD or corn oil (vehicle). To confirm the induction of cytochrome P450 isozymes (CYPs) by TBDD treatment in each strain, we initially measured CYP1A1 and 1A2 protein levels and their catalytic activities by western blot and alkoxyresorufin O-dealkylation (AROD) assay, respectively. We then performed the proteome analysis using two-dimensional electrophoresis coupled with matrix-assisted laser desorption/ionization time-of-flight/ time-of-flight (MALDI-TOF/TOF) mass spectrometry to identify the differentially expressed proteins between TBDD-treated and control groups in each strain and between control C3H/*lpr* and MRL/*lpr* mice. Network and pathway analyses of the differentially expressed proteins were further carried out to understand their protein-protein interactions and biological significance.

Results and Discussion

Results showed that TBDD treatment increased CYP1A1 and 1A2 protein expression levels in both strains, but a more prominent induction was observed in C3H/*lpr* mice than in MRL/*lpr* mice. This supports that C3H/*lpr* was more sensitive to dioxins than MRL/*lpr*. We successfully identified 40 up- and 17 down-regulated proteins by TBDD treatment in C3H/*lpr* and 7 up- and 10 down-regulated proteins in MRL/*lpr*. The proteins induced in C3H/*lpr* were involved in the metabolism of tryptophan and its metabolites as endogenous AhR ligands, suggesting that AhR is more activated by

accelerated production of endogenous AhR ligands in TBDD-treated C3H/*lpr* than in TBDD-treated MRL/*lpr*. We also identified that proteins responsible for reducing the oxidative stress such as superoxide dismutase and peroxiredoxins were up-regulated by TBDD treatment in C3H/*lpr* mice.

Conclusion

The present study reveals that the high dioxin-susceptibility of C3H/*lpr* strain may be associated with more activation of AhR signaling by endogenous AhR ligands and more efficient elimination of oxidative stress.

Keywords: dioxin, proteome, susceptibility

TP09-03

Simultaneous quantification of drug transporters, CYP enzymes, and UGTs in human liver microsomes by high-microflow LC-MS/MS.

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Introduction and Objectives

Drug transporters, cytochrome P450 (CYP) enzymes, and uridine 5′-diphospho-glucuronosyltransferases (UGTs) play an important role in the absorption, distribution, metabolism, excretion, and toxicity (ADMET) of drugs administered to animals and humans. The expression levels of these proteins in various tissues (e.g., liver and small intestine) and cells (e.g., hepatocyte and intestinal epithelium) in vivo or in vitro can be informative for prediction of the pharmacokinetic and safety profile of developing drugs. To meet an increasing demand for multiplexed analysis at ADMET research, the LC-MS/MS based strategy for protein quantitation, called quantitative targeted absolute proteomics (QTAP), is expected as a replacement of conventional immunoassays.In the present study, we aimed to develop the simultaneous quantification method of drug transporters, CYP enzymes, and UGT enzymes in human liver microsomes (HLM) using a high-microflow LC/MS/MS.

Methods

HLM was denatured and solubilized with sodium deoxycholate and N-lauroylsarcosinate. After reduction and alkylation, the samples were digested by lysyl endopeptidase and trypsin sequentially. Detergents were removed by liquid phase extraction, and samples was desalted by solid extraction. After dissolved in 0.1%TFA/water, the samples at 1 µg or 5 µg protein were injected to LC-MS/MS. All digested samples were analyzed using a triple quadrupole mass spectrometer (LCMS-8060; Shimadzu) coupled with a conventional HPLC (Nexera X2; Shimadzu). LCMS-8060 was modified using an ESI capillary tube with thin inner diameter (50 µm i.d.) for high-microflow analysis (Flow rate: 30 µL/min). The LC separation was performed using a trapping column (InertSustainSwift C18 non-metal cartridge column, 1.0 × 10 mm; GL Science) and a separation column (InertSustainSwift C18, 0.1 × 10 mm; GL Science). Quantifications of proteins were estimated from relative intensity to stable isotope-labeled internal standard peptides.

Results and Discussion

In this study, we constructed a high-microflow LC-MS/MS system and developed a simultaneous quantification methods of 20 drug transporters, 15 CYP enzymes, and 9

peptide sample was 3.0 fold greater than that of conventional flow LC-MS/MS (200μL/min). SRM transitions and CEs of tryptic fragments from target proteins were predicted using Skyline software (MacCoss Lab, U. Washington), and optimal MRM conditions were narrowed down by measuring synthetic peptides. Using the established MRM method, we measured HLM samples in various protein amounts. As a result, all of target proteins were detected at 5 μg injection of total HLM protein. Correlation coefficient between quantitative values (fmol / total HLM proteins) in 5 μg and 1 μg total proteins was 1.0.

Conclusion

Drug transporters, CYP enzymes, and UGTs in HLM were successfully measured by a high-microflow LC-MS/MS.

Keywords: Drug transporters, CYP enzymes, UGTs, human liver microsomes, High-microflow LC-MS/MS

TO10-01

The ProteomeXchange Consortium: 2016 update

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Introduction and Objectives

The overall aim of the ProteomeXchange (PX) Consortium (http://www.proteomexchange.org) is to enable data sharing in the field by providing a common framework and infrastructure for the cooperation of mass spectrometry proteomics resources (PMID: 24727771). PX defines and implements consistent, harmonised, user-friendly data deposition and exchange procedures among the members (PRIDE Archive, MassIVE, PeptideAtlas). Thanks to the success of PX, public availability of datasets in the proteomics field has become the norm, and as a result, reuse of public proteomics data is now flourishing.

Methods

In the current data workflow, PRIDE Archive (EMBL-EBI, Cambridge, UK) and MassIVE (University of California San Diego, USA), although focused in tandem MS/MS experiments, can store data coming from any type of proteomics approach, while PeptideAtlas (Institute for Systems Biology, Seattle, USA) provides a repository for Selected Reaction Monitoring experiments called PASSEL. It is expected that the new jPOST repository (various Institutions, Japan) will join the Consortium during 2016, demonstrating PX's unifying role in the proteomics community. The PX members actively recommend the use of the open data standards developed by the Proteomics Standards Initiative (PSI), including for instance mzML, mzIdentML and mzTab.

Results and Discussion

The implementation of PX has resulted in a rapid increase in publicly available proteomics datasets – by May 2016 around 4,000 PX datasets had been submitted to any of the three existing PX partners, totaling over 265 TBs. The main common access point is ProteomeCentral (http://proteomecentral.proteomexchange.org), which provides the ability to search datasets in all participating PX resources. It is possible for everyone to get subscribed to the announcements of new datasets as they become publicly available, via e-mail, RSS or using Twitter (@proteomexchange).

Conclusion

PX is actively changing the "culture" in the field, together with some funding agencies and scientific journals, by promoting and enabling efficient sharing of proteomics data in the public domain.

Keywords: Computational proteomics, data repositories, open data.

TO10-02

proBAMconvert: organizing MS identifications in a genome-centric fashion enables proteogenomics and proteomics integration.

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Introduction and Objectives

The proteomics BAM (proBAM) file format is designed for storing and analyzing peptide spectrum matches (PSMs) within the context of the genome and is developed in the frame of the HUPO Proteomics Standard Initiative (http://www.psidev.info/proBAM). It builds on the widely used SAM (Sequence Alignment Map) and BAM (binary SAM) formats designed for encoding alignment information of sequencing reads to a genome. The proBAM format is compatible with the BAM format such that most software applications that can read a BAM file and display sequencer reads will also be able to display identified peptides in the context of genome mapping. The objective is to have a flexible tool (proBAMconvert) enabling the mapping and visualization in a genomic context of MS-based proteomics data, thus allowing data integration and analysis of both MS-proteomics and NGS-based genomics/transcriptomics/translatomics data in proteogenomics studies.

Methods

The proBAMconvert tool converts peptide identifications (mzIdentML, pepXML and mzTab file formats) to the proBAM file format by mapping the identified peptides/PSMs to a reference genome making use of an annotation of choice (GENCODE, Ensembl, RefSeq,…). To enhance the ease of use, proBAMconvert is available as a stand-alone application as well as a python command line interface (CLI). While the stand-alone application requires no prerequisites, the python CLI may be more suited for advanced users and server use. The GUI stand-alone version is ported to both OSX (Mac), Windows and Unix.

Results and Discussion

The proBAMconvert was successfully applied to convert MS shotgun data and N-terminal COFRADIC MS data of a HCT116 WT-DKO model (DKO, where two DNA-methyltransferases, dnmt1 and dnmt3b are both knocked out). Conversion of the protein-centric MS data allowed the smooth integration and visualization with other genome-centric expression datasets (transcriptomics from RNAseq and translatomics from RIBOseq), studying the effect of DNA methylation on all levels of expression. Furthermore, our integration enabled to determine a quantitative correlation between all levels of expression.

Conclusion

This free and easy-to-use proBAMconvert tool should enhance the communication between the proteomics and genomics worlds. Proteogeonomics is rapidly becoming an interesting research field, addressing questions that cannot be solved solely by either proteomics or transcriptomics/translatomics. That is why this proBAM file format and supporting tools such as proBAMconvert are deemed necessary.

TO10-02

Keywords: Proteogenomics, Protein Standard Initiative, proBAM, MS, NGS

TO10-04

Adipose tissue pathways in obesity: Iberian pig as large animal model of metabolic disorders

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Introduction and Objectives

Obesity and associated metabolic disorders incidence is dramatically increasing worldwide. Genetic background, food excess and lack of exercise became, through obesity, the major cause of metabolic disorders associated to lifestyle. This leads to an increased risk to develop further alterations as metabolic syndrome, type-2 diabetes, cardiovascular diseases and many others.The Iberian breed (Sus scrofa meridionalis) has evolved to survive to food scarcity. It has developed a high potential for fat accumulation under its skin and among the muscular fibres, due to a polymorphism of the leptin receptor gene (LEPR) with effects as insatiability and obesity. Such state in human medicine is called leptin resistance and Iberian pigs develop a condition similar to the human metabolic syndrome and are a suitable animal model for studies on leptin resistance, obesity, metabolic syndrome, and type 2 diabetes.In order to study the mechanisms behind this extreme efficiency in energy storage and fat accumulation it has been decided to study the differential protein expression of visceral and subcutaneous fat tissue of this animal model between two different experimental groups: a group of control pigs with normal weight and a group of obese pigs developing metabolic disorders.

Methods

Differential protein expression has been studied through 2D electrophoresis coupled to mass spectrometry and bioinformatics analysis. In the study were included 6 biological replicates for each experimental group and both visceral (VAT) and subcutaneous (SAT) adipose tissues have been analyzed. Differentially expressed proteins have been analyzed through MALDI TOF MS.

Results and Discussion

It has been found the differential protein expression of 12 proteins between VAT of obese and control animals and 11 proteins among SAT comparison. None of the proteins identified were found to be commonly shared between the comparisons of (VAT) and (SAT). This demonstrates the high rate of metabolic differences among the two different metabolic profiles during this disorder.GO bioinformatics analysis performed through FunRich software highlighted that, among VAT comparison between lean and obese pigs, there were mostly differentially expressed the proteins related to the pathway of

inflammasome and phosphatidilinositol messenger. Among SAT comparison between groups it was found that, most of differentially expressed proteins were related to energy storage.

Conclusion

According to obtained results, both (VAT) and (SAT) underwent to different metabolic pathways strictly related to obesity. In particular, results seem to demonstrate that VAT is increasing its metabolism in the direction of glucose and energy intake in order to overcome the impaired function of SAT because of the surplus of energy storage.

Keywords: Obesity, Metabolic disorders, visceral adipose tissue, subcutaneous adipose tissueAcknowledgementsWork supported by Piano “Sviluppo UNIMI” Linea 2 Azione A to A.S.

TO10-06

Proteome alterations in the porcine endometrium during embryo implantation.

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Introduction and Objectives

In mammals, an efficient exchange of molecular signals between the embryo and the maternal environment plays a crucial role for implantation and the development of early embryos as well as for the maintenance of pregnancy. So far, only a few molecular signals involved in this process were identified. To address the underlying biochemical processes in pigs at the protein level, we performed a quantitative proteome study with endometrial tissue samples (n=5 per group) from non pregnant and pregnant (day 14) gilts.

Methods

For proteome analysis we applied an nano-LC-MS/MS iTRAQ 4-plex approach, complemented by 2D-DIGE. Results were validated by selected reaction monitoring (SRM).

Results and Discussion

In total we identified 1359 proteins, to our knowledge so far the largest dataset of porcine endometrial proteins. The biological significance of the dataset is indicated by the identification of proteins related to important signalling pathways like the integrin signalling pathway or the Jak-STAT signalling pathway.The quantitative analysis revealed 13 proteins to be less abundant and 19 proteins to be more abundant in the endometrium of pregnant animals. Several of the affected proteins are already known to play an important role in embryo maternal communication in other species, e.g., the Signal Transducer and Activator of Transcription 1 (STAT1), a protein mediating the cellular response of cells to interferons (IFNs) or the aldose reductase (AKR1B1) for which a key role in the synthesis of endometrial prostaglandin F is supposed. Several other proteins showing abundance alteration between pregnant and non-pregnant endometrial tissues were not described previously and represent new interesting targets for further functional studies addressing their role during early pregnancy. To confirm the quantitative results, we established SRM assays for the 5 proteins AKR1B1, farnesyl diphosphate synthase (FDPS), STAT1, Tryptophan-tRNA ligase cytoplasmic isoform a (WARS), and the retinol binding protein 4 (RBP4). In all cases, the SRM data were in accordance with the data from the iTRAQ experiments demonstrating the reliability of the presented proteomics data. To address and visualize abundance alterations among protein isoforms, a 2?D Fluorescence Difference Gel Electrophoresis (2D-DIGE) approach was used, including internal pooled standards for inter-gel matching and normalization. In accordance with the iTRAQ experiment, AKR1B1 intensities increased in samples from pregnant animals. Interestingly, 6 different spots could be assigned to AKR1B1 and probably reflect different

isoforms ongoing with a shift in molecular weight and isoelectric point. These spots were increased in intensity to a different extent.

Conclusion

The porcine endometrium proteome at day 14 is significantly affected by the presence of embryos. The identified proteins represent valuable targets for future studies to further decipher the molecular network underlying uterine receptivity.

Keywords: Reproduction, iTRAQ, DIGE, uterus, embryo

TP10-01

A QPrEST Resource for Targeted Plasma Analysis

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Introduction and Objectives

Targeted proteomics methods, such as Selective Reaction Monitoring (SRM), in combination with spike in of stable isotope labeled standards is the method of choice for precise peptide quantification in complex mixtures. However, the elaborative process of establishing those assays is challenging and assay development relies on identifying optimal proteotypic peptides from multiple shotgun MS runs. Thereafter, a smaller set of synthetic peptides are often acquired and used for optimization and robust method performance. Here, we propose a workflow for high throughput targeted assay generation as we have utilized the vast resource of already existing individually purified recombinant human protein fragments originating from the Human Protein Atlas Project (www.proteinatlas.org) to generate a multiplex Parallel Reaction Monitoring (PRM) assays towards 59 human plasma proteins. Protein epitope signature tags (PrESTs) are 50-150 amino acid long protein fragments identical to a part of a human protein sequence, which upon protein digestion releases proteotypic peptides. Up to date, over 45,000 protein fragments covering 18,000 unique human protein-coding genes are available within the Human Protein Atlas resource. This toolbox allows for a streamlined pipeline to generate targeted assays, as proteotypic peptides from PrEST sequences are used to generate custom spectral libraries that can be used as a foundation for MS based protein quantification.

Methods

In this pilot study, we have investigated if the PrEST resource can be used to establish multiple PRM assays towards human plasma proteins. The process included screening and validation of each peptide in multiplex before they were used to absolutely quantify plasma proteins of different clinical importance across multiple individuals. One scheduled PRM method was used to absolutely quantify proteins in plasma samples derived from 100 healthy individuals, sampled at two different time points. Raw plasma was spiked with stable isotope labeled (SIL) QPrEST standards before trypsin digestion.

Results and Discussion

In total, 232 precursors were monitored by a scheduled PRM method using a 30-minute LC gradient for peptide separation. Finally, 58 plasma proteins ranging 5 orders of magnitude were successfully quantified in multiplex. The early addition of SIL protein standards compensates for quantification biases introduced throughout the sample preparation and also for enzyme efficiency differences across the amino acid sequence, as the SIL standards mimics the endogenous amino acid sequence around the cleavage site as well as they are cleaved together with the target protein.

Conclusion

This pilot experiment clearly illustrates the usefulness of the PrEST resource for the

TP10-01

development of novel targeted assays towards proteins in complex mixtures.

Keywords: Targeted Mass SpectrometryParallel Reaction MonitoringStable Isotope Labeled StandardRecombinant Protein FragmentsAbsolute QuantificationPlasma Profiling

TP10-03

High-throughput production of heavy isotope-labeled Protein Epitope Signature Tags to use as internal standards in Mass Spectrometry

ANNE-SOPHIE SVENSSON[*,1]; Hanna Tegel[1]; Mathias Uhlen[1]

[1]*Human Protein Atlas, Sweden*

Introduction and Objectives

Protein Epitope Signature Tags (PrESTs) produced in the Human Protein Atlas (HPA) project have been shown to be a valuable source as isotopic standards for quantitative Mass Spectrometry. The HPA project has generated a large clone library that can be used as a resource to produce these isotopic standards in a high-throughput workflow.

Methods

The isotopic standards, called quantitative PrESTs (QPrESTs), are expressed recombinantly in an E.coli strain auxotrophic for lysine and arginine. Cultivation is performed in minimal auto-induction media in deep well plates. To incorporate heavy isotope-labeled versions (15N and 13C) of arginine and lysine in the protein sequence, heavy isotope-labeled arginine and lysine are added to the medium together with the light versions of the remaining 18 amino acids. After cultivation, the cells are lysed and the QPrESTs are purified with immobilized metal affinity chromatography. Each QPrEST is then analyzed with SDS-PAGE and Mass Spectrometry, and then accurately quantified by Mass Spectrometry using a non-labeled absolutely quantified protein. Thereafter, known amounts of QPrEST can be spiked into unknown biological samples as standards. The endogenous protein concentration is then calculated from the ratio of heavy to light peptides detected by Mass Spectrometry.

Results and Discussion

Per year, 1000 QPrESTs can be produced within the Human Protein Atlas project.

Conclusion

The Human Protein Atlas project has developed a high-throughput production pipeline for QPrESTs to use as internal standards in Mass Spectrometry.

Keywords: Human Protein Atlas, Internal standards, Isotope labeling, Mass Spectrometry

TP10-04

Development of an all-recombinant intact protein standard for LC MS application development and system suitability testing

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Introduction and Objectives

In recent years, interest in intact protein analysis by HPLC, LC-MS, and MS/MS has increased significantly. This can be attributed to both improvements to LC and MS hardware, instrument control software, and data processing software, as well as conceptual shifts in how we can best address and answer biological questions given these emerging commercially available capabilities. Having witnessed the explosive growth of bottom-up proteomics and the subsequent evolution of high-quality, widely accessible standards to normalize platform performance in time and space, and assist with method development for new applications, we recognize a similar need for the Top-down proteomics field. Here, we describe the development of a multi-purpose intact protein standard for LC, LC-MS, and LC-MSMS quality control and application development.

Methods

Protein standards were expressed in E. coli or purchased from Sigma. Multiple proteins were screened and seven were selected that 1) evenly covered a MW range of 12kD – 68kD, 2) presented clean, modification and adduct-free ESI spectra, and 3) whose ESI charge state distributions covered a wide m/z range from 500-2000. Mixing ratios were adjusted such that all seven proteins could be detected simultaneously in a single infusion MS experiment (R&D standard). Quality and stability of selected proteins was verified by SDS-PAGE, UV HPLC, and infusion or LC-MS using an Orbitrap mass spectrometer at both high and low resolution settings.

Results and Discussion

The initial R&D standard, which included cytochrome c (~12kD, charge envelope below 1000m/z), RNAse A (~14kD, charge envelope above 1000m/z), myoglobin (~17kD, charge envelope under 1000m/z), trypsin inhibitor (~20kD, charge envelope over 1000m/z), carbonic anhydrase (~29kD, charge envelope under 1000m/z), enolase (~47kD, charge envelope under 1000m/z), and BSA (~66kD, charge envelope over 1000m/z) met our needs with respect to providing a standard sample that covered a large MW range, a wide m/z range, varying LC-retention characteristics, and consistent MSMS performance. To extend utility of the sample to the broader intact protein analysis community, improvements to long term stability

regulations. As such, new non-animal, recombinant protein candidates were synthesized, affinity captured, and purified[h1] [VR2] . Affinity tags were removed and proteins were dialyzed into MS-compatible buffer. Selected candidates were fully characterized by UV HPLC and LC/MS/MS, and accelerated stability tests were performed on those which met purity, MW, and m/z distribution requirements. The new pre-commercial mixture will be presented here.

Conclusion

A widely-applicable non-animal, affinity tag-free, high quality intact protein standard for LC-MS quality control and application development.

Keywords: Intact/ Top Down protein analysis; animal free protein standard mixture

WO02-01

A proteogenomics approach to reveal molecular mechanisms of COPD

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Introduction and Objectives

COPD is a complex and heterogeneous disease that is influenced by many genetic, environmental factors and smoking. Several genetic and gene expression studies have contributed to our understanding of the molecular mechanisms underlying COPD development. However, these studies did not result in concrete scientific breakthroughs in the understanding of the molecular mechanism of the disease nor in improved treatment options. Present study aims to improve understanding of disease mechanisms by completing the large information collected at genomics and transcriptomics levels with deep proteome information.

Methods

Frozen human lung tissue samples were used from 10 ex-smoking stage IV COPD patients and 8 ex-smoking non-COPD controls. The trypsin digested protein extracts were analysed with 1D- and 2D-LC-MS/MS approaches while RNA sequencing was performed with polyA-selected RNA fraction. For each sample, we reconstructed and quantified transcript isoforms including known and new isoforms caused by genetic alterations, alternative splicing, small indels and post-transcriptional RNA modifications. Patient specific protein reference databases were used for peptide and protein identification using PEAKS allowing identification of novel patient-specific protein forms. Quantitative evaluation of the proteomics dataset was performed with spectral counting and single MS1 ion intensity using TAPP Pipeline. Meta-analysis was applied to combined 1D- and 2D-LC-MS/MS datasets and linear regression analysis based on a negative binomial distribution correcting for age, gender, unwanted variation and interaction between age and gender was used for differential expression analysis.

Results and Discussion

From 17,430 genes that showed appreciable expression on RNA level we identified 124 genes with decreased and 226 genes with increased expression in COPD lung tissue compared to control with FDR level of 0.05. The lung proteome analysis resulted in the identification of 795 peptides unique to protein forms that does not exists in Uniprot. Several high abundant new peptides were identified in COPD patients that match to novel isoforms of smooth muscle tissue proteins. We identified 33 proteins with decreased and 76 proteins with increased levels in COPD lung tissue compared to control. The combined

analysis of the transcripts and proteins showed 11 genes with increased and 3 genes with decreased expression at both levels, while 2 genes showed different direction of expression levels between the two molecular levels.

Conclusion

Using a novel proteogenomics approach in COPD we identified 795 new peptides that match to unique patient specific protein forms in lung tissue, of which the new protein forms from smooth muscle tissue are only present in COPD lungs. Many of the differentially expressed genes were already known to play roles in COPD pathogenesis, while others are newly identified component of the molecular mechanism of COPD.

Keywords: Proteogenomics, COPD, proteomics, transcriptomics

WO02-02

Launch of MissingProteinPedia: Accelerating Discovery of the Human Proteome Project's "Missing Proteins"

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Introduction and Objectives

The Delphic maxim "know thyself" is inscribed on the steps of the ancient Greek Temple of Apollo. Despite the exponential growth of data since 2003, when the human genome read nature's genetic blueprint for building human, we still do not have a comprehensive description of what it means to be human in strictly molecular terms (i.e., genome + proteome + transcriptome + metabolome). The Human Proteome Project (HPP) currently supports this grand challenge by finding high-stringency community-based MS evidence for the ~20,000 proteins expressed by the human genome. The long-term aims of the HPP are twofold. Firstly, to complete, in a stepwise manner, the protein 'parts list' of Homo sapiens (i.e., the draft human proteome) by identifying and characterizing at least one protein product and as many post-translational modifications (PTMs), single amino acid polymorphisms (SAAPs) and splice variant isoforms (SVIs) as possible for each protein-coding gene. Secondarily, to transform proteomics so it is complimentary to genomics across the clinical, biomedical and life sciences.

Methods

The Delphic maxim "know thyself" is inscribed on the steps of the ancient Greek Temple of Apollo. Despite exponential data growth since 2003, when the human genome provided the genetic blueprint for being human, we still do not have a comprehensive description of what it means to be human in strictly molecular terms. The Human Proteome Project (HPP) currently supports this grand challenge by finding high-stringency MS evidence for ~20,000 proteins expressed by the genome. Its long-term aims are twofold. Firstly, to complete, the protein 'parts list' of Homo sapiens by identifying and characterizing at least one protein product and many post-translational modifications (PTMs), single amino acid polymorphisms (SAAPs) and splice variant isoforms (SVIs) as possible for each coded protein. Secondarily, transform proteomics to be complimentary to genomics across clinical, biomedical and life sciences.

Results and Discussion

Here, we focus on the HPP's "missing proteins" defined as the neXtProt protein evidence (PE2-4) proteins, characterising factors that render them unobservable by high-stringency MS. We manually re-analyse all publicly available MS data for the largest missing protein family. We elaborate and encourage on the need for shared high-stringency metrics and re-analysis of all available MS evidence, a common language and broadening of communal data capture to include lower stringency non-MS data.

Conclusion

WO02-02

We launch MissingProteinPedia, a Wikipedia-like listing of missing proteins that is searchable to a compendium of accessible/modifiable pages to allow scientists to deposit/accumulate all types of “omics” scientific data (including PubMed) into a single repository. This could act as a series of clues to where, where, why, how these proteins are expressed in normal, stressed and diseased human cells/tissues, with the sole aim of accelerating HPP discovery.

Keywords: Human Proteome ProjectMissing Proteins

Proteogenomics of human cancer cell lines: coding variants identified by shotgun proteomics

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Introduction and Objectives

Proteogenomics is an emerging field aimed to the integration of multiomics data. In the cancer genome, many protein-coding sequences are dramatically changed by somatic mutations. We used customized exome data and high-resolution shotgun proteomics data to identify coding variants in human cancer cell lines. The important technical question was if the target-decoy search using customized genomic database could correctly identify variant peptides versus chemical modifications which occurred naturally or as procedure artifacts.

Methods

Publicly available genome data were processed to form the customized variant databases for target-decoy search. Data-dependent acquisition shotgun LC-MS/MS data obtained using Orbitrap were downloaded from public resources or kindly provided by authors and re-searched using customized databases. Own proteome data for HEK-293 cell line were obtained with Orbitrap QExactive LC-MS/MS analysis. All searches were done by two search engines. Searches were performed in one-stage mode, where wild-type and customized databases were pooled together, with 1% FDR at the peptide level.

Results and Discussion

First, exome data by Abaan et al. (2013) and proteomics data by Moghaddas Gholami et al. (2013) for cancer cell lines from NCI-60 panel were combined. Using own coding variants for each cell line as a positive control and variants from other cell lines as a negative control, we have found that 80-90% variants for individual cell line search were identified correctly, when the intersection between results of two search engine was used. Thus, such a database search was able to identify variants in shotgun proteomics data. When the pooled variant database was enlarged to include all cancer variants collected by The Cancer Genome Atlas, the real FDR was increased to 30% or more. Further, we identified coding variants of HEK-293 cell line, which had thoroughly characterized proteome. Shotgun proteome data published by Geiger et al. (2012), Chick et al. (2015), and obtained in this work for HEK-293 were searched against the customized genomic database generated using exome data published by Lin et al. (2014). Overall, 112 unique variants were identified at the proteome level out of ~1,200 coding variants annotated in the exome. In all considered shotgun data sets, the variant peptides were at the ratio of 1: 2.5 less likely being identified than the wild-type ones compared with the corresponding theoretical peptides.

Conclusion

WO02-03

Shotgun proteomics, with some limitations, was well suited for identification of cancer protein variants using customized genome sequences. Variant peptides were identified 2. 5-fold less likely than wild-type peptides, most probably, because many coding mutations in cancer were never expressed at the proteome level.

Keywords: Proteogenomics, cancer cell line, database search, shotgun proteome, coding variant

WO02-04

Proteogenomic Profiling of Neoantigens for Personalized Cancer Immunotherapy

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Introduction and Objectives

In recent years, cancer immunotherapy has become an essential element of cancer therapies. Indeed, activation of immune systems by immune checkpoint blocking drugs (e.g. Nivolumab, an antibody against programmed cell death protein 1 (PD-1)) exert a dramatic curative effect on various types of tumors. Cancer peptide vaccine is also an emerging strategy of cancer immunotherapy which induces anti-tumor immune responses activated by administration of neoantigens (peptides possessing cancer specific mutations). The aim of this study is establishment of personalized diagnostics to determine the optimal cocktail of neoantigens using immuno-proteogenomics technology.

Methods

We established a comprehensive neoantigen profiling technology by integrating whole exome sequencing and mass spectrometric immunopeptidome analysis. The whole exome sequences of esophageal cancer cell line TE-11 or surgically-resected endometrial cancer tissue were analyzed by the next generation sequencer (NGS). The TE-11 cells possess HLA-A*24:02 allele which is most frequently observed in Japanese population. Nucleic acid sequences were translated into amino acid sequences and used for database search analysis in subsequent immunopeptidome profiling. Following immunoaffinity purification of MHC class-I molecules, HLA-presenting peptide antigens were isolated by acidic elution and size exclusion chromatography. The resulting peptides were analyzed by LC-MS/MS, searched against individually-constructed NGS-based protein database, and finally quantified on the Expressionist proteome server (Genedata AG, Swiss).

Results and Discussion

Among 3,504 identified HLA-presenting antigen peptides (derived from 1,369 proteins), a variety of predicted neoantigens were successfully detected by LC-MS analysis, including a TP53 fragment with R110L substitution, for example. Importantly, our comprehensive immunopeptidome analysis revealed that length of HLA-A-presenting antigens was strictly restricted to 9 amino acids (~80%) with some discriminative consensus sequences. To enhance the detection sensitivity of antigen peptides from a small portion of tissue samples, we found and confirmed that pre-treatment of interferon gamma (IFN-γ) induced 10-fold upregulation of HLA-A expression level, allowing more sensitive profiling of immunopeptidome.

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Conclusion

Thus, by integrating genomic and proteomic analysis technologies, we can directly grasp which and how much amount of neoantigens are really presented on cancer cells for individual patients. If the Trans-OMICS neoantigen diagnostics could be routinely applied to clinical cancer therapy, we'd be able to provide patients with better personalized precision cancer immunotherapies in the future.

Keywords: Proteogenomics, Cancer immunotherapy, Neoantigen

WO02-05

Missing genes and supplementary tissues in the Human Protein Atlas

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Introduction and Objectives

Within the Human Protein Atlas Project (HPA) there is an ongoing quest to explore the basic building blocks constituting the human body. This is done by systematically exploring all proteins with immunohistochemistry approaches analyzing the majority of human tissue types and comparing with transcriptomic data. Last year all protein-coding genes were categorized by the HPA using RNA-seq data from 32 different tissue types, defining the genes with general expression from the tissue specific genes. Now, 5 more tissues are added and 37 different tissue types is used for the classification. However, more than 1000 genes are still not detected on RNA level in these tissues.

Methods

Uniprot was used to get a better understanding of what type of targets is in this list of not detected genes; potential pseudo gene, uncharacterized genes, olfactory receptors and ligands, keratins, interferons and other semi-known targets. But also secreted proteins, proteins related to seeing, hearing, brain functions and skin. The main reasons for not being detected seems to be either a complexity when it comes to RNA and protein localization, such can be the case for secreted proteins and brain related genes. Or simply the fact that we still have not analyzed the relevant tissue type, the case for ear, eye and tooth.

Results and Discussion

Three tissues, new to the HPA, were analyzed for relevant target proteins; eye, mammary glands and pituitary gland. The criterion for relevance was mainly based on previously published data or external transcriptomic data. More than 100 antibodies are utilized on eye samples and thus provide a spatial protein profile for targets related to seeing. We have also started to investigate brain, skin and adrenal gland for supplementary details. The brain is a complex organ with regions and sub fields as well as advanced networks transporting proteins across the brain, for these reason larger samples of brain areas was used for profiling instead of the standard TMA format. The TMA approach is not suited to capture the complexity in all tissues. Smaller structures like hair follicles or sweat glands in skin are difficult to include in a standardized 1mm sample, additional tissue samples were therefore used for investigation. The adrenal gland is an organ small enough to fit onto one slide and was used to improve the organ overview for several genes.

Conclusion

By expanding the tissues types used for analysis and adding supplementary samples we were able to better understand genes related to the eye, mammary glands, pituitary glands, brain, adrenal gland, skin and hair.

Keywords: Missing genes, missing proteins, Human Protein Atlas, RNA-seq, IHC, protein expression profile

WP01-01

Identification of Proteomic and Proteogenomic Biomarkers of Prostate Cancer in Seminal Plasma

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Introduction and Objectives

In our search for novel prostate cancer biomarkers, we focus on the proteome of seminal plasma (SP), a proximal fluid suitable for discovery of novel markers and development of non-invasive diagnostics. Using mass spectrometry, we previously identified over 3,200 proteins in SP of men with various urological disorders including prostate cancer. Here, we will introduce our proteomic and proteogenomic platforms for biomarker discovery in SP.

Methods

Our proteomic platform integrates profiling of SP by shotgun mass spectrometry followed by verification and validation of candidates by SRM and immuno-SRM assays (Karakosta et al. Mol. Cell. Proteomics, 2016). In addition, we utilize genomic data of the Cancer Genome Atlas in search for proteogenomic biomarkers, such as mutated proteins. To facilitate our diagnostic strategy, we consider as candidates only those proteins that were previously identified in the SP proteome.

Results and Discussion

To select proteomic biomarker candidates, we combined proteins identified through five approaches. First, we mined gene expression microarray data and identified 66 differentially expressed genes. Second, we identified by shotgun proteomics 1,276 proteins in SP from patients with negative biopsy, low-grade and high-grade prostate cancer, and selected 61 differentially expressed proteins. Third, we profiled proteins in the secretome of androgen-dependent and androgen-independent cell lines, and selected 8 differentially expressed proteins. Fourth, we identified in the Human Protein Atlas 50 prostate-specific proteins. Finally, we included 17 androgen-regulated proteins secreted into SP and compiled a list of 160 putative candidates. Following that, we developed a multiplex SRM assay, verified 83 proteins and validated 22 proteins in SP samples obtained from patients with prostate cancer (n=156) and negative biopsy (n=67). The most promising individual biomarker TGM4 (prostate-specific transglutaminase 4) predicted PCa on biopsy with AUC=0.66. As a result, we proposed a panel of SP markers for differentiation between prostate cancer and negative biopsy.To select proteogenomic biomarker candidates, we mined the Cancer Genome Atlas Data for the missense mutations translated to protein level. Of 971 mutated genes, only 250 proteins were found in SP and only 3 proteins had recurrent missense mutations measurable by SRM in SP. Thus, in spite of their high diagnostic specificity (theoretically 100%), proteogenomic biomarkers due to their low frequency have very low diagnostic sensitivity (1-2%) for detection of prostate cancer.

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Conclusion

SP is a promising fluid to discover novel markers of prostate cancer. Here, we proposed a proteomic panel for non-invasive differentiation between prostate cancer and negative biopsy in SP. Linking genomics and proteomics will be crucial to facilitate the new generation of genomics-guided proteomic studies and search for novel prostate cancer biomarkers.

Keywords: Prostate cancer, seminal plasma, proteogenomics, biomarkers, SRM assays

Search Pipeline of Single Amino Acid Variants using neXtProt Database.

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Introduction and Objectives

Single amino acid variants (SAAVs) are known to occurr from somatic variation in tumorous cells as well as germline variation in egg and sperm. The lack of method to find SAAVs using reference database led us to develop a search pipeline using home-made neXtProt-SAAV database which contains about 2.5 million number of variants from 20, 055 human protein coding genes.

Methods

The downloaded RAW files from ProteomeXchange repository of three kinds of proteogenomic studies from lung cancer tissue, liver cancer cell lines and testis tissue, were transformed by RawConverter, and analyzed by our Integrated Proteomic Pipeline including ProLuCID, DTASelect, and ProteinInferencer with global false discovery rate (FDR)<1% at protein level. From the search result, we mined new SAAVs with FDR < 1% at peptide level.

Results and Discussion

In the first case study from lung cancer tissue, we mined more than one hundred SAAVs, where 5.5 times (22/4) more SAAVs identified using neXtProt-SAAV DB than published results. In the second case study from the liver cancer cell lines, total about 700 SAAVs were mined, where more than half of them have corresponding wild type peptides identified. In the third case study from human testis tissue, we mined more than 1,000 SAAVs, where more than half of them have their corresponding wild type peptides identified.

Conclusion

Using SAAV-specific FDR (<1%) calculation, we made neXtProt-SAAV database to mine new SAAVs. We found our search pipeline is informative and effective for SAAV mining from human tissues and cell lines, and will continue to compare between SAAV data and their corresponding rSeq data for further validation study.

Keywords: Proteogenomics, C-HPP, single amino acid variants, neXtProt-SAAV

WP01-03

Proteogenomic approaches to discovery of alternatively spliced proteins in hepatocellular carcinoma cell lines

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Introduction and Objectives

Alternatively spliced proteins (ASPs) become a major cause to increase the diversity of human proteome and affect many biological mechanisms. Identification of ASPs is also one of the major goals of the Chromosome-centric Human Proteome Project (C-HPP)1. Although protein sequence database is required for mass spectrometry (MS)-based peptide identification, many of them cannot fully support cell/tissue-specific ASPs derived from mRNA splicing variants. Thus, we attempted to construct proteogenomic pipeline which can produce protein sequence database for MS-based ASP identification from RNA-seq. We then applied this pipeline to identify differentially expressed proteins and ASPs present in the human carboxylesterase 1 (hCE1) overexpressed (ox-) Hep3B cell lines. Given that hCE1 is known as one of the emerging diagnostic markers of hepatocellular carcinoma (HCC) 2 with multiple metabolic functions, we wish to particularly identify those ASPs in hCE1-ox Hep3B cell lines.

Methods

Extracted RNAs obtained from each Hep3B-Origin, Hep3B-Mock and Hep3B-hCE1-ox cell lines were subjected to RNA seq by Illumina HiSeq2500 sequencer. To make cell line specific protein sequence databases from RNA-seq results, TopHat2 was used to assign RNA-seq reads against reference genome (hg38) and then CuffLins are used for measuring transcript expression levels. Between TopHat and CuffLins, we added novel splicing junction extracting filter. We selected the transcript with FPKM>=1 and one of transcript's exon must be part of known protein coding regions. After selection was made, we translated them on a 3-frame shift. After translation, proteins without starting/stop site are removed and also short proteins (less than 15 amino acid length). MS/MS spectra of protein of each cell line were obtained using Orbitrap MS/MS and identified following HPP Data Interpretation Guidelines Version 2.0.1.

Results and Discussion

Using the RNA-seq data from each cell line, we can make cell line specific ASP DBs. This DB contains total 222,383 novel ASPs which have not previously annotated in Ensembl. The numbers of novel ASPs are 196,646 assembled in Hep3B, 199,772 in Hep3B-Mock and 191,170 in Hep3B-hCE1-ox, respectively, marking the first comprehensive collection of ASPs in HCC cell lines. The newly identified ASPs will not only help us understand how hCE1 exerts its multiple biological functions in HCC but also identify those proteins involved in hepatocellular carcinogenesis.

Conclusion

We developed the proteogenomic pipeline which can make the sample-specific novel ASP sequence databases from RNA-seq dataset. This can be applied to identification of hCE1 regulated proteomes including ASPs. We anticipate that this approach can identify

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novel ASPs from the biological samples which are also applied to discovery of sample specific changes in splicing variants in the levels of RNA and ASP expression. (This work was supported by a grant from the Korean Ministry of Health and Welfare (HI13C2098 to Y.K.P.).

Keywords: c-hpp, proteogenomics,HCC

Multiplexed Mass Spectrometric Screening of EGFR Mutation in Non-small-cell Lung Cancer

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Introduction and Objectives

High throughput and systematic sequencing of cancer genome has enabled the comprehensive characterization of somatic mutations. However, the expression level from transcription to translation is not always quantitatively correlated. Mass spectrometry (MS)-based proteomics approach is a powerful tool for reliable identification of peptides/proteins which heavily relies on the database searching against protein sequence database. The onco-proteogenomics analysis of mutated proteins, which integrates cancer proteomics with genomics data, could facilitate identification of cancer-specific protein mutation in clinical samples. Using EGFR as a model, the goal of this study is to develop a MS-based multiplexed identification platform for screening tumor-specific mutations on oncoproteins.

Methods

The non-small cell lung cancer (NSCLC) cell lines with different EGFR subtypes were used a model in this study. The in-silico digestion was applied with different proteases (e. g. trypsin, Asp-N, Glu-C, Lys-C, and Pepsin) in parallel or in combination for generating detectable EGFR mutated peptides. The EGFR was purified by immunoprecipitation with an antibody against the extracellular domain of EGFR, followed by parallel proteases digestions and LC-MS/MS analysis. Mutated EGFR peptides were identified using multiple search engines against the customized protein sequence databases. All matches were filtered to 1% false discovery rate.

Results and Discussion

We constructed two customized protein sequence databases composed of 40 EGFR variants, EGFR-interacting proteins and the entire reference proteome from UniProtKB and STRING analysis with high confidence score. The in-silico digestion suggested the combination of multiple enzymatic digestion would facilitate the identification of mutated EGFR peptides. The result detected the peptides from EGFR exon 19 deletion (E746-A750) and their corresponding wild-type encoded from heterozygous EGFR Del19/WT in PC9 cells. The sequential digestion of Asp-N and Pepsin was applied to simultaneously distinguish the point-mutated peptides encoded from EGFR L858R/WT in H3255 cells. Only the wild-type amino acids at position 719 and 790 in EGFR were identified in both PC9 and H3255 cells, which is consistent with genomic DNA data.

Conclusion

WP01-04

By developing the MS-based screening platform which integrates affinity purification, multiple enzymatic digestions, MS detection and database searching against the constructed mutated protein sequence databases, we were able to identify the wide-type and mutated EGFR peptides in a panel of NSCLC cell lines. We expect that the MS-based strategies can precisely determine the status of somatic mutations at protein level and will be a promising platform to assess multiple mutation proteins in clinical samples.

Keywords: Lung cancer, proteogenomics, mass spectrometry, EGFR

Protein expression landscape of mouse embryos during pre-implantation development

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Introduction and Objectives

Pre-implantation embryo development is an intricate process orchestrated by maternally inherited proteins and newly synthesized proteins following zygotic genome activation. These events must be precisely regulated and function successively to ascertain the development of an embryo into functional blastocyst. Although many genomic and transcriptomic studies have enriched our understanding of genetic and epigenetic regulations underlying this process1-5, the protein expression landscape remains unexplored owing to limited materials.

Methods

Here, we collected over 8000 mouse embryos of each developmental stage (zygote, 2-cell, 4-cell, 8-cell, morula, blastocyst) to perform tandem mass tag (TMT)-based quantitative mass spectrometry and identified nearly 5000 proteins in each stage. I

Results and Discussion

n-depth analysis indicates that protein expression profiles of zygote, morula and blastocyst show apparent difference from 2- to 8-cell embryos due to the maternal-totipotent-differentiation transition. Analysis of protein phosphorylation led to extraction of critical kinases and signal transduction pathways governing early embryo development. We further identified novel factors and proved that they play important roles in determining pre-implantation embryo development through RNA interference. Finally, combined analysis of transcriptomic and proteomic data reveals coordinated control of RNA degradation, transcription and translation in early embryos, and additionally identifies novel exon junction-derived peptides.

Conclusion

Taken together, our study not only provides an invaluable resource for further mechanistic studies of each individual protein, but also suggests novel players and biological processes that might govern pre-implantation embryo development.

Keywords: embryo proteome, TMT, quantitative proteomics, proteogenomics

WP01-06

Have small proteins been overlooked? A proteogenomics approach using ribosome profiling, MS and bioinformatics.

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Introduction and Objectives

Small open reading frames (sORFs) that encode peptides that are 100 amino acids or fewer in length are underrepresented in genome annotations. In the last decade several of these so-called micropeptides have been functionally characterized: hemotin (Pueyo et al., 2016), myoregulin (Anderson et al., 2015), and tal/pri (Kondo et al., 2010) to name a few. The search for these micropeptides was nourished with the advent of ribosome profiling (RIBOseq). This next generation technique recovers and subsequently sequences the ±30 nt RNA fragments captured within translating ribosomes thus providing a 'snap-shot' of what is being translated in a cell, rather than what is expressed in a cell. Mass spectrometry and new bioinformatics approaches, are being used to support annotation of these novel features. Our objective is to compile an public resource of putative sORF-encoded polypeptides (www.sORFs.org).

Methods

The RIBOseq data are mapped to the reference genome using the PROTEOFORMER pipeline (Crappé et al., 2015), only translation products between 10-100 amino acids long are retained. Currently three different species are supported (human, mouse and fruit fly), but many are to follow in the near future. The coding potential of the identified sORFs is assessed in multiple ways based on (1) sequence conservation (PhyloCSF), (2) ribosome profiling data using different scoring algorithms (FLOSS, ORF-score), (3) sequence variation presence using the Ensembl variation database, (4) sequence homology using BlastP matching against the non-redundant protein database and (5) mass spectrometry identification using the PRIDE ReSpin toolset. This latter automated set-up enables to reprocess PRIDE datasets and was set-up in a two stage search approach: a filtering search identifying all spectra at a 1% FDR rate at the PSM level against UniProt-KB including isoforms, and the cRAP library, and a follow-up search of the non-validated spectra against a sequence database containing the sORF peptides.

Results and Discussion

Currently www.sORFs.org harbors 266,342 sORFs identified in human, mouse and fruit fly. From these 266,343 sORFs, 4620 human, 292 mouse, and 1030 fruit fly sORFs were predicted as coding applying stringent thresholds described in literature (ORFscore > 6, PhyloCSF > 0 and a good FLOSS classification). The PRIDE ReSpin pipeline acquired evidence for 407 sORFs resulting from the human stringent sORFs dataset (preliminary results).

Conclusion

Although the attention to these missed small proteins has grown significantly, this research field is still in its infancy. Building a comprehensive public repository based on ribosome profiling to map this overlooked category of small proteins will certainly help

their characterization. Furthermore, new visualization tools and intuitive querying interfaces will enable wet lab researchers to question this pool of information built from novel NGS, MS and bioinformatics techniques.

Keywords: Proteogenomics, sORF, micropeptide, missed protein, ribosome profiling, MS

A comprehensive proteogenomic workflow reveals novel insights into leukemogenesis.

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Introduction and Objectives

Here we describe a comprehensive proteogenomic analysis of the mechanisms underlying the poorly understood leukemogenic activity of MLL-translocations. We combined deep proteomic quantitation of cell surface (>1000 proteins) and global proteins (>6000 proteins) with transcriptome RNA-sequencing. This comprehensive quantitative approach identified genes involved in leukemogenesis that are regulated by MLL fusion protein expression and revealed novel pathways and targets. Functional validation using targeted CRISPR/Cas9 gene disruption has also identified a number of novel protein biomarkers that regulate leukemia biology and predict for poor survival outcome in human AML patients.

Methods

We have used an in vivo mouse model of acute myeloid leukemia (AML) that is controlled by regulating expression of a common MLL-translocation gene, MLL-AF9. AML cells underwent both global and specific cell surface protein expression profiling using SILAC quantitative proteomics to identify proteins that are expressed during regulation of MLL-AF9. We extended this analysis by profiling changes to the transcriptome using RNA-sequencing. Bioinformatic analysis was performed to compare changes in protein and RNA expression before comparison to human AML expression array and survival data to identify proteins and pathways of diagnostic and therapeutic interest. Genes of interest were then functionally validated in primary AML cells using targeted CRISPR/Cas9 gene disruption.

Results and Discussion

Our results demonstrate that the cell surface expression profile of AML cells is unique from either RNA or global protein expression. Utilizing the RNA-sequencing data to inform our protein database resulted in the identification of novel proteins that are enriched in AML cells. Combining our proteomic and genomic datasets allowed the validation of several known targets in AML and also identified novel pathways and targets that are up-regulated when MLL-AF9 is expressed. These pathways include epigenetic regulators, cell signaling and cell-cell or cell-extracellular matrix interactions. We have extended our analysis by comparing the gene expression signature from our in vivo mouse models to human AML patient expression and survival data resulting in the identification of proteins that can be used as biomarkers to predict high risk and poor survival outcome in AML patients. We have functionally validated these genes using targeted CRISPR/Cas9 gene disruption and identified a number of proteins that contribute to AML survival and proliferation. The results from this study not only identify mechanisms by which MLL-AF9

regulates leukemogenesis, but also demonstrate the potential identification of clinical biomarkers and novel drug targets.

Conclusion

We have undertaken a comprehensive proteogenomic analysis of leukemia which revealed proteins that are of biological and clinical significance in human disease.

Keywords: Acute Myeloid Leukemia, cell surface, SILAC, biomarker discovery, proteogenomic

WP01-08

Proteogenomic Monitoring and Assessment of Increased Thermogenesis in response to β-Adrenergic Signaling in Ahnak Deficiency

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Introduction and Objectives

Systems-wide profiling of thermogenesis has almost always entailed RNA and DNA analysis by sequencing techniques. Robust interpretation of discreet biological domains remains a significant challenge in the face of complex biochemical regulation processes. Here, we report that AHNAK deficiency promoted browning and thermogenic gene expression in WAT in response to the CL-316243.

Methods

To find out an important role of AHNAK in the regulation of thermogenesis and lipolysis in WAT via β-adrenergic signaling, we performed in-depth proteogenomic profiling of Ahnak-/- vs wild-type mice. We analyzed 24 tissue samples in response to β-Adrenergic Signaling in Ahnak Deficiency mice and reached a quantitative depth of >1,000 proteins. Furthermore, we derived a signature candidates of browning and thermogenesis, which differ the response after treatment, through self-organization map (SOM) - based classification and feature selection.

Results and Discussion

Remarkably, only some proteins of the signature were associated with and reflected on the mRNA level. Through the integrated analysis of genomic and proteomic data, we demonstrated that ADRB3 agonist CL-316243 increased thermogenesis and activated PKA signaling in Ahnak deficiency mice compared to wild-type mice.

Conclusion

Overall, these findings suggest an important role of AHNAK in the regulation of thermogenesis and lipolysis in WAT via β-adrenergic signaling. These mouse phenotype features revealed by our work provide novel insights that may ultimately translate to development of phenotype-specific therapeutics.

Keywords: Proteogenomic, Ahnak, omics

proBAMsuite: a bioinformatics framework for genome-based representation and analysis of proteomics data

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Introduction and Objectives

Mass spectrometry based shotgun proteomics enables integrative analysis of proteomic and genomic datasets. However, the de facto community standards for proteomic data representation defined by the Human Proteome Organization (HUPO) Proteomics Standards Initiative (PSI) do not intuitively embed genomic information thus hinder such analyses. To address this challenge, we developed a new framework, proBAMsuite, in which a central component is the protein BAM (proBAM) file format for organizing peptide spectrum matches (PSMs) in the context of the genome, to facilitate genome-based representation and analysis of proteomics data.

Methods

The central component of proBAMsuite is the proBAM file format. The fundamental difference between proBAM and BAM is that PSMs replace sequence reads as the basic data unit in the proBAM format. Novel mandatory fields are introduced in the alignment section to accommodate unique features of proteomics data such as PSM scores, charge states, and protein modifications. We developed the R package proBAMr to map peptides back to the genome. proBAMr generates SAM files, which are subsequently converted to the binary BAM format and indexed for fast access using SAMtools. We developed another R package proBAMtools to perform various analyses based on the proBAM files. proBAMtools includes functions for genome-based proteomics data interpretation, protein and gene inference, count-based quantification, and data integration.

Results and Discussion

We demonstrate the utility of proBAMsuite by applying it to three recently published proteomics datasets. Firstly, the interpretation of proteomics data in relate to the genome is intuitive and straightforward. With well wrapped functions proBAMtools reports coverage of the whole human coding genome, classifies peptides according to their mapping property, etc. Secondly, PSMs can be easily re-annotated using user-specified gene annotation schemes and assembled into both protein and gene identifications. proBAMtools supports both protein-level inference and gene-level inference. Thirdly, using the genome as a common reference, proBAM meets critical needs in proteomics and proteogenomics data integration. proBAM alleviates the compatibility problem by bringing different proteomics datasets into the same coordinate system, i.e. the genome, thereby allowing data integration without re-searching the raw data. Finally, proBAM files can be readily visualized in genome browsers. All three datasets are made available in a JBrowse-based genome browser (http://bioinfo.vanderbilt.edu/proteogenomics), which allows communication of proteomics data to a general audience beyond the proteomics community. Peptide evidence for alternative transcript isoforms, exon-exon junctions, and mutations can be easily retrieved and visualized under this framework.

WP01-09

Conclusion

proBAMsuite provides genome-based representation of proteomics data, thus introduces novel data analysis, visualization, and interpretation opportunities.

Keywords: Proteogenomics, data format

GAPP: a proteogenomic software for genome annotation and global profiling of posttranslational modifications in prokaryotes

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Introduction and Objectives

While the number of sequenced prokaryotic genomes is growing rapidly, experimentally verified annotation of prokaryotic genome remains patchy and challenging. Proteogenomics is defined as the use of proteomic data, often derived from MS, to improve and refine genome annotation. It has emerged as a promising and indispensable approach to genome annotation and been applied for genome annotation including identification of novel genes, correction and validation of predicted genes in various organisms. Over the last few years, a number of automated software, including several visualization and database-building tools, have been developed for integration of MS-based proteomic data into genome databases. However, there is still a lack of automated software for proteogenomic analyses that incorporate both genome annotation and proteome-wide PTM analysis. To fill this gap, we developed a one-stop open source software termed GAPP.

Methods

GAPP is developed in Java and Perl, and the executables are distributed for Windows platforms. The source code and custom databases are free for noncommercial use and available at https://sourceforge.net/projects/gappproteogenomic/. Three pregenerated search algorithms X!Tandem, MSAmanda, and MSGF have been integrated into the GAPP tool for proteogenomic analysis. The unrestricted search algorithm MODification via alignment (MODa) is already integrated as a part of the standard distribution for PTM discovery. Moreover, integration with the results from other existing search algorithms is easily assembled into this tool.

Results and Discussion

To test the effectiveness and versatility of GAPP for annotating prokaryotic genomes, we analyzed proteomic data from the Helicobacter pylori strain 26695 (H. pylori). Previously, Müller et al. identified 71% of the predicted proteome using a typical proteogenomic approach. In this study, we used GAPP to perform an in-depth proteogenomic analysis of H. pylori using publicly available proteomic data. Our results confirmed 82.5% of predicted H. pylori gene products, identified 40 novel protein coding genes, and corrected four existing gene models with regard to translation initiation sites. In particular, GAPP revealed a large repertoire of PTMs using the same proteomic data and provided a rich resource that can be used to examine the functions of reversible modifications in this human pathogen.

Conclusion

GAPP is a one-stop proteogenomic software for prokaryotic genome refinement and global identification of PTM events from the same experimental proteomic datasets. It is easy to configure and run in one command line. This software allows concurrent querying

WP01-10

of proteomic and genomic databases to refine genome and identify diverse PTM events. This includes MS data and database preprocessing, database searches, FDR calculations, statistical result integration, biological interpretation, and global PTM discovery.

Keywords: Proteogenomics, High-accuracy tandem mass spectrometry, Genome annotation,Posttranslational modifications, Prokaryotes

Identification for protein-level evidence of genomic variants in cancer cells using new proteogenomic approach

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Introduction and Objectives

Variations in the coding sequence of genes causing amino acid codon alterations may lead to protein misfolding, polarity shift, improper phosphorylation and other functional consequences. Since expressions of such variants are often suppressed by cellular quality control systems, it is required to confirm their protein-level expression. The goal of our study was to identify genomic variants at the protein level, and to monitor the expression of such variants from DNA to protein.

Methods

Generally, proteomic data in proteogenomics are acquired based on shotgun proteomics, using liquid chromatography coupled to mass spectrometry (LC-MS/MS). Shotgun proteomics are usually performed by data dependent acquisition method (DDA) for identification of peptides. This method has a limitation in identifying peptides from highly complex samples, due to poor peptide reproducibility and automated ion selection. As an alternative to this disadvantage, various methods have been reported, such as DDA with inclusion list (Inclusion) and data independent acquisition method without precursor ion selection (PAcIFIC). However, limitations of these methods are that they require time-consuming analysis and a demand for large amount of sample. In these methods, the running time is very often spent in analyzing insignificant peptides. By combining these methods, we have developed a proteogenomic strategy that complements the disadvantage of each method. We named the strategy as Sequential Targeted LC-MS/MS based on Prediction of peptide pI and Retention time (STaLPIR). The STaLPIR consists of two precursor ion dependent acquisition methods (DDA and Inclusion), and one precursor ion independent acquisition method with target list (TargetMS2).

Results and Discussion

We present an analysis of nonsynonymous variants at the protein level by using our STaLPIR method on gastric cancer cells. Briefly, we integrated the entire exome sequence data and STaLPIR data. Subsequently, we selected a set of 296 nonsynonymous variants and confirmed the expression of 147 variants at the protein level, with different expression pattern of variants according to allele type during expression of DNA to protein. Integrated analysis of DNA, mRNA and protein suggests that protein expression level of the nonsynonymous variant is regulated either before or after translation, according to influence of the variant on protein function. Surprisingly, despite the rise of studies on variants using proteogenomics, few have attempted to

address the expressed feature of variants at the protein level.

Conclusion

Our approach shows improved peptide identification, and has the potential for the unbiased analysis of variant sequence as well as corresponding reference sequence. The STaLPIR approach can also be applied to getting protein-level evidence of other genomic variations such as in-del, genes fusion, and alternative splicing.

Keywords: proteogenomics, genomic variation, proteomics, genomics, transcriptomics

Proteogenomic Monitoring and Assessment of Increased Thermogenesis in response to β-Adrenergic Signaling in Ahnak Deficiency

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Introduction and Objectives

Systems-wide profiling of thermogenesis has almost always entailed RNA and DNA analysis by sequencing techniques. Robust interpretation of discreet biological domains remains a significant challenge in the face of complex biochemical regulation processes. Here, we report that AHNAK deficiency promoted browning and thermogenic gene expression in WAT in response to the CL-316243.

Methods

To find out an important role of AHNAK in the regulation of thermogenesis and lipolysis in WAT via β-adrenergic signaling, we performed in-depth proteogenomic profiling of Ahnak-/- vs wild-type mice. We analyzed 24 tissue samples in response to β-Adrenergic Signaling in Ahnak Deficiency mice and reached a quantitative depth of >1,000 proteins. Furthermore, we derived a signature candidates of browning and thermogenesis, which differ the response after treatment, through self-organization map (SOM) - based classification and feature selection.

Results and Discussion

Remarkably, only some proteins of the signature were associated with and reflected on the mRNA level. Through the integrated analysis of genomic and proteomic data, we demonstrated that ADRB3 agonist CL-316243 increased thermogenesis and activated PKA signaling in Ahnak deficiency mice compared to wild-type mice.

Conclusion

Overall, these findings suggest an important role of AHNAK in the regulation of thermogenesis and lipolysis in WAT via β-adrenergic signaling. These mouse phenotype features revealed by our work provide novel insights that may ultimately translate to development of phenotype-specific therapeutics.

Keywords: Proteogenomic, thermogenesis, lipolysis, Ahnak, CL, Adipocyte

WP01-13

A rigorous proteogenomics workflow to discover functional novel-coding loci and single amino acid variants

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Introduction and Objectives

Cancer cells are characterized by driver mutations. In addition, non-coding RNAs and pseudogenes have also been shown to play functional roles in cancer development. Despite this, conventional proteomics is limited to canonical proteins.

Methods

We present a rigorous and comprehensive proteogenomics workflow to identify novel peptides from undiscovered protein-coding loci and variant peptides coded by SNPs and mutations.

Results and Discussion

This workflow was validated using a cancer cell line (A431) supported with whole genome sequencing and RNA-seq data and used to explore five normal human tissue types. Our proteogenomics analysis showed that a number of pseudogenes are translated, hence forming novel human coding paralogs, and moreover the quantitative analysis reveals tissue specific regulation patterns. As part of the workflow, we provide a tool, SAAValidator, for automated inspection of variant peptide spectra and demonstrate that it substantially increases the confidence of identified variant peptides.

Conclusion

Altogether, the here described proteogenomics workflow can be used as pipeline to discover novel and variant peptides for improved annotation of protein coding regions and detection of potential neo-antigens in cancer.

Keywords: Proteogenomics, cancer, pseudogene, novel coding loci, single amino acid variants

WP02-01

Human Personal Omics Profiling (hPOP)

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Introduction and Objectives

The influence of genome variation, modulated by lifestyle and environment, triggers an extensive phenotypic variability found in human populations. Profiling and quantification of large cohort of diverse individuals, provides important information for better understanding the relationship of individual's proteome with their background, medical history and lifestyle.Recent advances in high throughput technologies allow profiling of thousands of analytes within a single experiment. These measurements could potentially be used to diagnose disease early, monitor treatment progression and stratify patient groups to ensure each individual obtains the treatment best suited to their needs. This personalized approach to medicine would include continuous monitoring of thousands of parameters over a whole lifetime. However, in order to be able to interpret such data, we need to have a better understanding of the underlying natural variation of biological molecules in large crowds. Only if we know the natural ranges of individual analytes, the expected responses to perturbations and the long-term trends in their levels, can we draw meaningful conclusions from comprehensive personalized profiling.

Methods

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become one of the most powerful techniques in profiling human proteome. Data independent acquisition with "Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra" (SWATH) provides data-independent fragmentation of all precursor ions entering the mass spectrometer in isolated window. Continuous stepping of isolation window covers the whole m/z range of interest. This allows repeated analyses of each window during the elution of a single chromatographic peak and therefore a deeper fragment ion map of the sample.

Results and Discussion

As a pilot phase of our study, we have collected blood, urine and stool samples from 31 healthy individuals who attended 2016 US HUPO conference. These participants vary in different ages, nationality, ethnicity and backgrounds. We are planning to extend the project to several hundred participants in the upcoming international HUPO conference. Using plasma and PBMCs of subjects we are able to look at the variation of proteome of this diverse crowd and relate it to their food/exercise/stress habits and their medical history. The protein profile of these individuals can be studied parallel to their DNA/RNA sequence, metabolome, transcriptome and metagenome and therefore personal omics of each individual can be profiled.

Conclusion

Large dynamic range of protein concentrations plasma make plasma proteome technically challenging and time-consumable to study. We performed several optimizations on

WP02-01

current SWATH mass spectroscopy method and after depletion of first 14 abundant proteins we can now quantify more than 450 proteins in human plasma sample with coefficient of variation (CV) less than 20% and false discovery rate (FDR) of 1% in only 60 minutes.

Keywords: SWATH Mass Spectroscopy

Monitoring oxidative stress and progression to cell death: from secretome to blood diagnosis

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Introduction and Objectives

Oxidative stress is perhaps the main cause of cell death involved in a large variety of disorders. However, oxidative stress (de)regulation may occur through diverse mechanisms leading to different responses, which can be reflected in the secreted molecules. Under controlled conditions, this may be reflected by the molecules secreted which can play key roles in the regulation of many physiological processes and thus, be good predictors of the cellular physiological state. As a result, the secretomes obtained under controlled conditions can be is an important source of potential biomarkers, more likely to be reflected in biological fluids. Therefore, the aim of the present work was to obtain a comprehensive panel of markers from the secretome analysis which can be used to distinguish the changes caused by oxidative stress regulation or induction of cell death, and thus be able to monitor indicators of cell death progression in blood.

Methods

A cell model was treated with different stimulus of hydrogen peroxide: (i) to induce oxidative stress without cell death, and (ii) to induce oxidative stress with cell death. The newly generated secretomes spiked with the proper internal standards, were analyzed by a quantitative mass spectrometry approach (SWATH-MS). Additionally, cell viability assays were conducted to correlate with the MS data. The markers identified in the previous approach were then validated in cerebrospinal fluid (CSF) and serum from 6-hydroxydopamine (6-OHDA) injected animals, a model of Parkinson's disease.

Results and Discussion

A large number of molecules were quantified between control and mild oxidative stress conditions, from them 119 up- and 115 downregulated proteins, and 519 up- and 921 downregulated metabolite features were highlighted by allowing a clear distinction between the conditions. Since no impact in cellular integrity was detected, these markers can be considered indicators of stress previous to cell death. In addition, 24 proteins markedly increasing in the cell death condition were detected, being good extracellular indicators of cell death. From the identified panels of markers, 3 reveal to be able to distinguish the 6-OHDA injected animals from the controls in both CSF and serum samples.

Conclusion

An integrative approach was introduced and successfully applied in the identification of

oxidative stress biomarkers in cellular secretome, allowing the creation of libraries of proteins and metabolites that can be used to interrogate other samples for routine analysis. To transpose the proposed method to clinical diagnosis, these potential biomarkers were further validated in CSF and plasma from animal models subjected to an oxidative stress insult, revealing to be capable to distinguish the two groups. Finally, these three markers will be further validated in plasma of patients with neurodegenerative disorders, where they will be used to evaluated the progression of the disease and the respective therapy.

Keywords: Extracellular Biomarkers, Oxidative Stress, SWATH-MS

WP02-03

Meta-analysis of omics profiling to reveal translational regulation of chronic hypoxia stress in colon cancer cells

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Introduction and Objectives

Hypoxia plays a central role in most solid tumor with a poor clinical prognosis owing to its multiple contributions to chemoresistance and radioresistance, angiogenesis and vasculogenesis, tumor invasion and metastasis, and anti-apoptosis. Dysregulation of protein expression has been associated with the development of cancer, and it is needed to be investigated in many different cancers during hypoxia.

Methods

Here we present a comprehensive omics profiling of hypoxia-induced gene regulation for the first time. Quantitative mass spectrometry (MS) analysis of hypoxic proteome/secretome was performed in colon cancer cells.

Results and Discussion

A total of 5700 proteins were quantified from proteome and 722 proteins were quantified from secretome under hypoxic conditions. These proteome datasets were taken for further meta-analysis with transcriptome and translatome datasets. MS analyses were verified by Western blotting to confirm candidate proteins that were isolated from hypoxic versus normoxic conditions. More than 1000 proteins were compared with their transcription and translation levels, and 49 proteins were differently regulated at the translational level during hypoxia.

Conclusion

Finally, we provide evidence that hypoxia-induced translational activation promotes extracellular matrix organization, extracellular exosome and protein processing in endoplasmic reticulum in colon cancer cells. These pathways seem to be associated with an increased risk of tumor invasion and metastasis. Altogether, this dynamic working model may facilitate the development of new anti-cancer therapeutics for the treatment of human colon cancer.

Keywords: Hypoxia, Transcriptome, Translatome, Proteome, Secretome

Extracellular Vesicles - A novel class of biomarkers

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Introduction and Objectives

Extracellular Vesicles (EVs) are present in nearly all body fluids like blood plasma, urine, saliva, breast milk fluid and cerebral spinal fluid. They are key players of various intercellular pathways and contain a specific composition of lipids, proteins and genetic information. The most abundant EVs in the circulation are derived from platelets (PLTs). PL-EVs are involved in vascular and metabolic diseases, neurodegeneration, chronic inflammation, autoimmunity, and cancer. Activated PLTs and PL-EVs play a crucial role in atherosclerosis, enhancing thrombosis and atherosclerotic plaque formation.To investigate the total content of these PL-EVs, we used multiple omics techniques.

Methods

PL-EVs were isolated by differential gradient ultracentrifugation into 5 distinct platelet derived vesicle subfractions (PL-EV F1-F5) and platelet exosomes (PL-EXs) and characterized by Nanoparticle Tracking Analysis, Flow Cytometry, proteomic/lipidomic mass spectrometry and miRNA profiling.

Results and Discussion

Isolated PL-EVs subfractions show overlapping particle sizes of 180-260 nm, but differed molecular composition. Less dense, intermediate and dense PL-EVs respectively are enriched in lipidomic and proteomic markers for plasma membrane, intracellular membranes/platelet granules and mitochondria. The Parkinson related protein, alpha-synuclein, accumulates in early fractions F1-F2, while the amyloid beta precursor protein, the hallmark protein for Alzheimer's disease can be found in Fractions F3-F4. The apolipoproteins ApoE and ApoJ are predominantly found in F3-F5. MiRNA profiling indicates enrichment of neurological disease-relevant miRNAs in PL-EVs.

Conclusion

The different lipid and protein compositions of PL-EV subfractions suggest their unique cellular origins, partly overlapping with platelet granule secretion. Dense PL-EVs might represent autophagic vesicles released during platelet activation and apoptosis. Segregation of alpha-synuclein and amyloid beta precursor protein, ApoE/J into less dense and dense PL-EVs, respectively, show their differential carrier role of neurological disease-related cargo.

Keywords: early BiomarkerAlzheimer´s Diseaseextracellular vesicles

WP02-05

Integrative Multi-Omic Analysis of a Single Immune Cell Type

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Introduction and Objectives

Cellular function and diversity are orchestrated by complex interactions of fundamental biomolecules including DNA, RNA and proteins. Technological advances in genomics, epigenomics, transcriptomics and proteomics have enabled massively parallel and unbiased measurements. Such high-throughput technologies have been extensively used to carry out broad, unbiased studies, particularly in the context of human diseases. Nevertheless, a unified analysis of the genome, epigenome, transcriptome and proteome of a single human cell type to obtain a coherent view of the complex interplay between various biomolecules has not yet been undertaken. Here, we report the first multi-omic analysis of human primary naïve CD4+ T cells isolated from a single individual.

Methods

Blood samples were obtained for isolation of hematopoietic cells after obtaining informed consent from a healthy subject. Peripheral blood was collected in sodium heparin containing vacutainer blood collection tubes and peripheral blood mononuclear cells (PBMCs) were enriched by Ficoll gradient. Naïve CD4+ T cells were isolated using magnetic beads following manufacturer's instructions (Miltenyi Biotec #130.094.131) and assessed using FACS Calibur, with the average purity of each isolation being ~95 %. Resting memory cells were isolated by enriching pan resting CD4+ T cells followed by CD45RO depletion of resting memory CD4+ T cells, with the average purity of each isolation also being >95 %.

Results and Discussion

Integrating multi-omics datasets allowed us to investigate genome-wide methylation and its effect on mRNA/protein expression patterns, extent of RNA editing under normal physiological conditions and allele specific expression in naïve CD4+ T cells. In addition, we carried out a multi-omic comparative analysis of naïve with primary resting memory CD4+ T cells to identify molecular changes underlying T cell differentiation. This analysis provided mechanistic insights into how several molecules involved in T cell receptor signaling are regulated at the DNA, RNA and protein levels. Phosphoproteomics revealed downstream signaling events that regulate these two cellular states. Availability of multi-omics data from an identical genetic background also allowed us to employ novel proteogenomics approaches to identify individual-specific variants and putative novel protein coding regions in the human genome.

Conclusion

We utilized multiple high-throughput technologies to derive a comprehensive profile of two primary human cell types, naïve CD4+ T cells and memory CD4+ T cells, from a single donor. Through vertical as well as horizontal integration of whole genome sequencing, methylation arrays, RNA-Seq, miRNA-Seq, proteomics, and phosphoproteomics, we

WP02-05

derived an integrated and comparative map of these two closely related immune cells and identified potential molecular effectors of immune cell differentiation following antigen encounter.

Keywords: Multi-Omics, Naive CD4+ cell

The Dynamic Responses of Gene Expression in Meiosis I prophase of Mouse Spermatocytes

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Introduction and Objectives

Male infertility mainly resulted from abnormal development of sperm. While the meiosis occurs exclusively in gametogenesis and is the key step of sperm maturation, the study focusing on such meiosis in mammalian is difficultly implemented due to technique barriers in separation and collection of enough cells of different stages in meiosis. We therefore for the first time initiated the project to efficiently collect the spermatocyte at meiotic phases in mouse and closely monitor the dynamic responses of gene expression during meiosis progress.

Methods

The spermatogenesis in mouse were synchronized to enrich the stage specific cells. The spermatocytes at four meiotic phases, leptotene, zygotene, pachytene and diplotene, were separated and collected by flow cytometry after digestion of the testis tissues. The transcriptomes in these spermatocytes were profiled using RNA-Seq with Hi-seq4000 and their quantitative proteomics using iTRAQ labelling were surveyed by Q-Exactive mass spectrometer.

Results and Discussion

The total transcripts at each stage of meiosis showed similar sizes, and the unique transcripts at each stage were less than 5%. A total of 12954 protein coding genes were identified, in which 4162 genes were significantly differentially expressed between any two stages. The proteomic data exhibited that total of 5116 proteins were identified, in which 1460 were differentially expressed. Over 90% of the identified proteins were also identified at transcriptional level, however, the abundance correlation for the two expression levels was only 0.068, indicating that the transcriptional abundance lacks indicative meaning to the translational abundance and vice versa. The functional enrichment analysis of the relatively upregulated genes in the four stages revealed that the genes associated with meiosis or spermatogenesis only enriched in the pachytene spermatocytes at transcriptional level, while the genes associated with the meiosis or spermatogenesis such as synapsis, DNA replication initiation, meiotic nuclear division were enriched in all the stages of the meiosis I prophase at proteome level. It seems that the proteome abundance changes were more closely responded to the meiotic development. Notably, several cilium movement, sperm motility, axoneme assembly and cilium assembly related genes were significantly upregulated during meiosis I prophase, suggesting that some genes were at the “prepared” status in advance for their following functions in sperm maturation.

Conclusion

We successfully collected enough mouse spermatocytes at four different stages of

WP02-06

meiosis I prophase. By exploring both the transcriptome and proteome quantitative profiling, we found that there was only a few of genes specifically expressed at one stage, while nearly 30% genes were differentially expressed between the neighbour stages. Dynamic analysis further demonstrated the abundance changes of transcripts and proteins followed a strict sequential order during spermatogenesis.

Keywords: meiosis, proteome, transcriptome

WP02-07

Towards an in-depth overview about increasing arginine production in C. glutamicum by rational strain design using metabolomics and proteomics

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Introduction and Objectives

C. glutamicum is a gram negative bacterium used for the biotechnological production of amino acids and other primary metabolites. Arginine is a glutamate-derived amino acid of commercial importance in cosmetic and pharmaceutical industries and as a food additive. Here we highlight that combining rational strain design with metabolomics and proteomics is a powerful tool to increase production of desired metabolites in a biotechnological, bacterial workhorse.

Methods

Three mutant strains were compared to wildtype C. glutamicum extracts. Metabolomics and proteomics data have been acquired on an impact II QTOF-MS (Bruker Daltonics) instrument using data dependent MS/MS methods. The novel MetaboScape software was used for processing of metabolomics data, whereas MaxQuant was used to process proteomics data. Mapping of detected changes to the arginine pathway enable data interpretation in a biological context.

Results and Discussion

Metabolomics and proteomics data have been acquired on a Q-TOF instrument to gain deeper insights into the changes introduced by rational strain design to increase arginine production in C. glutamicum. Metabolomics data processing and interpretation resulted in the tentative identification of an unknown compound — more abundant in the mutant strains — as glutamylvaline by molecular formula generation and database query. Several known compounds in the arginine biosynthetic pathway could be automatically identified and quantified. Proteomics data revealed significant changes of proteins involved in the arginine biosynthesis pathway. Mapping alterations detected via metabolomics and proteomics on biochemical pathway maps enabled quick formulation of hypotheses for the observed changes in the biological context.

Conclusion

Our results demonstrate that combination of non-targeted omics techniques enables in-depth investigation of changes in C. glutamicum caused by rational strain design to increase production of desired metabolites.

Keywords: Q-TOFProteomics &MetabolomicsNon-targetedPathway mapping

WP02-08

Quantitative Proteomics and Whole Transcriptomics Sequencing of Progeria-derived Cells Point to a Key Role of IGF Signaling Pathway in Premature Aging

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Introduction and Objectives

Mutations in LMNA gene are the causal agent of subset of genetic diseases affecting mesoderm tissues called laminopathies. Among them, Hutchinson Guilford Progeria Syndrome (HGPS) or Progeria is a fatal disease with a very low incidence characterized by a typical clinical picture of elderly pathologies. HPGS-affected patients begin to show symptoms of accelerated aging at age of two, and typically die in the second decade of life due to cardiovascular deficiencies. HGPS is due, in most cases, to a point mutation (G608G) in LMNA gene encoding Lamin A and C, major structural components of the nuclear lamina. The mutation causes the occurrence of a cryptic alternative processing site in Lamin A, generating a truncated isoform, Progerin -PG-. Accumulation of PG promotes defects on nuclear structure, replication, chromatin organization and stem cell differentiation. Proteomics provides tools to globally analyze cellular activity at protein level. Besides, this proteomic profiling will allow the elucidation of connections between broad cellular pathways and molecules that were previously impossible to predict using only traditional biochemical analysis. However, so far, the results obtained must be orthogonally validated with other approaches. Next generation sequence (NGS) technology, together with novel methods of pattern recognition and network analyses, has revolutionized cellular pathways. Our aim for this study was to combine both shotgun proteomics and NGS to unravel new molecular pathways modulated in HGPS.

Methods

Three Progeria-derived cell lines and their correspondent family-related healthy controls where subjected to quantitative proteomic (iTRAQ) analysis in a LTQ-Orbitrap Velos (Thermo). Raw data was processed using Proteome Discoverer software. Next Generation Sequencing was done in parallel on an Illumina HiSeq 1500 platform. The raw RNA-Seq reads for each sample were aligned to the reference human genome (hg19) using Bowtie2 and TopHat. Functional annotation was performed using the ToppGene Suite 22. ELISA validation was done using a commercially available kit (Abcam).

Results and Discussion

Surprisingly, both proteomic and genomic massive data indicates a modulation of Insulin Growth Factor-Like signaling pathway in HGPS-derived cell lines. Specifically, protein levels of IGFBP3, an IGF modulator, appears to be down-regulated in PG-expressing cells. The results have been orthogonally validated by ELISA. Besides, the effect on the IGFBP3 production of some well-known regulators of the Insulin Growth Factor signaling pathway, like curcumin and rapamycin has been studied in cell culture. Results indicate

that both factors stimulate IGFBP3 secretion to the culture media.

Conclusion

The combination of high sensitive proteomics and genomics techniques has allowed us to unravel an unexpected new role of IGF signaling on premature aging, opening a new scenario for future therapies.

Keywords: Aging, Progeria, Nucleus, Quantitative Proteomics.

Transcriptomic and proteomic verification of predicted scheme of ATRA-induced HL60 cell line differentiation

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Introduction and Objectives

Methods

We matched components of the predicted scheme with genome wide transcriptomic data and shotgun mass-spectrometric data at different time points after ATRA treatment to prove their expression in HL60 cells at the mRNA and protein level. We used genome wide transcriptomic data and target mass-spectrometric approach (PRM and SRM) to obtain quantitative data. Along with molecules of predicted scheme we investigated LYN, FGR, VAV1 and PRAM1 using the target mass-spectrometric approach.

Results and Discussion

Conclusion

Using transcriptomic and proteomic approach we verified the predicted scheme of ATRA-induced HL60 cell line differentiation, which could represent overcoming p53 deletion in HL60 cells. Key molecule PARP1 and transcription factor HIC1 are the most prominent components of the predicted scheme. Differentially expressed molecules LYN, FGR, VAV1 and PRAM1 could be the parts of the signaling pathway which is involved in ATRA-induced differentiation. The mentioned above molecules could represent a new target for leukemia drug therapy.

Keywords: Transcriptomics, proteomics, HL60 cell line, ATRA-induced differentiation

WP02-10

THE RELATIONSHIP BETWEEN URINE PEPTIDOME AND PROTEIN MISFOLDING DURING PREECLAMSIA

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Introduction and Objectives

Preeclampsia (PE) is a pregnancy complication characterized by high blood pressure and proteinuria. The disorder usually occurs in the third trimester of pregnancy and gets worse over time. PE increases the risk of poor outcomes for both the mother and the baby. New methods for PE prediction and early diagnosis are required to prevent disease complications. The aim of the study is further application of urine peptidome as a promising approach for the research in the field of preeclampsia, particularly in differentiation of mild and severe PE. Urine peptidome was studied by high performance LC-MS/MS in combination with two extraction methods based on solid phase extraction (SPE) and size-exclusion chromatography (SEC). Complementary search engines such as Mascot, PEAKS, MaxQuant were used and for qualitative and quantitative data analysis.

Methods

In this study thirty urine samples from women with mild and severe preeclampsia and the control group were prepared using size-exclusion chromatography. All patients provided written informed consent; all procedures and study methods were approved by the Commission of Biomedical Ethics at V.I. Kulakov Research Center for Obstetrics, Gynecology and Perinatology. Peptide extraction was performed by solid-phase extraction (SPE) and/or gel-filtration (GF).LC-MS/MS experiments were performed for each sample in fourfold on a nano-HPLC Agilent 1100 system (Agilent Technologies, Santa Clara, CA, USA) in combination with a 7-Tesla LTQ-FT Ultra mass spectrometer (Thermo Electron, Bremen, Germany) equipped with a nanospray ion source (in-house system).

Results and Discussion

Conclusion

Urinary peptidome study by high resolution mass spectrometry coupled to high performance liquid chromatography can give a clue to the failure in proteins folding in case of preeclampsia. Our results revealed unique identifications (correlate to alpha-1-antitrypsin, collagen alpha-1(I) chain, collagen alpha-1 (III) chain, and uromodulin, for instance) that can potentially serve as early indicators of PE.The work was carried out under the support of RFBR grants no.14-08-01236 A, no.16-54-21011_SNF_?, the part of the study related to peptides extraction and identification was supported by Russian Science Foundation grant no.16-14-00181.

Keywords: Preeclamsia, Urine peptidome, LC-MSMS

WP02-11

Diversity of HNF4A function in regulating growth and invasion of HCC

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Introduction and Objectives

Hepatocellular Carcinoma (HCC) is one of the most common human cancers with a poor prognosis. The major obstacles to survival are metastasis and recurrence after HCC resection. Metabolic switch is one of the hallmarks of cancer cells, while HNF4A is a highly expressed master transcription factor in hepatocyte regulating metabolism and differentiation. The roles HNF4A plays in hepatocarcinogenesis and metastasis were still not well understood because of conflicting result from different studies. Here, we report a potiential mechanism of HNF4A in HCC progress.

Methods

HNF4A level in HCC cell lines with different metastasis potiential were compared by WB. HNF4A stable overexpression or know-down cells were established in HCCLM3 and Huh7 cell lines respectively. Proliferation, invasion and colony formation assays of HCC stable cell lines were performed in different glucose conditions. Target TFs and genes of HNF4A under different glucose conditions were detected by TFRE-MS and ChIP-seq. The HNF4A function in HCC progress in vivo under different blood glucose level were compared by subcutaneous xenograft model in balb/c mice.

Results and Discussion

HNF4A inhibited the MAPK pathway and suppress the proliferation, invasion and colony formation of HCC cells in glucose sufficient condition, however, under glucose deficient condition or treated with 2-DG, HNF4A activated MAPK pathway and enhanced the proliferation and invasion of HCC cells both in vitro. and in vivo. Multiomics research by TFRE-MS and ChIP-seq and system biology analysis showed that there were different patterns for HNF4A target TFs and genes under glucose deficient or sufficient condition, and this diversity of HNF4A function in regulating growth and invasion of HCC was related to the activity of AMPK pathway. Also, after blocking AMPK pathway, HNF4A would not promote the proliferation and invasion of HCC even under glucose deficient condition.

Conclusion

HNF4A is a metabolism regulator which inhibit MAPK pathway and depress proliferation and metastasis under AMPK inactive condition and it would turn to activate MAPK pathway and promote the proliferation and metastasis of HCC cells depending on the activation of AMPK. HNF4A would play crucial roles in metabolic switch of cancer cells and development of HCC.

Keywords: HNF4A; HCC; reversible function; Multiomics research; AMPK

WP02-12

Towards spatially resolved, multiplexed (up to 800 plex) digital characterization of protein and mRNA abundance in tissue

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Introduction and Objectives

Access to technologies, scientific trends, and individual preferences are some of the factors that have influenced the relative prevalence of DNA, RNA, and protein measurements among oncology biomarker signatures. Indeed, many peer-reviewed publications espouse the benefits of specific assay and analyte types for identification of pathways of interest or prediction of treatment responses, for example. The genomics and proteomics fields have both experienced significant technical progress with the introduction of next-generation techniques, and yet neither alone will be sufficient to solve the biggest open problems within biomarker discovery and drug resistance research. This is especially true in studying the Tumor Microenvironment (TME). The tools to assess spatial heterogeneity of proteins and nucleic-acids in tissue slices continues to be limiting. Current techniques like immuno-histochemistry (IHC) and fluorescent in situ hybridization (FISH) are inherently limited in utility because it has been difficult to quantify the abundance of multiple protein/nucleic-acids across a wide dynamic range. Here, we report the development and validation of a spatially-resolved protein and RNA detection platform with the potential to simultaneously quantify up to 800 targets with >5 log10 of dynamic range from a single formalin-fixed paraffin-embedded (FFPE) slide. We demonstrate validation of this technology by characterization of a panel of immune proteins expressed in breast cancer biopsies, and we also demonstrate spatially resolved detection of RNA.

Methods

Results and Discussion

Conclusion

Keywords:

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Proteomics E-Learning and Outreach Initiatives: An Effort from Indian Proteomics Community

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Introduction and objectives

Web-based educational portals are getting world-wide popularity and are playing an important role in modern education system. The power of information technology (IT) has brought the e-learning and open learning resources with easy access worldwide. In India, Sakshat Virtual Labs and National Programme on Technology Enhanced Learning (NPTEL) have initiated the virtual labs and e-learning program in Proteomics, respectively. "Virtual Proteomics Laboratory (VPL-http://iitb.vlab.co.in/?sub=41&brch=118)" and "Clinical Proteomics Remote Triggering Virtual Laboratory (CPRTVP-http://iitb.vlab.co.in/?sub=41&brch=237)" are two virtual proteomics initiative started from IIT Bombay. These initiatives have provided practical experience of basic proteomics experiments, including gel-based proteomics, mass spectrometry based workflows and basic bioinformatics tools. In addition, virtual lab has also provided an interface to access the remote system equipped with proteomic tools and softwares to get practical experience on proteomic workflows.

Methods

1. Both "Virtual Proteomics Laboratory" and "Clinical Proteomics Remote Triggering Virtual Laboratory" were hosted by "Sakshat portal" from MHRD, Govt. of India.
2. National Programme on Technology Enhanced Learning (NPTEL) was another innovative thought of MHRD program and hosted by "Indian institute of Technology Madras" on NPTEL portal.
3. NPTEL-MOOC course is an update from NPTEL team and the video lectures, assignments, quizzes and discussion will be done on NPTEL-MOOC portal and the video lectures were uploaded in youtube for public use.
4. The proteomics documentaries were uploaded in public domain via Youtube and also uploaded on the respective personal websites.

Results and Discussion

MHRD has started NPTEL program to provide online video lecture series in proteomics. Recently, NPTEL has started Massive Open Online Course (MOOC) which is fundamentally a web-based platform for distance learning in science and engineering using recorded video lectures, notes, assignments and quiz series, thereby providing the participants a periodical self-assessment. As many as three proteomics courses

have been launched by IIT Bombay so far, namely; Proteins and Gel-Based Proteomics (http://nptel.ac.in/courses/102101049/) and Mass Spectrometry based proteomics (http://nptel.ac.in/courses/102101050/). The NPTEL-MOOC proteomics courses have received enormous response from the Indian as well as the global community. Nearly 1000 students were registered for each course and 15% students have cleared exams with certificate. MOOC courses are not only beneficial for the participant's but this interactive platform also gives them extensive networking opportunities to interact with the experts in this field.

Conclusion

Apart from e-learning initiatives, scientific documentaries are getting into the world of modern science to convey the ideas, opinions, suggestions and future endeavours of the scientific programs by the world experts. "Proteomics: the code of life" is one such proteomic documentary where world proteomics experts have shared their views on how the transformation of proteomics has happened from genomics and how the technology and tools have been developed over the period. In summary, it explains the journey of proteomics from genomics and the contributions of proteomics in functional biology to decipher innovative solutions in last two decades.

Keywords: Virtual labs, NPTEL-MOOC, proteomics, e-learning initiatives

Proteome and transcriptome analysis of retinoic acid-induced differentiation of human leukemia HL-60 cells

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Introduction and Objectives

Acute myeloid leukemia (AML) is characterized by blockade of differentiation of progenitor cells; this results in accumulation of immature myeloid cells in bone marrow and peripheral blood. In clinical practice, the strategy of reduction of the number of these nonfunctioning cells is based on the use of chemotherapy and differentiation therapy. The example of AML is promyelocytic cell line HL-60. All-trans retinoic acid (ATRA) causes HL-60 differentiation into granulocytes and this makes the HL-60 cell line a good model system for studying differentiation of human myeloid cells, as well as for development of approaches for differentiation-based treatment of leukemia.

Methods

In this study, the systematic analysis of changes in ATRA-induced differentiation of HL-60 cells was carried out at various time intervals in range of 0.5 to 96 hours. Transcriptome analysis of mRNA isolated from HL-60 cells at different time points was performed using whole genome microarray technology (Agilent Technologies). Proteomic experiments were performed using high-resolution OrbiTrap Velos mass-spectrometer followed by label-free quantitation with SPIRE software. Functional analysis of up- and down-regulated genes/proteins as well as identification of master regulatory molecules and pathways construction upstream of above genes/proteins was carried out using GeneXplain platform (http://platform.genexplain.com).

Results and Discussion

Transcriptome analysis revealed expression about 14600 genes. Among them 18, 51, 159, 231 and 1449 genes were identified as being differentially expressed after induction of differentiation for 30 min, 1 h, 3 h, 24 h, and 96 h in comparison with control (time point 0), respectively. Quantitative the label free analysis of LC-MS/MS proteomic data allowed to find 122, 169, 199, and 275 proteins with altered expression at time points 3 h, 24 h, 48 h, and 96 h, respectively. The procedure of functional classification provided groups of genes involved in differentiation of myeloid cells at various time points. These gene groups of interest and proteins with altered expression underwent further analysis to identify transcription factors and master regulatory molecules important for HL-60 cells differentiation. As a result, the schemes of regulatory pathways which demonstrate the signaling for activation of cell differentiation and/or for cell proliferation inhibition, as well as for survival/apoptosis of HL-60 cells during differentiation were obtained.

Conclusion

Combination of the high-throughput experimental techniques together with the computational analysis allowed to reveal molecules important for differentiation. Obtained data can be useful for further understanding of the molecular mechanism of HL-60 cells

differentiation and for development targeted differentiation therapy. The study was partially supported by the grant of RSF (Russian Science Foundation) # 14-25-00132.

Keywords: Omics technologies, HL-60 cells differentiation, regulatory pathways

A Systems Biology Approach to Dissect Acetylation-dependent Cancer Vulnerabilities

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Introduction and Objectives

Lysine deacetylases (KDACs) catalyse the removal of acetyl groups from histone and non-histone proteins. (De)acetylation of histone proteins mediates epigenetic regulation of gene expression, whilst non-histone protein acetylation influences protein stability and intracellular localisation. Many KDAC inhibitors are emerging as promising agents for the treatment of different types of tumours. To investigate the multivalent role of acetylation in this context, KDAC function was inhibited in Jurkat T cells by two KDAC inhibitors: trichostatin A (TSA) and romidepsin (RD). The latter is a clinically-approved drug for treatment of blood cancer.

Methods

The global cellular response to RD and TSA treatment was monitored under mild, non-apoptotic drug-treatment conditions to depict relative changes in mRNA, protein abundance, and protein acetylation. To quantitate global proteome changes, a TMT 6-plex experiment comprised of cell duplicates treated with DMSO (control), TSA and RD. Tryptic peptides were individually labelled with the TMT tags 126 to 131. Acetylated proteins were additionally immunoprecipitated with anti-acetyl lysine antibody in triplicate and quantitated with a label-free approach. In both cases, the samples were fractionated prior to analysis by LC-MSMS on an Agilent 1200 series HPLC coupled to a hybrid LTQ Orbitrap Velos. Finally, the mRNA from the same biological samples was isolated and analysed on an Illumina HiSeq 2500 sequencer to assess changes in mRNA expression.

Results and Discussion

Approximately 1200 genes and 700 proteins were significantly regulated ($p<0.05$) after KDACi treatment compared to the DMSO control. Overall, proteins involved in cell differentiation, cell growth and early apoptotic events were up-regulated, whereas the proteins involved in proliferation and cell cycle progression were down-regulated. Interestingly, RD treatment resulted in well-correlated changes in gene and protein expression, whereas TSA treatment showed no correlation. This result suggested a mechanistically different cellular response to RD and TSA. The majority of the proteins with altered acetylation, however, were not significantly changed in abundance; indicating the major role of non-histone protein acetylation in regulating protein activity and localisation rather than stability/degradation. Moreover, specific changes in this PTM occurred on proteins crucial for cell-cycle progression and cell survival, with a unique set of protein-acetylation changes mediated by two distinct drug treatments.

Conclusion

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A systems biology approach is necessary to investigate the role of RD and TSA at the transcriptomic (epigenetic) and (post)translation level. Moreover, acetylation changes on proteins that are unaltered in abundance provides an additional layer of information to characterise the mechanism-of-action of such drugs and will undoubtedly contribute to deciphering the role of this PTM in cancer.

Keywords: acetylation, cancer, Jurkat T cells, lysine deacetylase inhibitors

WP02-16

Lipidomic Analysis of Risk Factors for Chronic Total Occlusions after Percutaneous Coronary Intervention

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Introduction and Objectives

Arterial plaque which contains large amount of lipid deposition, is the main component of chronic total occlusion's stemming. In case of a low success rate and poor prognosis for the percutaneous coronary intervention (PCI) of chronic total occlusion (CTO) patients, very limited data about lipid composition during perioperative period of CTO-PCI is available up to now. This study aimed to investigate the risk predictors of CTO-PCI patients during perioperative period using lipidomic technology.

Methods

Large-scale lipid qualitative analysis of CTO-PCI patients' plasma (samples of plasma of pre-operation, 24 hours after PCI and 72 hours after PCI, and plasma samples of normal people as control group are collected)

Results and Discussion

Shotgun lipidomics were performed and more than 1500 kinds of lipid molecules have been identified and quantified. There are 11 differential lipid molecules including phospholipids and triglycerides were found. The lipids have a significant change in 24 hours after CTO-PCI may suggest a side-effect of PCI. The results may explain the mechanism of CTO through lipid metabolism and may predict the risk factors of the prognosis of CTO-PCI. Moreover, the emotivity of CTO will also be discussed, those who has a high level of phosphatidylserine PS (18:0/18:1) are tend to be total occlusion.

Conclusion

Shotgun lipidomics were performed to qualitative analysis of CTO-PCI patients' plasma in perioperative period and more than 1500 kinds of lipid molecules have been identified and quantified. 11 differential lipid molecules including phospholipids and triglycerides were found a significant change in perioperative period. And the result showed that who had had a high level of phosphatidylserine PS (18:0/18:1) were tend to be total occlusion

Keywords: Chronic total occlusion / Percutaneous coronary intervention / Lipidomics / MS / Risk factors

WP02-17

Systematic Identification of Arsenic-Binding Proteins Reveals that Hexokinase-2 is Inhibited by Arsenic

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Introduction and Objectives

Arsenic trioxide (ATO) is highly effective for treating acute promyelocytic leukemia (APL). And ATO has also been found to be effective against many other hematologic malignancies and solid tumors, including chronic myelocytic leukemia (CML), lung cancer, hepatocellular carcinoma, melanoma, renal cell carcinoma, colorectal cancer, etc. However, while its mechanistic effects in APL are established, its broader anti-cancer mode of action is not understood. Thus we hypothesized that there are many more ATO binding proteins that remain to be discovered.

Methods

We applied a human proteome microarray containing 16,368 proteins with arsenic-binding proteins discovery. Combined with western blot, biolayer interferometry (BLI), and streptavidin agaroseaffinity assay, we then validated the bindings between arsenic and the target proteins. Subsequently, we detected the changes of cell growth and metabolites levels in SGC7901 upon ATO treatment, by using flow cytometry and GC/LC-MS. In addition, applying MALDI-TOF mass spectrometry, we determinedthe arsenic binding sites on target protein.

Results and Discussion

Using a human proteome microarray, we identified 360 proteins that specifically bind arsenic. Among the most highly enriched proteins in this set are those in the glycolysis pathway, including the rate-limiting enzyme in glycolysis, hexokinase-1 (HK1). Biochemical assay showed the activity of highly homologous hexokinase-2 (HK2) could be inhibited by arsenic, and metabolomics analysis showed that G6P, the direct product of hexokinase was significantly down-regulated in gastric cancer cell line SGC7901 when treated with arsenic. Furthermore, overexpression of HK2 rescued cells from arsenic-induced apoptosis. Meanwhile, we found that Cys256 and Cys704 are the arsenic binding sites, which are in 10Å range to the active sites of HK2. This also explains why arsenic could inhibit the activity of HK2. Besides glycolysis pathway, arsenic binding proteins were also observed in mitochondria function, cellular glycosylation, nucleotide metabolism, response to oxidative stress (glutathione metabolism), and so on. These findings are in good agreement with other arsenic related systems biology studies.

Conclusion

In summary, taking advantage of a human proteome microarray, we have performed a comprehensive survey of the direct targeting proteins of arsenic, which provides a valuable resource for future study. By correlating the proteomic microarray data with functional studies and metabolomic analysis, our results strongly implicate glycolysis, and HK2 in particular, as a key target of arsenic. Moreover, the arsenic-binding proteins identified in this work are expected to serve as a valuable resource for the development of

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synergistic anti-tumor therapeutic strategies. And the strategy that we developed could be readily adopted for the study of other potentially therapeutically interesting compounds.

Keywords: arsenic trioxide | human proteome microarray | glycolysis | hexokinase-2

WP02-18

Integrative Systems Biology Investigation of Fabry Disease

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Introduction and Objectives

Fabry disease (FD) is a rare X-linked recessive genetic disorder caused by a deficient activity of the lysosomal enzyme alpha-galactosidase A (GLA). It is characterized by intra-lysosomal accumulation of globotriaosylceramide (Gb3) and other related sub-types of glycolipids (e.g. glycosphingolipids) in many cell types, including blood vessels throughout the body, which initiates a cascade of events, starting with the disruption of basic metabolic processes at the cellular level and progressing to inflammatory events and cell death. This affects the system as a whole with an increase of renal, cardiac, cerebrovascular, and skin complications. This study describes FD at a systems level using a systems biology approach, in which molecular data sourced from multi-omics studies is extracted from the literature and integrated as a whole, in order to reveal the biochemical processes and molecular pathways potentially affected by the dysregulation of differentially expressed molecules and in this way provide new insights that describe the pathophysiology of this rare disease.

Methods

We performed a meta-analysis of peer-reviewed publications including high-throughput omics technologies (i.e. transcriptomics, proteomics and metabolomics) regarding FD naïve patients and undergoing enzyme replacement therapy (ERT). Information concerning the description of the experimental setup, clinical data, and differentially expressed molecules in FD from human data sets was extracted from publications and manually curated. Reduction of molecular redundancy within the gathered data was achieved by using a specific ontology to tie together and harmonise multi-level omic studies based on gene and protein clusters. We applied the following analysis workflow: functionality tag clustering, gene ontology (GO) and pathway term clustering, molecular clustering based on protein-protein interactions (PPI's), regulatory networks, metabolic networks, mapping of the molecular features into existing pathway maps and as well into in-house built pathway maps and lastly merging results and provide an overall interpretation in the context of FD.

Results and Discussion

Data statistics: we extracted data from 10 large-scale, data rich publications including six proteomics studies, one transcriptomic study, one next-generation sequencing study, one peptidomic study and one metabolomic study characterising differently expressed molecules in blood and urine. Contrasting regulation of acute-phase

response proteins of the groups of naïve (up-regulation of ORM1, ORM2, ITIH4, SERPINA3 and FGA) and ERT (enzyme replacement therapy) (down-regulation of FGA, ORM1 and ORM2) patients as potential hallmarks for distinction of naïve patient and patient undergoing ERT. The most relevant associated pathways were the regulation of lipid metabolism by peroxisome proliferator-activated receptor alpha (PPARalpha) for the naïve patients groups and formation of fibrin clot (blood coagulation cascade) for ERT patients group.

Keywords: Anderson-Fabry Disease, omics, data integration, disease modelling

WO03-01

Quantitative proteomics depicts the landscape of cysteine redoxome for nitric oxide-mediated myocardial protection against ischemia-reperfusion injury

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Introduction and Objectives

Myocardial infraction (MI) caused by coronary occlusion is a major health challenge around the world. To date reperfusion during percutaneous coronary intervention remains the only established clinical practice to reduce the acute MI. However, the burst of reactive oxygen species (ROS) generated at the onset of reperfusion also leads to additional damage to ischemic cardiomyocytes. Cysteine (Cys) oxidation and subsequent degradation of cardiac proteins may account for ischemia-reperfusion (IR) injury. It was further proposed that S-nitrosylation (SNO) can protect Cys against oxidation, thus preventing the myocardium from subsequent reperfusion-induced damage. However, a precise delineation of such process in vivo remains elusive. The objective of this study is to address how nitric oxide (NO) supply may shift the landscape of reversible Cys redoxome in the mouse heart subject to IR, leading to alleviation of myocardial injury.

Methods

Myocardial ischemia was achieved by ligation of the left anterior descending (LAD) artery in the mouse heart. For cardioprotection, S-nitrosoglutathione (GSNO) was given to the mouse immediately after LAD artery ligation. Reperfusion was subsequently performed to generate cardiac injury. For analysis of reversible Cys redoxome, total heart extracts were reacted with iodoacetamide, which blocks non-modified Cys residues. S-nitrosylated Cys and the rest of reversibly modified Cys were sequentially reduced by ascorbate and TCEP, followed by labeling with isobaric iodoTMT1 and iodoTMT2 tags, respectively. Peptides containing formerly modified Cys were selectively enriched by resins coupled with anti-TMT antibody, and subjected to mass spectrometry (MS) analysis.

Results and Discussion

GSNO treatment prevented the mouse heart from IR injury as evidenced by obvious reduction of MI and cardiac troponin-I release to the blood. Among heart samples subjected to ischemia, IR and IR supplied with GSNO, 785 reversibly modified Cys sites located on 402 proteins were identified and quantified. Our data demonstrated that GSNO treatment promoted S-nitrosylation of cardiac proteins significantly in the heart exposed to IR. Remarkably, with the supply of GSNO, IR-induced reversible Cys modifications other than SNO, presumably including Cys oxidation and glutathionylation, were greatly decreased. We selected a few candidates out of identified proteins and validated the protective effect of SNO modification on IR injury using cultured cardiomyocytes.

Conclusion

Employing the workflow of MS analysis for quantitative Cys redoxome in vivo, we have shown that S-nitrosylation prevents myocardial proteins from IR-induced Cys oxidation. We have further illustrated the critical function of a single Cys SNO site in protection of

WO03-01

cardiomyocytes against IR injury.

Keywords: Quantitative proteomics, cysteine redoxome, S-nitrosylation, myocardial protection, ischemia-reperfusion injury

WO03-02

Proteomic analysis of S-sulfhydration by ultrafilter-assisted functional supramolecular polymer capture

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Introduction and Objectives

Protein S-sulfhydration is a newly defined oxidative posttranslational modification and plays an important role in H2S-mediated signaling pathways. So far botin switch technique (BST) is the most popular method for the detection of protein S-sulfhydration, by which thiols in proteins were firstly blocked with S-methyl methanethiosulfonate (MMTS), and persulfides (-S-SH) were then reacted with biotin-HPDP, finally captured by streptavidin beads. However, due to the silimar chemical reactivity, it is difficult to distinguish thiols and persulfides by using MMTS as a alkylating reagent. Therefore, it is questionable for the current BST method to enrichment and detection of S-sulfhydration, which might result in many erroneous results. To solve these problems, in our recent study, a ultrafilter-assisted functional supramolecular polymer capture S-sulfhydration approach is proposed.

Methods

by this approach protein digests were firstly reacted with iodo-funtionalized supramolecular polymer (Mw=58 kDa), and then placed onto a ultrafilter device (Mw cutoff=10 kDa), after the removal of disulfide peptides and unmodified peptides, the S-sulfhydrated peptides could be released with dithiothreitol.

Results and Discussion

We applied this method for the quantitative analysis of protein S-sulfhydration from SILAC labelled SHSY-5Y cells treated with or without Na2S, and we quantified 131 S-sulfhdrated peptides with high confidence, corresponding to 108 proteins, most of which were related with Ras-related proteins and ATP/GDP-dependent enzymes.

Conclusion

All these results demonstrated our developed method is of great potential for the proteomic analysis of S-sulfhydration.

Keywords: quantitative protoeomics, S-sulfhydration, selective enrichment.

WO03-03

TRPC6-dependent S-nitrosylation in Duchenne Muscular Dystrophy

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Introduction and Objectives

Duchenne muscular dystrophy (DMD) is a muscle disorder that induces severe skeletal and cardiac dysfunction. To date, the treatment of this disease has focused mainly on improving skeletal muscles. However, it has been reported that cardiac function may even worsen if left untreated. It is yet unclear whether the mechanisms of DMD cardiac disorder are similar to those of skeletal muscle dysfunction, and thus, if a dual-targeted therapy can be developed. Recently, a Ca^{2+} channel, transient receptor potential canonical channel type-6 (TRPC6) was reported to play a major role in DMD cardiomyocytes, inducing greater Ca^{2+} influx, adverse force generation and arrhythmia. Calcium dysregulation was thought to stimulate oxidant stress and alter control of nitric oxide (NO); the latter itself altered in DMD by disruption of NO synthase localization, at least in skeletal muscle. The altered NO signaling presumably switches protein S-nitrosylation (SNO), an oxidative post-translational modification. Here, we identified and quantified SNO-modification and assessed cardiac abnormalities in dystrophic models.

Methods

8-10 month-old C57/BL6, DMD, TRPC6 knock-out DMD mice (n=10-13/cohort) were generated and used. SNO-proteins from hearts were detected with our established dual-labeling strategy, using TMT-switch assay coupled to mass spectrometry analysis. Cardiac function of mouse models were assessed from the pressure-volume loop in response to acute increase in afterload induced by transverse aortic constriction.

Results and Discussion

Proteomics analysis identified over 1200 SNO sites on >500 proteins in these DMD and TRPC6-deleted DMD hearts, including novel sites specific for DMD, for example, SNO sites on low abundant ryanodine receptor. Quantification across the three cohort samples revealed SNO on 60 % sites were up-regulated in DMD while 40 % were down-regulated, compared to WT. Of these, the SNO levels in DMD were reversed by TRPC6-deletion in more than half of the sites. The SNO level of 77% sites and 52 % were reversed back among the up- and down-regulated SNO sites, respectively. As an example, SNO on Cys190 of tropomyosin alpha-1 increased in DMD but significantly went back down with TRPC6-deficiency (WT: 6.8, DMD: 17.5, TRPC6-deleted DMD: 9.4, $p<0.01$). This result suggests TRPC6-dependent SNO modulation in dystrophy. In the pressure-overloaded DMD hearts, contractile and stroke work were reduced and impaired diastolic function reflected by isovolumic relaxation constant was notable but these responses were reversed in TRPC6-deleted hearts ($p<0.05$). These support our hypothesis that hyper-active TRPC6 amplifies DMD pathophysiology via protein SNO-modulation.

Conclusion

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In conclusion, our study determined TRPC6-dependent SNO-modification in DMD and the contribution of TRPC6 to cardiac functional abnormality of DMD in vivo. Our integrated analysis of the two approaches supports the impact of TRPC6 via the SNO modifications in dystrophic heart.

Keywords: Redox; S-nitrosylation; Muscular dystrophy

WO03-04

Proteomic and redox proteomic analyses reveal a dual ROS-regulation of glucose uptake in adipocytes

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Introduction and Objectives

The accumulation of ROS is thought to contribute to various human diseases, including type 2 diabetes. The relationship between ROS and glucose uptake is very complicated, and the mechanism is still unclear.

Methods

In this study, we used BCNU and auranofin to specifically inhibit glutathione system and thioredoxin system to build cellular ROS models, and performed proteomic and redox proteomic analyses. After that, biological validation was done.

Results and Discussion

In our cellular ROS models, we found ROS would increase basal glucose uptake but decrease insulin-responsive glucose uptake. To better understand that we performed SILAC-based quantitative proteomics and quantified 6182 proteins in total. Based on their temporal profiles, differentially expressed proteins were clustered into different groups, and some of them were tightly associated with glucose uptake changes. Those positively correlated proteins were enriched in proteasome pathway while negatively correlated ones were mainly enriched in oxidative phosphorylation pathway. We also performed redox proteomic analysis of the same cellular models using iodoTMT isobaric labelling strategy. Totally we quantified 15133 oxidized cysteine-sites and over 3000 sites presented negative correlation with glucose uptake changes, which were enriched in TCA cycle, Ribosome pathway and quite a few metabolism related pathways. To further uncover ROS's influence on glucose uptake we performed WBs and results showed ROS did negatively affect PI3K-Akt signalling, although Akt was still well-phosphorylated, indicating Akt's potential key role in mediating this regulation. Taking advantage of in vitro kinase assays we confirmed that ROS did reversibly regulate Akt's activity but would not affect its phosphorylation, suggesting this regulation is independent of well-known phosphorylation-based mechanism. After re-mining our large-scale omic data, we found the oxidation of 4 Akt cysteines (Cys60, Cys77, Cys124 and Cys311) increased, among which Cys60 and Cys77 within PH domain presented significance. We did another IP-MS analysis of native Akt digestion and discovered that after oxidation there would be two disulfide bonds (Cys60-Cys77 and Cys297-Cys311) forming within PH domain and kinase domain respectively, which proved to be intramolecular rather than intermolecular by non-reduced gel. Then we mutated these PH domain cysteines and overexpressed them in adipocytes, we found mutant C60S could efficiently alleviate ROS-induced glucose uptake inhibition, compared to wild-type. Interestingly, this improvement was mainly derived from increasement of basal glucose uptake rather than insulin-responsive glucose uptake.

WO03-04

Conclusion

Taken together, this study revealed a novel dual ROS-regulation of glucose uptake in adipocytes, which positively affected basal glucose uptake while negatively affected insulin-responsive glucose uptake, and Akt was involved in this dual influence as a key mediator.

Keywords: Proteome; Redox proteome; ROS; Glucose uptake; Akt.

WO03-05

Identification of protein estrogenization as a redox post-translational modification by shot-gun proteomics and activity probe with dimethyl labeling

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Introduction and Objectives

Proteins, covalently modified by catechol estrogens (CEs) referred to as estrogenized proteins, are potential biomarkers for estrogen homeostasis and cancers. However, serum proteins endogenously modified by CEs, the modification sites, and functional changes of proteins by the modification remain elusive. We aim to establish protein estrogenization as a redox post-translational modification and develop feasible analytical platforms to identify cellular targets of estrogenization.

Methods

In this study, LC-MS-based shotgun proteomics was applied to identify site-specific protein estrogenization in human blood via a systematic approach and stringent validation. Furthermore, affinity enrichment and stable isotope dimethyl labeling was developed and applied to identify potential protein targets of estrogenization in tissue samples. Furthermore, structural and functional impacts of protein estrogenization were characterized by molecular modeling and bioassays.

Results and Discussion

Endogenous estrogenization of serum proteins in diabetic patient blood was identified with high confidence by LC-MS and confirmed by comparisons with estrogenized protein standards.[1] Chemical reactivity of CEs towards cysteine, lysine and histidine residues on insulin and the structural and functional changes of insulin by estrogenization were further characterized. Our results indicate that CEs, namely 2- and 4-hydroxyl estrogens, were thermodynamically and kinetically more reactive than the catechol moiety. CEs actively cleaved the inter-chain disulfide linkage and modified the exposed Cys7 in both the A chain and the B chain of insulin, as well as modifying His10, and Lys29 in the B chain. Estrogenization on these sites may block the receptor-binding pockets of insulin.[2] Insulin signaling and glucose uptake levels were lower in MCF-7 cells treated with modified insulin than in cells treated with native insulin. A click chemistry-based activity probe coupled with stable isotope dimethyl labeling was developed. Dozens of potential protein targets of estrogenization in rat liver tissue including enzymes, transporters, and co-factors were identified.1. C-M Fang, et al. “Identification of Endogenous Site-specific Covalent Binding of Catechol Estrogens to Serum Proteins in Human Blood” Toxicological Sciences 148(2), 433–442 (2015).2. M-C Ku, et al. “Site-specific covalent modifications of human insulin by catechol estrogens - Reactivity and induced structural and functional changes” Scientific Reports 6, 28804; doi: 10.1038/srep28804 (2016).

Conclusion

We establish protein estrogenization as a metabolic post-translational modification via redox process. Our data demonstrate that protein estrogenization may alter normal cell

WO03-05

physiology and worth further investigations.

Keywords: redox modificationpotein estrogenizationclick chemistrystable isotope dimethyl labeling

WP03-01

Multiplexed Isobaric IodoTMT-Switch Approach to Identify and Quantify the Changes in the Cardiac Redox-Environment

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Introduction and Objectives

Ischemia/reperfusion (I/R) induces cardiomyocyte death and reactive oxygen species (ROS) generation. Reversible redox modifications of Cys thiols in the forms of protein S-ni trosylation (SNO) and S-glutationylation (SSG) are widely implicated in myriad pathophysiological processes including cardiomyopathy and cardioprotection. Although the reversible cysteine modifications of cellular proteins have been reported as a key mechanism, but the underlying mechanism remains elusive. Herein we apply our recently established iodoTMT-switch method to proteomic samples prepared from I/R injury model for a quantitative analysis of dynamic cysteine modification sites sensitive to oxidative stress.

Methods

For in vitro experiments, embryonic rat cardiomyocytes (H9c2 cells) were exposed to hypoxia/reoxygenation (H/R) in control medium with or without S-nitrosoglutathione (GSNO). For in vivo study, ischemia was created in mice by ligating the left anterior descending coronary or ischemia followed by reperfusion for studying myocardial injury. The non-modified cysteines were first irreversibly alkylated with iodoacetamide, and the S -nitrosylated thiols were reduced with ascorbate prior to conjugation with iodoTMT1 tags, then other reversible modified thiols were reduced and labeled by TCEP and iodoTMT2 tag, thus allowing the selective enrichment of peptides containing formerly reversibly modified cysteines by anti-TMT resin. Peptides were injected into a trapping column with 0.1% formic acid (FA) for 10 min and then separated by a C18 BEH column with a segmented gradient in 150 min from 5% to 35% acetonitrile at a constant flow rate of 300 nl/min connected to the LTQ-Orbitrap Elite hybrid mass spectrometer.

Results and Discussion

GSNO attenuates lactate dehydrogenase (LDH) leakage in cell model of H/R, and significantly reduced myocardial infarct size and cardiac troponin-I (cTnI) levels in mouse model of myocardial I/R injury. Collectively, we have quantitative dynamic change in various Cys-redox modifications from H9c2 cells and mouse model undergoing H/R and I/R with and without adding GSNO, indicative of individual differences in susceptibility to S -nitrosylation or S-glutathionylation. In total, 785 SNO/SSG modified modification sites from 402 proteins were identified and quantified (FDR < 0.05%). We thus demonstrate how quantitative analysis of various Cys-redox modifications occurring in biological samples can be performed precisely and simultaneously at proteomic levels. Our proteomic and functional data suggest that cathepsin B inhibition has cardioprotective effects and support a beneficial role of cathepsin B inhibition in the treatment of heart

failure after myocardial infarction.

Conclusion

We established MS-based workflow for quantifying the relative ratio of SNO versus other forms of reversible cysteine modifications based on sequential tagging with isobaric iodoTMT tag for system-wide Cys-redoxomic analysis.

Keywords: S-Nitrosylation, S-Glutathionylation, iodoTMT

WP03-02

Identification of Carbonylated Proteins Caused from Oxidative Stress in Hepatocyte line: Preliminary Study on Pathology of Fatty Liver Disease

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Introduction and Objectives

Oxidative stress is harmful to cells, organs as well as biomolecules such as proteins. Addtionally, Oxidative stress is relevant to etiology of many non-communicable diseases. Liver is the vulnerable organ to oxidative damage since it functions as physiological detoxifier. Exposure to xenobiotics and radicals from dietary could contribute the oxidative stress liver leading to pathological conditions and liver dysfunction, eventually. One of the well-studied effects of oxidative stress is protein oxidation, especially protein carbonylation. Typically, the proteins that have been carbonylated will turn to be dysfunctional and they will be degraded, subsequentially. Protein carbonylation has been proposed as one of promising etiological mechanism of various diseases, for instance, Alzhimer's disease, diabetes, non-alcoholic fatty liver disease, etc. Therefore, this study aims to identify carbonylated proteins that caused from oxidative stress in liver cell line by using redox proteomics approach.

Methods

In this study, HepG2 cells were treated with menadione, an oxidative stress inducer, markers of oxidative stress were compared with untreated cells. Superoxide anion production and lipid peroxidation product, well-studied markers, showed significantly elevated level after treatment with menadione. Carbonylated proteins were extracted from untreated and menadione-treated HepG2 cells and visualized using Oxy-blot kit in 2D-gel electrophoresis. Differentially expressed and carbonylated proteins were identified with mass spectrometry.

Results and Discussion

Surprisingly, most of the proteins were less carbonylated in oxidative stress condition, including fructose-bisphosphate aldolase A, a key enzyme in glycolytic pathway. This protein was up-regulated and decarbonylated in menadione-treated lysate, which affect the energy metabolism of the cells.

Conclusion

In conclusion, up-regulation and decarbonylation of this protein under oxidative stress might be a compensatory pathway for cells to survive under the stress condition and further studies may be needed for a better understanding of protein decarbonylation and cell survival.

Keywords: Oxidative stress, Protein carbonylation, Redox proteomics, HepG2, fatty liver disease

WP03-03

Thio-tag tip method by using zinc(II)–cyclen-attached agarose beads for enrichment of cysteine-containing biomolecules

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Introduction and Objectives

Thiol groups in biomolecules such as glutathione and cystein-containing proteins are sensitive to redox conditions and can function as redox switches, which turn intracellular signal transduction on or off in response to various extracellular stimuli or stresses. Recently, thiol-containing small molecules have attracted much interest due to their vital biological functions and as important biomarkers for diagnosing diseases. Profiling of thiol-containing molecules is analytically challenging because of their characteristic activities over wide ranges of concentrations in biological systems. Here we demonstrate practical examples for separation of cysteine-containing biomolecules.

Methods

A macrocyclic tetraamine zinc(II) complex, Zn^{2+}–cyclen (cyclen = 1,4,7, 10-tetraazacyclododecane, we call it “Thio-tag”), is capable of reversibly binding anionic ligands. Among biologically relevant anions, thiolate anions have an extraordinarily high affinity toward Thio-tag. We prepared “Thio-tag tip”; a 200-µL micropipette tip containing 10 µL of a hydrophilic cross-linked agarose beads attached with Thio-tag. We showed a basic separation protocol for thiol-containing molecules.

Results and Discussion

Thiolate-selective binding to Thio-tag tip was confirmed using a solution of a mixed sample of six N-acetylamino acids. The reference compounds were all eliminated in the flow through and the washing fractions, whereas most of AcCys was eluted in the elution fractions. We attempted to apply this method to the separation of a Cys-containing peptide (CP) from a tryptic digest of beta-casein. The recovery of CP in the E1 fraction was 80% and CP remaining in the Thio-tag tip was eluted in the E2 fraction, resulting in total recoveries of more than 90%. Almost all of the reference peptides were eliminated in the FT, W1, and W2 fractions. Under optimal conditions, the thiol capacity was nearly 30 nmol of N-acetylcysteine per Thio-tag tip containing 10 µL of thiolate-affinity agarose beads. All steps for the separation (binding, washing, and eluting) are conducted using aqueous buffers at room temperature. The entire separation protocol requires less than 15 min per sample.

Conclusion

We have demonstrated a novel procedure for simple and efficient separation of thiol-containing biomolecules by using Thio-tag tip method based on Zn^{2+}–cyclen coordination chemistry in aqueous solution. The total time required for sample collection in the elution fractions was less than 15 min, much quicker than the thiol-selective covalent chromatography technique using reversible disulfide bond formation. Furthermore, the beads are sufficiently stable to permit multiple use and long-term

storage for one year. The method could therefore be successfully used for enrichment of various intact thiol-containing biomolecules with adequate recovery.

Keywords: Thiolate-affinity chromatography, Thiolate recognition, Zinc(II) complex, Macrocyclic tetraamine

Characterization of total thiol redox status in human fibroblasts using MS-based quantitative approach iodoTMT

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Introduction and Objectives

The majority of protein thiols function as sensing thiols. These are not essential for protein activity, however their modification (oxidation/reduction) affect protein activity and thus regulate the cell function (Jones, 2011). To describe the number of such cysteine modifications and the complex of the regulatory system remains a challenge. We have established a new mass spectrometry (MS)-based quantitative approach using iodoacetyl-based cysteine reactive isobaric tandem mass tags (iodoTMT) for mapping of all reversibly modified and unmodified protein thiols in model system of human fibroblasts treated with hydrogen peroxide.

Methods

To test the quantification accuracy long lifespan BJ human fibroblast cells were lysed, proteins were reduced with TCEP and labeled by two distinct labels (iodoTMT130 and iodoTMT131). Labeled protein mixtures were desalted using Zeba desalting columns and mixed in known ratio (1:3), digested and analyzed by nano LC MS/MS UltiMate 3000 RSLCnano system (Dionex) with QExactive Plus mass spectrometer (Thermo Scientific). For further sequential iodoTMT experiment mapping, the BJ human fibroblast cells were treated with 1mM H2O2 for one hour and lysed after 2h of recovery. First, natively reduced cysteine thiols were labeled with iodoTMT126 then the samples were desalted on Zeba columns, reversibly modified thiols were reduced with TCEP and labeled with iodoTMT127. Such labeled samples were digested and analysed as described above. Data were evaluated in MaxQuant with Andromeda search engine and statistical analysis was done in Perseus.

Results and Discussion

Our preliminary results indicate that 80 proteins had significantly ($P<0.005$) changed redox state in H2O2 treated cells compared to untreated cells, while the total redox state of the whole cell remained unchanged pointing to regulation and accommodation capabilities of the cell.

Conclusion

For the first time we used an iodoTMT set of labels for detection and quantification for total redox changes of protein thiols.GA15-03379S

Keywords: redox proteomics, iodoTMT, cysteine thiols

TO04-01

Molecular Imaging of Protein in Tissues Using Ambient Ionization Top-Down Mass Spectrometry

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Introduction and Objectives

Determination of spatial distribution of molecules in tissues are essential to the understanding of human diseases. MALDI-IMS (imaging mass spectrometry) is widely used for tissue imaging especially for proteins. However, MALDI-IMS takes hours for sample preparation and requires high vacuum environments. Alternatively, strategies that allow soft ionization at atmospheric pressure were developed in the past decade. Among those ambient ionization methods, desorption electrospray ionization (DESI) IMS provides an ability to obtain distributions of metabolites and lipid species in tissues. However, DESI-IMS has so far never reported on protein imaging in tissue. Here we demonstrated that for the first time, using nanospray desorption electrospray ionization (nanoDESI) IMS, protein imaging in tissues are obtained at atmospheric pressure with only minimum sample preparations.

Methods

A nanoDESI source was interfaced onto a x-y stage that we previously used for DESI-IMS, which allows automatic 2-D scanning with <5 micrometer step-size. Various types of tissues, including brain, kidney, and different cancerous tissue samples were sections for direct nanoDESI measurement. The mass spectrometry data in the sections were collected in continuous scanning mode for imaging. Electrospray ions generated by nanoDESI throughout the sections were introduced to LTQ-Orbitrap XL to obtain high resolution mass spectra so as to resolve protein signals of multiple charges. Protein ions were analyzed using top-down approaches on sections using the same platform. Adjacent tissue sections of cancers were stained for further pathological analysis.

Results and Discussion

Multiple-charged proteins of mass up to 15 kDa were successfully measured and visualized by nanoDESI Orbitrap IMS on different biological samples. In an adult mice brain section, expression of proteins including ubiquitin, β-thymosin, myelin basic protein, and hemoglobin were spatially mapped and characterized. We also determined the location of methylation on myelin basic protein. The capability of visualizing molecular heterogeneity is complementary to pathological evaluations. For example, in MYC-induced lymphomas, we observed an array of truncated proteins in the region where normal thymus cells were infiltrated by tumor cells, in contrast to

healthy tissue. The identification of the proteins were performed directly on the sample surfaces via top-down tandem mass spectromentry analysis. Parent ions as well as fragment ions were collected in high resolution using LTQ-Orbitrap. In additional to large molecules, endogenous compounds including lipids and small metabolites could also be imaged in the lower mass window simultaneously.

Conclusion

We have presented a new methodology for visualizing proteins of modest molecular weight in tissue sections using a readily implemented ambient ionization technique. This is for the first time protein imaging akin to MALDI-IMS are acquired with ambient ionization on tissue sections.

Keywords: Imaging mass spectrometrytop-down

TO04-02

3D MALDI imaging mass spectrometry using next generation technologies - Reconstruction of a molecular imaged epididymis

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Introduction and Objectives

Previous proof-of-principle experiments demonstrated the feasibility of 3D imaging mass spectrometry (IMS). When expanded three-dimensionally, IMS can be used to determine volumetric molecular distributions in different regions of an organ. The common approach to 3D IMS is the analysis of serial sections in 2D followed by computational generation of a final 3D dataset. Here, we focused on key aspects, including consistent sample preparation, accelerated acquisition and computationally intensive analysis of big datasets. Rat epididymis was chosen as a model because of its small size, medium anatomical complexity and relevance for studies in the field of spermatogenesis and male infertility.

Methods

Epididymides from adult rats were embedded in CMC, frozen in isopentane and stored at -80°C. An entire organ was cryo-sectioned. HCCA matrix application was conducted using an ImagePrep (Bruker Daltonics). MS acquisition was conducted on a rapifleX MALDI Tissuetyper (Bruker Daltonics) in linear positive mode at 30 µm raster width. Data was then analyzed using SCiLS Lab 3D (SCiLS) to generate a tridimensional organ representation.

Results and Discussion

We present a complete, standardized workflow including tissue collection and sectioning, matrix deposition and molecular image acquisition for 3D IMS. The rapifleX instrument allows analysis of large number of sections to reconstruct an entire organ in 3 dimensions. The high number of replicates has been used to demonstrate the importance of consistency in sample preparation and acquisition to allow the reconstruction of a 3D model. We have developed a pipeline of innovative computational methods enabling the registration of serial sections and the reconstruction of a 3D molecular image model. Spatial segmentation was used to detect prominent spatial features. The segmentation reflects the anatomy of the organ and represents the maturation of proteins through the epididymis. A second unsupervised multivariate approach, probabilistic latent semantic analysis (pLSA), was also applied, which also represents the anatomy of the epididymis and the maturation/alteration of proteins through the epididymal tube. In contrast to the spatial segmentation approach, the pLSA is able to also uncover overlapping distributions. Subsequently, we were able to discover co-localized molecular masses for the major anatomical regions.

Conclusion

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We now aim at exploiting this 3D molecular image to study the molecular events underlying the final maturation of spermatozoa during their transit through the epididymis. The fully automated image registration of two different 3D datasets into one reference coordinates systems will allow the simultaneous correlation of molecular information with histology. The approach has significant potential to enhance the study of physiological and pathophysiological processes, particularly those involving structural changes or those in which the spatial relationship of features is important.

Keywords: Sperm maturation, epididymis, 3D MALDI imaging

TO04-03

Localization and Identification of Peptides from Tissue using high-speed MALDI TOF/TOF mass spectrometry

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Introduction and Objectives

MALDI Imaging is an analytical method for the detection of potential biomarkers directly from tissue sections that has gained popularity in life science research over the past decade. A key challenge in the established workflow is the identification of candidate peaks detected in differential imaging experiments, e.g. cohorts of samples displaying different clinical health states. We previously introduced a workflow which combines the localization of peptides in tissue by MALDI imaging with the identification of peptides by LC-MALDI. Total analysis time is a bottleneck of this approach. Therefore, we established both the imaging and LC-MALDI components on a MALDI-TOF/TOF instrument with 10 kHz laser, reducing acquisition time by a factor of 5-10.

Methods

Tissue sections (fresh-frozen or FFPE) were mounted on ITO-slides. After digestion, one section was coated with matrix using a robotic sprayer and imaged on a rapifleX MALDI TissuetyperTM (Bruker Daltonik, Germany). Peptides were extracted from the serial section in 0.1% TFA, separated by nano-LC (using an 80 min gradient split into 384 fractions). The LC-MALDI analysis was conducted on a rapifleX MALDI TissuetyperTM in TOF/TOF mode. Imaging and LC-MALDI data were combined based on intact peptide masses after statistical recalibration of all spectra using the imageID software, resulting in annotated and localized peptides. In addition, selected peptide IDs where confirmed by on-tissue MS/MS.

Results and Discussion

We consistently achieved acquisition rates of >10 spectra per second, reducing image acquisition time to only 1-2 hours per section and considerably less at reduced spatial resolution (e.g. 45 minutes at 50 µm pixel size). In contrast to previous platforms, which typically require acquisition times 5-10 times longer, this is compatible with the remaining workflow, which requires 30 minutes for enzyme and matrix application and ~2 hours for digest incubation. For the LC-MALDI analysis, acquisition time in MS mode is negligible. Our workflow results in medium complex mixtures of low abundant peptides with ~1000-5000 compounds that require between ~0.4-2 h to analyze in TOF/TOF mode. We typically achieved ID rates of 80% resulting in >100 identified proteins. Not all identified peptides were detected in the imaging runs, which typically had between 200-300 peaks detected consistently in more than 1% of all pixels. Of these, up to 80% could be matched to an ID result in the LC-MALDI data. We validated our matches using two approaches. For selected peptides, we acquired MS/MS data directly from tissue to confirm peptide sequences suggested by the automated matching. For proteins identified with several peptides, we evaluated the co-localization of the corresponding ion images by calculating

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their Pearson correlation coefficients, assuming that peptides originating from the same protein should have similar distribution.

Conclusion

The Image ID workflow speed was increased to a level compatible with Biomarker Discovery.

Keywords: MALDI TOF-TOF

TO04-04

Monitoring ErbB1 and ErbB2 interaction and activation using engineered cell lines and Duolink proximity ligation assay by high-content imaging

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Introduction and Objectives

The ErbB receptor tyrosine kinase family of cell surface receptors signal through AKT, MAPK, and many other pathways that regulate cell differentiation, migration, proliferation, and apoptosis. Many ErbB family members are over-expressed in various forms of cancer, making them important drug targets. A method of monitoring ErbB expression and signaling by high-content screening analysis will enable researchers to screen potential therapeutic agents. ErbB family consists of four cell surface receptors: ErbB1/HER1 (aka, EGFR), ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4. Upon cellular stimulation in a variety of conditions, these cell membrane receptor tyrosine kinases can form homo- or heterodimers, become phosphorylated and internalized. In addition, ErbB proteins can be transported into the nucleus and act as both kinases and transcriptional regulators. Our aim was to investigate the dimerization and activation pattern of EGFR and HER2 by high-content imaging using genetically engineered breast cancer cell lines as model systems.

Methods

Using CompoZr Zinc Finger Nuclease technology, we have developed modified cell lines in which the ErbB1/EGFR and/or ErbB2/HER2 genes have been fluorescently tagged, resulting in endogenous expression of the fusion proteins. These cells have enabled us to monitor their activation and translocation processes. In addition, Duolink proximity ligation assay allows precise detection and quantification of proteins, protein-protein interactions, and protein modifications (e.g., phosphorylation) during the downstream signaling and translocation process in a variety of conditions.

Results and Discussion

To induce EGFR-HER2 interaction and activation, SKOV3 cells with fluorescently-tagged EGFR and/or HER2 were treated with EGF. A dramatic increase in dimerization of EGFR and HER2 was detected by Duolink in response to EGF exposure. In addition, activation of both EGFR and HER2 was detected by Duolink using anti-phospho-EGFR and anti-phospho-HER2 antibodies, respectively. Furthermore, when used in a high-content screening machine, the ability of various drugs to modulate the actions of EGFR and HER2 can be determined.

Conclusion

Thus, Duolink creates an ideal method for screening potential therapeutic agents that affect protein expression, protein-protein interactions, and/or protein modifications.

Keywords: Protein interaction, phosphorylation, Duolink, high-content image analysis

TO04-05

Molecular profile discrimination and mapping of skeletal muscle regeneration in rat crush model using MALDI imaging

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Introduction and Objectives

MALDI imaging is a powerful technique for determining, mapping and visualizing the distribution of endogenous biological molecules such as proteins, peptides or lipids across biological materials. As a result it is possible to obtain spatial localization of a variety of compounds. Softwares allow to discriminate variable/fluctuant compounds within a tissue section or between several tissue sections. This method of analysis presents a great interest for tissue engineering. It will allow to better understand tissue regeneration mechanism and give the means to check that the structure and molecular composition of the regenerated tissue is as close to a healthy tissue as possible. In the current study we used MALDI imaging to evaluate molecular profiles in injured skeletal muscles capable to regenerate spontaneously. The aim was to compare protein expression in healthy muscles (HM) and muscles harvested 2 or 7 days after a crush injury.

Methods

Crush was applied on soleus muscle of Rats (2 months old). Soleus muscles were harvested 2 and 7 days following injury and from control rats (healthy muscle); muscles were frozen in nitrogen and cryosections (10µm) were put on Indium Tin Oxide (ITO) coated glass slides. We used Sinapic Acid (SA)/aniline matrix and analyzed molecules with m/z between 2000 Da and 20000 Da. Image acquisitions performed with flexcontrol software; fleximaging software gave in situ molecular imaging of tissue sections, with spatial localization and ion signal intensity of each molecule. Comparative profile analysis and statistics were performed using SCiLS Lab software (Scils) and ClinPro tools (Bruker).

Results and Discussion

The resulting molecular images clearly discriminated regions within the tissue section that were consistent with injured and non-injured regions. These observations were consistent with the histological analysis. Injured areas, in muscles harvested 2 days after the crush, exhibited a specific low molecular weight profile compared to non-injured areas. This difference disappeared in the muscle harvested 7 days after the crush. The molecular profile analyzed with the software ClinPro tools identified 3 different expression profiles 1) some molecules present on day 2 were absent in healthy muscle and disappeared on day 7; 2) some molecules present on day 7 are also present in healthy muscles ; 3) molecules present on day 7 are absent on day 2 and in healthy muscle. The analysis showed the discrimination of 20 molecules found higher in the injured areas than in non-

injured and among them, three presenting a high level of discriminative profile.

Conclusion

In this study we confirmed the histological result that showed a non injured area and an injured area on soleus muscle in the rat crush model. This method has put forward, in this model, the quick transient expression of low molecular weight proteins during muscle regeneration. This approach will help understanding the molecular processes of skeletal muscle regeneration.

Keywords: MALDI imaging / Muscle injuries

WP04-01

Identification Algorithm of Proteins in 2DE gel image Basing on Matlab Development Environment

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Introduction and Objectives

An identification algorithm is designed for distinguishing proteins from background grayscale in a 2de gel image. The program is written in the environment of Matlab because Matlab itself is one of the most efficient and powerful mathematical software. A large quantity of functionalities built in Matlab are available to deal with images and show data in various forms, bar graph or thermograph for example.

Methods

In this identification algorithm, Gaussian average augment, Gaussian band-pass filter and other functions are used to enhance the quality of original figure for more efficient protein-identification. That is, with these processes, proteins in a gel image could be emphasized and easier to figure out.

Results and Discussion

In former algorithm, edge of a protein is always identified by contour line of the all grayscale, which may lead to the ring form if the center of protein is supersaturated. Different from this kind of algorithm, the algorithm designed here is based on pixel continuation to prohibit this phenomenon.

Conclusion

Saturated gray pixel and local maximum gray level are suspected to be centers of a protein. Classifying these suspected centers could form the only center of a single protein .The 3d view of each protein is given out to show whether the protein gray grad fits Gaussian distribution. This gives a criteria for whether the identified figure is a real protein or is mistaken.

Keywords: Identification algorithm; Matlab; 2DE gel image;

WP04-02

A new Mass Spectrometry Imaging method for evaluation of drug distribution in the Central Nervous System

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Introduction and Objectives

A detailed understanding of the spatial distribution of a drug in a brain is very helpful to definite the pharmacological effect of the Central Nervous System (CNS) in order to minimize side effects. In vitro autoradiography (ARG) is traditionally used to determine the localization of the drug in the brain tissue. Mass spectrometry imaging (MSI) is a powerful analytical technique that provides two-dimensional spatial maps of target compounds in a single experiment without the need to use labeled compounds. This study exemplifies the strength of the in vitro experimental MSI model that involves deposition of a drug onto tissue sections. The usefulness of in vitro MSI method was verified in comparison with ex vivo experiments using rat brain after single subcutaneous administration of the drug. The specific drug distribution in the rat brain tissue section was also evaluated with quantitative measurement.

Methods

Results and Discussion

Applying the in vitro MSI method to rat brain sections result in reproducible the levels of haloperidol obtained in the ex vivo MSI method. One micromolar and one hour were selected as optimal conditions for the in vitro MSI method. The In vitro MSI showed a higher concentration of haloperidol in the striatum as compared to the cerebellum.

Conclusion

The data shows that this simple experimental approach is successful when applied to investigate drug distributions in tissue sections. It is expected that the direct measurement of drug distribution in the tissues can give helpful suggestions for drug discovery strategy. In vitro MSI assay would be a valuable screening tool in the early drug discovery phase for the understanding the drug distribution.

Keywords: MALDI, Mass spectrometry imaging, CNS, Brain, Drug, Distribution

TO09-01

Quantitative proteome-wide profiling of the retromer cargo landscape

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Introduction and Objectives

Accumulating evidence suggests that retromer activity is disrupted in multiple neurodegenerative diseases, including Parkinson and Alzheimer's disease. The core retromer complex composed of VPS35, VPS26 and VPS29 functions together with sorting nexins to sort endosomal cargo to specific membrane structures including the plasma membrane and golgi. Proper retromer function is necessary to direct the localization of specific cargo proteins, however to date a comprehensive characterization of retromer cargo and its specific intracellular trafficking routes has not been performed. This study systematically identifies retromer cargo and elucidates defects in cargo sorting in retromer deletion mutants using quantitative proteomics.

Methods

We used multiplexed tandem mass tag-based quantitative mass spectrometry to quantitatively monitor retromer-dependent cargo trafficking to the plasma membrane, golgi and lysosome in VPS35 deletion mutants. Plasma membrane, golgi and lysosome extracts were isolated from WT and VPS35KO 293T and HeLa cells generated by CRISPR/Cas9 gene editing. Plasma membrane proteins were enriched using selective cell surface biotinylation followed by streptavidin enrichment. Golgi and lysosome fractions were isolated by sequential ultracentrifugation. Proteins were digested with LysC/trypsin, peptides labeled with TMT, pooled, fractionated using a high-pH reversed-phase spin cartridge and analyzed on an Orbitrap Fusion using SPS-MS^3.

Results and Discussion

Quantitative proteomics enabled the deep coverage of the plasma membrane, golgi and lysosome proteome in 293T and HeLa cells. 3850 and 3717 plasma membrane proteins were quantified in 293T and HeLa cells respectively, of which 394 and 428 were differentially regulated in WT and VPS35KO cells. Quantitative proteomics profiling of the golgi and lysosome in WT and VPS35KO 293T and HeLa cells identified 3978 and 3876 proteins, respectively, in the golgi and 4052 and 3976 proteins, respectively, in the lysosome. Notably, several autophagy receptors such as TAX1BP1 accumulated in the golgi in VPS35KO cells, suggesting a role of retromer in regulating nutrient homeostasis. To identify high-confidence retromer cargo, we focused on differentially expressed plasma membrane and golgi proteins consistently regulated in 293T and HeLa cells. These analyses revealed a subset of differentially regulated proteins that included previously characterized retromer cargo such as SORL1 and APP, involved in Alzheimer's disease, and PODXL, recently described as a potential risk factor for Parkinson disease, as well as novel retromer cargo including various transporters such as SLC16A3. Western Blot and immuno-fluorescence experiments confirmed trafficking defects of several high-confidence cargo in VPS35KO cells.

Conclusion

TO09-01

This study comprehensively characterizes the retromer cargo landscape using quantitative proteomics and increases our understanding of retromer-dependent protein trafficking.

Keywords: Retromer, subcellular proteomics, cargo trafficking, TMT

TO09-02

Refining the details in the tissue-based map of the human proteome

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Introduction and Objectives

The tissue-based map of the human proteome, released by the Human Protein Atlas project focuses on an integrated omics approach for in situ detection of human proteins down to the single cell level. In the quest for generating a complete tissue-based map of all human proteins and identifying missing proteins, the Tissue Atlas will continue to evolve in upcoming releases.

Methods

The Tissue Atlas utilizes quantitative transcriptomics at the tissue and organ level, combined with tissue microarray-based immunohistochemistry. Extended antibody validation strategies, including independent antibodies, internally and externally generated RNA-seq data and knowledge-based expert annotation were employed in order to generate a best estimate of the histological distribution and relative expression level of each protein.

Results and Discussion

While most proteins have been shown to be expressed in all tissues and relatively few are unique for one or a few tissues, almost 10% of all human proteins are not detected in any of the previously analyzed tissues. By expanding the tissue repertoire, more than 100 new genes were identified in e.g. retina and more specialized regions of brain. In-depth analysis was also performed on 100 testis enriched genes by expert annotation of proteins expressed at various stages of the seminiferous cycle during spermatogenesis.

Conclusion

The data and images in the online Human Protein Atlas (www.proteinatlas.org) represent a valuable resource in order to gain biological insight on human proteins. In order to refine the details in the Tissue Atlas, a large effort is put into extended antibody validation strategies, inclusion of more specialized tissues, as well as more detailed exploration of Human Protein Atlas images, including testis samples. The generated data undoubtedly constitute the basis for understanding of normal and pathological spermatogenesis and other biological processes. It is also likely to become useful for numerous spin-off projects in both basic and clinical research.

Keywords: Human Protein Atlas, spatial proteomics, integrated omics, immunohistochemistry, RNA-seq

TO09-03

Membrane Localization of Metabolic Enzymes and Metabolic Modulation in a Cell Division Mutant of Escherichia Coli Identified by Omics Approaches

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Introduction and Objectives

It is generally believed that membrane association of a protein could cause conformational changes thus modulating its function. In this study, we report that a membrane-associated protein oscillator, the E. coli Min system, may play a role in modulating the membrane interaction and function of proteins.

Methods

Inner membranes from the wild-type and Δmin mutant strains were analyzed to generate proteomics datasets based on NanoLC-nanoESI-MS/MS mass spectrometry using the isobaric tags for relative and absolute quantitation (iTRAQ) method. The data were processed and resulted in an inner membrane proteome of unique proteins with quantitation. Forty proteins of interest (POIs), that show significant difference in protein abundance of the mutant membrane, were isolated through statistical filtering.

Results and Discussion

Three important features were found associating with POIs. First, more than half were peripheral membrane proteins, suggesting that the Min system affects mainly reversible protein association with the inner membrane. Second, metabolic enzymes accounted for 45% of the POIs, and there was a change of metabolites in the related reactions that were identified by metabolomic analysis. Therefore, we hypothesize that the Min system could alter the membrane location of proteins to modulate their enzymatic activity. Third, 6 out of 10 selected POIs directly interacted with at least one of the three Min proteins as demonstrated by both two-hybrid and pull-down assays, confirming the correlation between POIs and the Min system as well as identifying novel interactions of the Min system.

Conclusion

The metabolic modulation could be an adaptive phenotype in the Δmin mutant, due to an imbalanced abundance of proteins on the inner membrane, suggesting a global physiological impact of the Min system in addition to the division site placement.

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Keywords:
Bacteria, Cell division, iTRAQ, Protein Targeting, Subcellular analysis, Metabolism, Protein-membrane interaction, the Min system

TO09-04

Determining Post-Translational Modifications of Nuclear Proteins

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Introduction and Objectives

Genomic DNA is packaged into nucleosomes that contain eight histone proteins to form compact chromatin domains. Chromatin changes its structure to regulate fundamental cellular processes such as DNA replication, transcription, DNA damage repair, and recombination. Histone proteins, DNA, and DNA-binding factors such as transcription factors regulate chromatin functions. In addition, post-translational modifications (PTMs) have critical roles in the functions of chromatin protein components. For example, histones are known to be acetylated, methylated, phosphorylated, and ubiquitinated. These PTMs cause chromatin structure changes to regulate transcription. However, little is known about PTMs of chromatin proteins other than histones and a few others. The enrichment of chromatin protein components is required for comprehensive proteomic analysis of such proteins by mass spectrometry. Chromatin enrichment for proteomics (ChEP) was developed to aid such analysis. However, it is not suitable for enriching samples for mass spectrometry. We have therefore optimized ChEP to facilitate the effective analysis of chromatin protein components by LC-MS/MS.

Methods

Chromatin proteins and DNA were cross-linked in vivo using formaldehyde. Following cell lysis, non-cross-linked proteins were washed away and chromatin was sheared by sonication. The samples were boiled to reverse formaldehyde-induced cross-linking. After digesting with trypsin, samples were enriched for phosphopeptides using titanium dioxide. Peptides were analyzed using an LTQ Orbitrap Velos mass spectrometer. Phosphoproteins and phosphopeptides were identified by MASCOT (1% FDR). Label-free quantitative analysis was performed with Progenesis QI software.

Results and Discussion

We identified a total of 1907 phosphopeptides derived from 688 phosphoproteins. By optimizing ChEP, the abundance of known phosphoproteins related to transcription and chromatin modification was increased relative to the total proteome.

Conclusion

By using ChEP combined with LC-MS/MS, we were able to detect more PTMs on

nuclear proteins than by conventional methods without concentration. Our results suggest that new knowledge concerning diseases can be obtained by optimizing ChEP. Using our improved ChEP method, we were able to analyze chromatin protein components from androgen-independent prostate cancer cells and identify their PTMs.

Keywords: Chromatin, Phosphorylation, Proteomics

TO09-05

Single-cell proteome profiling: Innovations in sample preparation

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Introduction and Objectives

Single cell analyses are becoming more common place with other measurement areas such as genomics and transcriptomics based on signal amplification. These results demonstrate that individual cells have unique characters and should be individually analyzed in some cases. It remains difficult to achieve single cell proteome analysis, especially as one cannot comprehensively amplify MS signals of whole digested peptides. Here we optimize all procedures to minimize losses, optimize signals, and finally achieve single cell proteome profiling.

Methods

Target cells were directly captured into capillary columns packed with immobilized trypsin beads. The whole proteins were extracted, reductively alkylated, and digested in the capillary column, and then the columns are directly connected to nanoLC-MS/MS system. The digested peptides were analyzed using QExactive or Triple TOF 5600. We utilized miniaturized capillary columns for peptide separation to achieve high separation efficiency and low flow rate. These conditions reduce ionization suppression effects in electrospray ionization, leading to enhanced MS signals. Peptide identification was performed against the NCBI nr or the UniProtKB/Swiss-Prot database at the 95 % confidence limit using Mascot software.

Results and Discussion

First, our In-capillary method was validated with respect to the efficiency of reductive alkylation and frequency of missed cleavages using small amount of HeLa cells. Only one peptide among 101 cysteine-containing peptides included non-alkylated cysteine residue. The number of peptides with one or two missed cleavage sites was comparable to conventional methods in bulk solutions. Furthermore, the number of identified peptide was three times higher using In-capillary method than conventional high-recovery method, indicating that our method is a promising tool to achieve the highest recovery of whole-cell proteins. In-capillary method was applied to various kinds of Aplysia Californica single neurons. We identified up to 621, 1054, and 556 proteins from single R2, R15, and metacerebral neurons, respectively. The percentages of overlapping proteins between different kinds of cells were lower (20-28%) than that between two MCCs (58%) and also that between duplicate MS analysis of pooled proteins from many cells (70-80%). These results suggest that the different proteome profiles between different kinds of cells and also between different single cells of the same cell strain can be detected on single cell level, meaning that we can evaluate the single cell heterogeneity in the same strain. We will further report on a novel sample preparation tool to achieve more sensitive single cell proteome profiling.

Conclusion

TO09-05

MS-based single cell proteome profiling appears achievable and cellular heterogeneity was analyzed on single cell level.

Keywords: single cell proteomics, highly sensitive LC-MS, In-capillary sample preparation

WP05-01

Standardization of mitochondrial preparations for the Human Proteome Project.

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Introduction and Objectives

The mitochondrial Human Proteome Project (mt-HPP) is a HUPO initiative led by the Italian Proteomics Association (ItPA) focused on human mitochondrial proteins. The functional and pathological correlations of the mitochondrial proteins with the nuclear encoded proteins is a fundamental task of the mt-HPP. An integrated view of the molecular actors leading the life and the functions of human mitochondria will represent a strong base for studying the mitochondrial alterations which occur in many human diseases. Eventually, all the data collected will be integrated within the Biology/Disease Human Proteome Project (B/D-HPP). In this view, we started a standardization project aimed at defining the most appropriate method for each cellular model and for the specific biological issue faced.

Methods

Fifteen labs have been involved. Ten ECACC cell lines have been selected (BJ, SH-SY5Y, U2OS, MDA-MB-231, NCI-H28, Hek293, HUVEC, THP1, HepG2, HeLa) and cultured mycoplasma free. Three methods for mitochondrial isolation have been chosen (differential centrifugation, sucrose gradient separation, a commercial kit based on surfactants) and samples prepared with different methods from the same cell line reached the same MS lab. Integrity of mitochondrial preparations were assessed by measuring the oxygen consumption rate and the activity of selected enzymes. Seven different mass spectrometers have been used (Bruker Maxis HD and Impact HD, Waters Synapt G2si, Thermo Fusion, Velos ETD and LTQ-Orbitrap-XL, Sciex TripleTOF 5600+) and all the data reached the same center for the data analysis with the PEAKS software. Protein identities observed in at least two out of three biological replicates (two technical replicates each) have been selected for further analysis. Identities were mapped on the mitochondrial functional proteome network (Fasano et al., EuPA Open Proteomics 2016).

Results and Discussion

The standardization project of the Italian mt-HPP consortium is still ongoing. At the moment, all the samples have been prepared and reached the MS labs. Complete datasets for three cell lines have been processed. On the average, about 80% of identified proteins belong to the functional proteome of the mitochondrion, i.e., proteins either localized in or associated to the mitochondrion. The proportion between these two categories is dependent of the enrichment method and of the cell line. In general, the commercial kit leads to highly purified, fully viable mitochondria, whereas centrifugal methods offer a compromise between high yield and a broader coverage of associated proteins.

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Conclusion

We may summarize, at the moment, that some preparations are more suitable for particular cell lines or particular scientific purposes. As an example, the sucrose gradient separation has normally a very low yield but a high purity, thus being appropriate for cell types rich in mitochondria and for investigations where a little contamination is required.

Keywords: Mitochondria; B/D-HPP.

WP05-02

A Mouse Tissue Transcription Factors Atlas

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Introduction and Objectives

Transcription factors (TFs) drive a variety of biological processes, ranging from embryo development to carcinogenesis.A global view of mammalian tissue TFs in proteome scale can directly point to tissue specific mechanism, and place the function of each TF in a whole organism perspective.

Methods

Here, we employed concatenated tandem array of consensus TF response elements (catTFRE)approach developed recently, to profile activated TFs in 24 adult tissues and 6 fetal tissues mouse organs/tissues.

Results and Discussion

An average of 290 TFs from a single tissue type and a total of 941 TFs were quantitatively identified on protein level, representing over 60% of mouse gene coding TFs. TF networks in blastoderm, system, and tissue resolutions were built up. Integrated omics analysis further dissected TF hierarchy in tissues, including expressed TF, specific TF, and tissue type maintenance TFs (ttmTFs). ttmTFs, defined in this database, are crucial in tissue functions and identities. The regulations of TF patterns, especially ttmTF groups, are directly correlated to physiology and pathology perturbations.

Conclusion

Our study provides a landscape of TF expressions and activities in mouse tissues that can serve as references of tissue transcriptional drivers, rendering better understanding of tissue development, differentiation, and function maintenance in mammal.

Keywords: Transcription factor/ Nuclearreceptor / TF response elements/Proteomics/Tissue atlas

WP05-03

Subcellular look at asthenozoospermia: Proteomic analysis of human sperm tail in asthenozoospermic patients

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Introduction and Objectives

Asthenozoospermia (AS) which is characterized by low sperm motility is one of the most common causes of male infertility. Although many intrinsic and extrinsic factors are involved in the etiology of AS, the molecular basis of this condition still remains unclear in many patients. In fact, sperm acquire motility by the action of a specific tail structure. So, an in-depth study of sperm tail can elucidate impaired sperm motility. In this study, we combined high throughput quantitive proteomics, based on Tandem mass Tags (TMTs) labeling along with subcellular fractionation in order to identify low-abundance proteins which would otherwise be undetectable by conventional methods.

Methods

This was a case-control study comprising 80 men who attended Royan infertility center for assisted reproduction. Semen samples were evaluated by computer assessed analyzer and divided into asthenozoospermic group (progressive motility <32%, N=40) and normozoospermic group (progressive motility >32%, N=40). Samples of four individuals were pooled and the tail fractions isolated by sonication and successive sucrose gradient. After confirming tail fraction purity, the extracted proteins were labeled with tandem mass tags (TMTs) followed by shotgun proteomics. Bioinformatic analyses were performed using DAVID. Candidate proteins were further validated by Western blot analysis.

Results and Discussion

We detected 2145 proteins in the tail fraction of human sperm where 189 and 290 proteins were respectively up and down regulated in asthenozoospermic patients compared to normozoospermic donors. The main down-regulated proteins were structural proteins as well as those involved in energy production pathways. Furthermore, we identified DDX3Y (ATP-dependent RNA helicase) and HSFY (Heat shock transcription factor Y linked), Y chromosome encoded proteins, being altered in the sperm of asthenozoospermic patients.

Conclusion

While it is known that Y chromosome encoded proteins play a critical role in male gonad development and spermatogenesis, our data highlight their significance in the function of mature spermatozoa.

Keywords: Sperm tail, Asthenozoospermia, Proteome, DDX3Y, HSFY

WP05-04

Major mitochondrial machineries are integral part of cell architecture comprising cytoskeleton and nuclear matrix

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Introduction and Objectives

In situ fractionation of cellular components using biochemical extractions was devised about three decades ago, as a means to prepare insoluble cell structures, such as nuclear matrix and cytoskeleton. Given its stability under stringent conditions and structure reminiscent of cell morphology, we propose to term this residual structure as 'cell architecture', the basic structural form of a cell. In order to investigate its protein constituents, we subjected HeLa cell architecture to proteomic analyses.

Methods

Results and Discussion

We have identified a total of 1425 proteins in cell architecture. Using the information in Gene Ontology (GO) database, we found that about 43% and 25% of these proteins are associated with the keyword 'nucleus' or 'cytosol', respectively. For example, we successfully identified several nuclear lamina proteins and nucleoporins, consistent with the expected composition of cell architecture. Surprisingly, we found 28% of cell architecture proteome is likely from mitochondria. We have defined the proteins removed with a four-step extraction procedure, and found that these four fractions have features distinct from the cell architecture, strongly arguing for the unique properties of cell architecture proteins. Major mitochondrial machineries, such as respiratory chain complexes, mitochondrial ribosomes and protein import complexes, are all part of cell architecture, which is corroborated by indirect immunofluorescence staining that analyzed mitochondrial proteins, such as MRPL41, COX4 and UQCRC1.

Conclusion

Our data suggest that cell architecture, the cellular base in conjunction with extracellular matrix, is a gigantic structure that also contains part of mitochondria.

Keywords: in situ fractionation; cell architecture; cytoskeleton; nuclear matrix; mitochondria; mass spectrometry; proteomics

WO04-01

Analysis of Plasma Membrane Proteomes of Gastric Cancer Cells Reveals that ASCT2 is involved in Cancer Metastasis by Targeting WNT/b-catenin Signaling

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Introduction and Objectives

Gastric cancer (GC) is one of the most frequent cancers and remains a significant cancer burden nowadays worldwide. Plasma membrane proteome represents an attractive source of potential molecular biomarkers and drug targets for therapeutic intervention of cancer. Here, we used an optimized biotin/streptavidin affinity method to isolate and enrich cell surface proteins from one normal gastric cell line and three gastric cancer cell lines. Membrane proteins were SDS-PAGE-separated and analyzed by repeated label-free LC-MS with LTQ Orbitrap, followed by a multi-engine database searching. We identified 4,158 proteins at a false positive rate of 0.6%. Almost half of them (2,019; 48.6%) were extracellular-exposed proteins, including 1,332 plasma membrane proteins and 687 extracellular matrix proteins or secreted proteins. There were also 1,005 cellular membrane-integral proteins. We identified 15 up-regulated proteins and 29 down-regulated proteins, including plasma membrane protein SLC1A5 (ASCT2), an amino acid transporter for glutamine. ASCT2 protein was higher in GC cell lines tested comparing with a normal GC cell line GES-1 and ASCT2 gene was upregulated in diffuse, intestinal and mixed type of GCs by interrogating Oncomine gene expression database. Increased ASCT2 protein or gene expression was significantly associated with poor prognosis of GC, as revealed by immunohistochemistry of a tissue microarray and data-mining of Oncomine data. Silencing of ASCT2 gene downregulated GC cell proliferation by suppressing mTOR/P70S6K signaling pathway. Furthermore, knockdown of ASCT2 attenuated CG cell migration, invasion and epithelial-mesenchymal transition. WNT/ -catenin activity (Tof-flash assay) and the expressions of the downstream effectors were also suppressed. While Ectopic expression of ASCT2 stimulated the downstream gene expressions of WNT/ -catenin pathway. These results suggested that ASCT2 was a potential biomarker and therapeutic target of GC. Taken together, our current analysis of GC plasma membrane proteome shed novel insight into the characteristics of GC cells and provide a wealth of potential therapeutic target for GC.

Keywords:

Gastric cancer / ASCT2 / Plasma membrane / Proteomics / Glutamine / Metastasis / Molecular marker

WO04-02

Tumour-dependent fibroblast activation: when a good neighbour turns bad

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Introduction and Objectives

Tumour-stromal signaling crosstalk in the tissue microenvironment is increasingly recognized as a driving force in metastatic dissemination. While the roles of endothelial and immune cells have been studied rigorously, how activated fibroblasts contribute to tumour metastasis remains sketchy. This work aims to (I) identify potential tumour-derived fibroblast activators; and (II) elucidate cellular changes in activated fibroblasts that support tumour propagation, using a 'multi-omics' approach.

Methods

To identify the first paracrine factors that initiate fibroblast activation, we screened the tumour secretome for proteinaceous activators. By coupling pulsed metabolic labelling with click chemistry and affinity enrichment, we avoided secretome artifacts due to serum starvation and high-density culture, and achieved 95% enrichment of secreted proteins from highly invasive breast carcinoma. Fibroblast-activating function of tumour-derived factors were validated using an elegant immuno-depletion/supplementation approach. Effects of activated fibroblasts on tumour migration and invasion were assessed with in vitro co-culture systems.

Results and Discussion

From this, we identified 348 secreted proteins with high confidence and extensively validated a two-component system of tumour origin that synergistically activates fibroblasts of the same patient, in a contact-independent manner. Activated fibroblasts exhibited striking changes in morphology and growth characteristics, with concomitant expression of activation markers. In co-culture systems, the presence of activated fibroblasts in media contact also significantly increased migration and invasion of paired tumour cells, suggesting that fibroblasts and tumour cells engage in two-way crosstalk both before and after activation. Analysis of activated fibroblasts by a combination of proteomics, phosphoproteomics and metabolomics approaches further revealed that the activated state is associated with higher nutrient uptake and sustained by a myriad of proteome and phosphorylative changes converging on the regulation of ROS signaling, antioxidant defense, inflammation and tissue repair. The activation process also drastically shifted the fibroblast secretion profile to an almost "tumour-like" one where essential growth and pro-metastatic factors, as well as mediators of oxidative damage, are now provided exogenously by the activated fibroblasts.

Conclusion

These findings demonstrate succinctly how tumour cells influence the stromal microenvironment and manipulate fibroblasts to support a metastatic niche. Data presented herein should contribute to deeper understanding of tumour-dependent fibroblast activation, and potentially reveal alternative paths towards tumour clearance by

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inhibiting this process.

Keywords: secretome; pulsed metabolic labeling; tumour microenvironment crosstalk; fibroblast activation; multi-omics analyses.

WO04-03

Analysis of Proteoforms in Membrane Protein Complexes by CID/ETD Top-Down Proteomics

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Introduction and Objectives

Despite their involvement in cellular processes of importance like homeostasis, inter-cell communication, cell adhesion and transport, our understanding of the function of membrane protein complexes in pathologies is often limited. Getting some insight into their chemical structure is a first step to get a better understanding of their biogenesis, function and regulation. Top Down proteomics can be a valuable tool to perform a qualitative and semi-quantitative analysis of the various proteoforms belonging to a complex. As those complexes are often fragile and/or hydrophobic, their analysis can constitute an analytical challenge. Therefore, we have developed a method for the efficient, reproducible and unbiased extraction of intact subunits from excised clear native gel bands for top down proteomics.

Methods

Excised gel slices were crushed in detergent solution to release and dissolve intact protein complexes. After protein extraction, the solution was exchanged with urea for detergent removal before direct LC MS/MS. LC-MS/MS Measurements have been performed on a maXis 4G ETD or a maXis II UHR-Q-TOF coupled to a nano-advance UHPLC (Bruker Daltonics) in both CID and ETD modes. The pre-concentration was done with a C4 PepMap300, 5µm, 300Å pre-column (Thermo Scientific) and the separation was performed on a PLRP-S column (0.15 x 150mm, 4000Å, Michrom) at a flow rate of 2 µl/min. Data was processed in Data Analysis (Bruker Daltonics). Protein identification and characterization have been performed with BioTools (Bruker Daltonics) and Mascot (Matrix Science) or TopPIC (Indiana University–Purdue University Indianapolis).

Results and Discussion

This approach has been applied to the studying of protein complexes of the mitochondrial oxidative phosphorylation (OXPHOS) system in Bos taurus. The OXPHOS enzymes are hydrophobic heteromeric complexes in the range of 0.2 – 1 megadalton that generate adenosine triphosphate to fuel cellular processes. A mitochondrial fraction was prepared from bovine heart and extracted protein complexes were subjected to high resolution clear native gel electrophoresis. OXPHOS complexes were isolated from gel slices. The reproducibility and recovery of the extraction method was evaluated by denaturing gel electrophoresis and top-down proteomics. Denaturing gel electrophoresis results showed that all subunits from each complex were extracted without any bias. The vast majority of subunits for each complex could be detected by top-down proteomics except for some large or hydrophobic subunits. Various post-translational modifications were found such as mitochondrial import sequence cleavage, acetylation, formylation, and

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phosphorylation. In-depth characterization of proteoforms by electron transfer dissociation and collision induced dissociation tandem mass spectrometry will be presented.

Conclusion

This Approach enables the characterization of proteoforms in membrane protein complexes to gain insight into their biology.

Keywords: Top Down, Proteoforms, UHR-Q-ToF, ETD

WO04-04

In silico characterization of the human lipid raft proteome

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Introduction and Objectives

Dynamic cholesterol and sphingolipid-enriched membrane microdomains, also called lipid rafts, regulate cell function by modulating molecular interactions. Despite the functional importance of lipid rafts in health and implication in disease, features that facilitate recruitment of proteins to lipid rafts are poorly understood. The objective of this study is to characterize the human lipid raft proteome based on the structural features of the proteins.

Methods

Lipid raft proteomics database RaftProt (1) was updated with new proteomics studies to generate the list of human lipid raft proteins. Human proteome and human plasma membrane proteome was used as comparison. Transmembrane helices were predicted using TMHMM (2) and TOPCONS (3). Protein lipidation annotation information was retrieved from SwissPalm (4), UniProtKB (5) and HumanPRENbase (6). Cholesterol-binding proteins were retrieved from Hulce et al (7).

Results and Discussion

The updated RaftProt contains a total of 4898 non-redundant human lipid raft proteins from 81 proteomics experiments on 54 cell/tissue types. Compared to the plasma membrane and total human proteome, palmitoylated and cholesterol binding proteins are selectively enriched in the human raft proteome. Based on the mode of lipid raft targeting, we classified the human lipid raft proteome into 3 subsets. Raft proteins with direct membrane interaction via transmembrane or lipid binding domains, or lipid modifications (2183) were classified as intrinsic raft proteins. Extrinsic components of lipid raft (1684) were defined as proteins that directly interact with integral raft proteins. The remainder of the raft proteome (1031) show no apparent features of raft targeting and could be contaminants during raft isolation. Protein-protein interaction network analysis showed a higher degree of interconnections among intrinsic and extrinsic lipid raft proteins compared to the plasma membrane. Furthermore, functional enrichment analysis hints at over-representation of receptors, transporters and cytoskeletal proteins in the intrinsic and extrinsic lipid raft proteome.

Conclusion

Our global integrative analysis begins to reveal the raft-targeting mechanisms and function of the human lipid raft proteome. REFERENCES1. Shah A et al. Nucleic Acids Research. 2015, 43:D335.2. Krogh A et al. Journal of Molecular Biology 2001, 305:567.3. Tsirigos KD et al. Nucleic Acids Research 2015, gkv485.4. Blanc M et al. F1000Research 2015. 4.5. Breuza L et al. Databases 2016, bav120.6. http://mendel.imp.ac.at/sat/PrePS/HumanPRENbase/7. Hulce JJ et al. Nature Methods 2013. 10:259.

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Keywords: Lipid raft, protein targeting, membrane proteome, lipid binding

WO04-05

Exosomal EphA2 transmits chemoresistance and predicts pancreatic cancer patient responses to therapy

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Introduction and Objectives

Resistance to therapy is a primary cause of treatment failure in most human cancers. Pancreatic cancer (PC), one of the most challenging malignancies to treat, is characterized by aggressive local invasion, early metastasis, and a high degree of resistance to therapy. Drug resistance is thought to be a major reason for treatment failure, indicating an urgent need to identify and apply predictive markers to segregate PC patients into personalized treatment regimens to minimize therapy resistance. Molecular and genomic research of PC suggests that varying responses to therapy may be attributable to tumor heterogeneity, implying that metastases may exhibit divergent responses due to clonal diversity. Tumor-derived exosomes contain proteins and nucleic acids that can serve as key mediators in cell-cell communications, increasing tumor progression and metastasis. We hypothesized that exosomal transfer of a resistance factor from a chemoresistant PC tumors might also be able to increase chemoresistance of more susceptible PC clones.

Methods

We therefore analyzed whether exosomes and specific exosomal proteins could transfer chemoresistance between heterogeneous pancreatic cancer (PC) cell lines. Exosomes isolated from three PC cell lines (PANC-1, MIA PaCa-2, and BxPC-3) with variable gembicitabine (GEM) sensitivity were tested for their capacity to transmit chemoresistance and then analyzed by comparative proteomics to identify candidate resistance factors.

Results and Discussion

Our results revealed that exosomes of chemoresistant PANC-1-derived exosomes were internalized by and increased GEM resistance of chemosensitive PC cells. PANC-1 exosomes were subsequently found to overexpress Ephrin type-A receptor 2 (EphA2), which is associated with therapy resistance in other tumor types, and chemoresistance transmission was inhibited by shRNA-mediated EphA2 knockdown in PANC-1 exosome donor cells, while direct treatment with recombinant EphA2 did not promote chemoresistance. Notably, circulating exosomal EphA2 levels were dramatically increased in mice bearing PANC-1 tumors, and in human PC patients who subsequently revealed poor responses to various chemotherapy and/or chemoradiation regimens.

Conclusion

Based on these results, we conclude that exosomal EphA2 can transmit therapy resistance between heterogeneous PC cells and may potentially serve as a non-invasive biomarker to predict treatment response in patients with pancreatic cancer. However, it remains to be seen if additional exosomal factors regulate resistance to other cancer

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therapeutic agents in pancreatic cancer or other cancer types.

Keywords: Exosome, EphA2, Cytotoxic resistance, Pancreatic cancer, Gemcitabine

WO04-06

Systemic perturbation of keratinocyte homeostasis by genetic loss of the extracellular matrix protein collagen VII

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Introduction and Objectives

Collagen VII is an extracellular matrix protein and the major component of anchoring fibrils. It is of vital importance for the functional integrity of the dermal-epidermal junction zone. Loss of collagen VII causes recessive dystrophic epidermolysis bullosa (RDEB) and may lead to a broad spectrum of clinical features: blister formation, abnormal wound healing, excessive scarring, often resulting in aggressive skin cancer.

Methods

To better understand molecular mechanisms underlying these diverse phenotypes we performed global transcriptomics and proteomics profiling of primary human keratinocytes comparing RDEB cells to healthy control cells. We investigated the impact of loss of collagen VII on cell homeostasis using RNAseq and SILAC-based quantitative mass spectrometry-based proteomics. The cellular proteome as well as the protein composition of the extracellular matrix were analyzed.

Results and Discussion

This approach enabled us to provide a detailed but also global image of the deregulation of molecular mechanisms in RDEB. Loss of collagen VII leads to distinctive, consistent changes on transcriptome and proteome level, such as the downregulation of direct and indirect binding partners of collagen VII and the upregulation of fibrotic markers. However, we also detected proteome specific intra- and extracellular changes, like the upregulation of S100 proteins, which are linked to inflammation and are used as respective biomarkers. Our analyses further suggest an upregulation of proteases, such as cathepsin B, on the protein level.

Conclusion

Increased cathepsin B activity in RDEB keratinocytes might be involved in ECM degradation providing a potential new druggable target for disease intervention

Keywords: extracellular matrix, collagen VII, MS-based proteomics, transcriptomics, RNAseq, recessive dystrophic epidermolysis bullosa, RDEB, cathepsin B, primary human keratinocytes, inflammation, fibrosis

WP06-01

Bio-inspired nanoparticles derived from immune cells: design, characterization, and understanding their cellular fate by proteomic tools

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Introduction and Objectives

Bio-inspired nanotechnology uses biological systems as inspiration to create novel drug delivery platforms. Bio-inspired nanoparticles (NPs) functionalized with cell membrane proteins are imbued with cell-like functions, allowing them to simultaneously evade immune surveillance and negotiate transport across biological barriers. From this standpoint, immune cells provide a valuable basis from which to build injectable carriers. Leukocytes freely circulate in the bloodstream and, thanks to their interaction with inflamed vasculature, accumulate selectively in diseased tissue. We have manipulated and exploited leukocyte membranes as a proteolipid material to formulate a new generation of biomimetic liposomes, called leukosomes.

Methods

Our approach leverages versatile assembly methods used for liposomes (our control) to synthesize stable, highly standardized NPs. Leukosomes were extensively characterized to elucidate their physicochemical properties through cryo-electron and atomic-force microscopy, and dynamic light scattering analyses. In addition, proteomic and flow cytometry studies were performed to characterize protein composition, status, and orientation on the carrier's surface.

Results and Discussion

Proteomic analysis showed the presence on leukosomes of over 180 leukocyte membrane proteins conserving their orientation, post-transcriptional modifications, functions, and cooperation with other proteins. Thanks to the functionalities of these proteins, leukosomes showed in vivo targeting of inflamed endothelium associated to localized inflammation and cancer (breast cancer and melanoma). Moreover, leukosomes exhibited lower accumulation in MPS organs (liver, spleen) and longer circulation times, compared to conventional liposomes. The protein corona adsorbed on leukosomes following incubation in plasma was also evaluated and compared to those adsorbed on liposomes. Upon intravenous injection, NPs are surrounded by biomolecules present in the blood that create a sort of "corona" around them. This confers a new biological identity on the particles that largely determines their biological fate. Understanding the interactions occurring at the interface between NPs and the surrounding biological system has become crucial to predicting and interpreting NPs biodistribution, targeting, and efficacy, and will, therefore, be needed to design more effective drug delivery systems. Our results revealed that the presence of leukocyte plasma membrane proteins within the vesicle's bilayer strongly affected the number, amount, and type of plasma proteins adsorbed.

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Conclusion

This approach could open new avenues for the development of next generation personalized treatments by using immune cell of patients as source of membrane proteins. To the best of our knowledge, this approach represents the first time (i) plasma membranes have been formulated into lipid nanovesicles using an established methods; (ii) bio-inspired NPs' protein corona has been investigated.

Keywords: nanomedicine, leukocyte, membrane proteins

WP06-02

Label-free Proteomic Analysis of Exosomes Secreted by HBV-inducible HepAD38 Cell Line

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Introduction and Objectives

Hepatitis B virus (HBV) infection is a major health problem worldwide. Recent evidence suggests that various viruses can manipulate the infection process by secretion of specific viral and cellular components into exosomes, small nanometer-sized (30-150 nm) vesicles secreted from various cells. However, the impact of HBV replication to hepatocytes produced exosomes has not been fully delineated. In this work, an HBV-inducible cell line HepAD38 was used to directly compare changes in the protein content of exosomes secreted from HepAD38 cells with or without HBV replication.

Methods

Exosomes were isolated from conditioned medium of HepAD38 cell cultures and the purity of exosomes were confirmed by transmission electron microscopy (TEM) and Western immunoblotting assays. Ion-intensity based label-free LC-MS/MS quantitation technologies were applied to analyze protein content of HBV-exosomes and HBV-free-exosomes.

Results and Discussion

In total, 1412 exosomal proteins were identified, in which 35 proteins were significantly altered. Strikingly, 5 subunit proteins from the 26S proteasome complex, including PSMC1, PSMC2, PSMD1, PSMD7 and PSMD14 were consistently enhanced in HBV-exosomes. Bioinformatic analysis of differential exosomal proteins revealed the significant enrichment of components involved in proteasomal catabolic process. Proteasome activity assays revealed that HBV-exosomes have enhanced proteolytic activity compared to HBV-free-exosomes. Furthermore, Human peripheral monocytes incubated with HBV-exosomes induced a significant lower level of IL-6 secretion compared to HBV-free-exosomes. Incubation in the presence of proteasome inhibitor MG132 decreases the difference in IL-6 induction. These results suggest that transmission of proteasome subunit proteins by HBV-exosomes might influence proteolysis activity in the recipient monocyte cells.

Conclusion

These new findings contribute to our knowledge about HBV infection and the role of exosomes from HBV-infected hepatocytes in transfering functional proteins to immune cells.

Keywords: HBV / exosomes / hepatocyte / proteasome / label-free.

Mining biomarkers for gastric cancer diagnosis by personalized membrane proteomics and multiple reaction monitoring mass spectrometry

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Introduction and Objectives

The membrane proteins hold promises for cancer detection because most FDA-approved cancer biomarkers are secreted membrane proteins. At present, however, the current biomarkers, such as CEA, CA 19-9, and CA 72-4, have low sensitivity and specificity for gastric cancer (GC) detection. In this study, we established discovery-through-verification pipeline for individualized tissue membrane proteomic profiling by label-free quantitation and validation of targeted membrane proteins of individual patients by MRM-MS assay to discover and verify biomarker candidates.

Methods

In discovery phase, membrane fractions were prepared from 24 pairs of tumor and adjacent normal tissues. Each batch of extracted membrane proteins were undergone gel-assisted digestion, high-performance LC-MS/MS analysis and a label-free quantitation by IDEAL-Q. In verification phase, targeted candidates were verified by MRM-MS approach by following steps: (1) transitions for each candidate were selected from our in-house membrane proteome spectral libraries, (2) selected transitions were further tested on mixed cell lines and tissues to evaluate their reliability, (3) the optimized collision energy and cycle time for each transitions were set up for scheduled MRM-MS analysis. Selected candidates with available antibody were further examined by WB, IHC, and tissue microarray (TMA).

Results and Discussion

To the best of our knowledge, we have provided the largest membrane proteome profile of GC to date. The analysis quantified 1746 proteins. The proteins satisfying criteria: (1) higher expression in more than 60% of all patients as well as in stage I and II patients; (2) evidence of presence in serum or secretion ability, were selected as the biomarker candidates. A total of 35 marker candidates were filtered and verified by optimized MRM-MS analysis. 8 candidate proteins were confirmed with significantly higher expressions in tumors. To further evaluate the clinical relevance of these candidates, 3 biomarker candidates with available antibodies were examined by TMA from GC patients. ROC curve revealed that these 3 candidates exhibited excellent discriminate between tumor and normal mucosa. To further pursue the possibility of non-invasive diagnosis from serum, we focused on membrane proteins which could secreted or shed from cancer cells and released to the circulation. these 3 candidates could be detected in serum with

significantly higher levels in cancer patients than the normal controls. These result implied the promise of these candidates as a panel of biomarkers for prediction and early diagnosis GC.

Conclusion

Our membrane proteomic pipeline not only identified clinically adapted biomarkers but also provide large number of membrane biomarker candidate proteins with elevated expression levels in the early stage of GC patients. The results demonstrated the power of tissue membrane proteomics for the discovery of valuable biomarker candidates for early diagnosis of GC.

Keywords: Gastric cancer, membrane proteomics, MRM-MS, biomarker

WP06-04

Comprehensive membrane proteome analysis for discovery of novel potential therapeutic targets against HTLV-1 associated disease

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Introduction and Objectives

Human T-lymphotropic virus type-1 (HTLV-1) is the causative agent of adult T cell leukemia (ATL) and HTLV-1 associated myelopathy/Tropical spastic paraparesis (HAM/TSP). Approximately 30 million people worldwide are infected with HTLV-1, in which 4% or 0.3% of them may develop ATL or HAM/TSP, respectively. In 2012, anti-CCR4 antibody (mogamulizumab) was approved for treatment of ATL and showed significant clinical response rate (50%), however, on the other hand, 100% of mogamulizumab-treated patients had adverse effects. What is worse is that no effective molecular-targeted therapy has been developed for mogamulizumab-resistant ATL and HAM/TSP patients so far. In this study, we performed comprehensive cell surface proteome analysis of HTLV-1 infected cells to identify novel therapeutic targets for ATL or HAM/TSP.

Methods

Peripheral blood mononuclear cells were collected from 69 individuals (ATL patients; n = 13, HAM/TSP patients; n = 21, asymptomatic HTLV-1 carriers; n = 21, and healthy donors; n = 14), from which HTLV-1 infected $CD4^+$ T cells were isolated by cell sorting system. To focus on cell surface proteome, glycopeptides were specifically enriched from tryptic digests of $CD4^+$ T cells using 96-well ConA lectin affinity plate (in house). Resulting glycopeptides were analyzed by LTQ-Orbitrap-Velos LC/MS. Protein identification, label-free quantification, and subsequent statistical analysis were performed on Expressionist proteome server platform (Genedata AG, Swiss). The expression level of an identified therapeutic target for ATL (GRAM) was further validated using independent set (ATL patients; n = 14, HAM/TSP patients; n = 10, and healthy donors; n =17) by means of flow cytometry. To investigate for functional significance of GRAM on development of ATL, proteome-wide interactome analysis was performed, followed by specific growth signaling pathway analysis.

Results and Discussion

Among 946 identified membrane proteins, 14 proteins were significantly overexpressed ($p < 0.05$ and fold change > 2.0) in ATL or HAM/TSP group compared to control group (healthy donors and asymptomatic HTLV-1 carriers). Significant upregulation of a candidate therapeutic target for ATL, GRAM, was clearly confirmed using independent validation set in flow cytometry analysis. Interestingly, GRAM expression level on acute type ATL patients' T cells (n = 9) was drastically higher than that of chronic type ATL

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patients (n = 5), indicating that overexpression of GRAM should be strongly associated with malignant transformation of ATL. Based on mass spectrometric interactome analysis for GRAM, we present a molecular basis of GRAM-driven progression of ATL by regulating intracellular growth signaling pathway.

Conclusion

These results suggested that our high-throughput cell surface proteome profiling technology could provide effective ways to identify novel targets for antibody therapy, which would be applicable for target discovery in any other diseases.

Keywords: HTLV-1 associated disease, membrane proteome, glycoproteomics, antibody therapy

WP06-05

A novel p53 pathway influences the colorectal cancer tumor microenvironment via exosomes

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Introduction and Objectives

Tumor-derived exosomes are important for cell-cell communication. And deregulation of TP53 gene is the critical genetic alteration during the multi-step progression of colorectal cancer (CRC). However, the role of TP53 in the control of exosome production in CRC is controversial and unclear.

Methods

The features of exosomes secreted from HCT116 TP53-wild type (WT), TP53-knockout (KO) and constructed TP53 (R273H)-mutant (MT) cells were observed by transmission electron microscopy and nanoparticle tracking analysis. A comprehensive proteomic analysis of exosomal proteins was performed using the iTRAQ-2D-LC-MS/MS strategy.

Results and Discussion

The exosomes from MT and KO cells showed significantly reduced sizes compared with those from WT cells. A total of 3437 protein groups with >=2 matched peptides were identified which were the so far largest exosomal protein dataset in CRC. Among them, hepatocyte growth factor-regulated tyrosine kinase substrate (HGS) was consistently down-regulated in the exosomes from MT and KO cells. Functional studies showed that low HGS levels were responsible for the decreased exosome size. TP53 regulated HGS expression and thus HGS-dependent exosome formation. Additionally, HGS expression was gradually increased concomitant with CRC carcinogenesis and was an independent poor prognostic factor.

Conclusion

In conclusion, a new HGS-dependent TP53 mechanism in tumor microenvironment remodeling was highlighted in CRC. HGS may serve as a novel prognostic biomarker and a candidate target for therapeutic intervention.

Keywords: olorectal cancer / tumor microenvironment / exosome / TP53 / HGS

WP06-06

Inflammatory signaling-derived exosome activate immune response in macrophages

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Introduction and Objectives

Exosome are secreted small vesicles with diameters of 30-100 nm from cells. These vesicles transfer protein, mRNA and miRNA to recipient cells to mediate many biological processes, including tumorigenesis, metastasis, drug resistance and immune response etc. However, whether the inflammatory signaling leads to the change of constituent of exosome and its roles in immune response remains to be determined.

Methods

The exosome were isolated by centrifuge from LPS, Nigericin treated or control bone marrow-derived macrophages. Then we used label-free quantification method by MS/MS to identify the components of exosome that induced with LPS, Nigericin treated or control cells. The differential expressed proteins were classified by Gene Ontology and statistical methods. The inflammatory related proteins were further verified by western blot.

Results and Discussion

Conclusion

We found that inflammatory signaling-derived exosome directly activate immune response in macrophages. The inflammatory signaling was amplified in neighbor cells in an exosome-dependent way. Block the transfer of exosome might be used to treat auto-immune diseases.

Keywords: exosome, inflammatory signaling, proteomics

WP06-07

Quantitative proteomics of fresh tissue-derived secretome reveal the molecular mechanisms of hepatocellular carcinoma tumorignesis and development

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Introduction and Objectives

Hepatocellular Carcinoma (HCC) is one of the most common malignant tumor, which is causing the second leading cancer-related death worldwide. Although surgical excision was demonstrated to be the first choice for HCC treatment, the incidence of HCC continues to increase and the prognosis was still poor. Most HCC is not diagnosed until the advanced stage of the disease, which are not suitable for surgical treatments. Therefore, early detection and treatment is the key to improve the therapeutic outcomes, reduce mortality and increase long-term survival rate of HCC.

Methods

Secreted proteins play important roles in the tumorigenesis, development, invasion and metastasis of HCC. In this study, cancerous, surrounding noncancerous and distal noncancerous tissues from 12 HCC patients with HBV infection were cultured in vitro, and the culture supernatant was used to analysis the tissue secretome of HCC through a iTRAQ-based quantitative proteomics approach. Meanwhile, biochemistry and molecular biology technologies were also used to explore the molecular mechanisms of the tumorigenesis and development of HCC.

Results and Discussion

In this high-quality secretory/releasing proteome of HCC, IBA57 was discovered up regulated in HCC group compaired with other noncancerous group, which could be used as a potential serum biomarker of HCC. The levels of IBA57 was evaluated by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). In addition, we found Caveolar-mediated Endocytosis Signaling was abnormal regulated in HCC group compaired with other noncancerous group, which might affect cell trafficking and fusion with early endsome, which may be involved in the occurrence and development of HCC.

Conclusion

Thus, this study provides a valuable resource of the HCC tissue secretome with the potential to investigate serological biomarkers. IBA57 could be used as novel biomarkers for the early detection of HCC. And Caveolar-mediated Endocytosis Signaling might be involved in the occurrence and development of HCC.

Keywords: Hepatocellular carcinoma (HCC); Quantitative proteomics; Tissue secretome; Early diagnosis biomarkers; 2D LC-MS/MS

WP07-01

Functional integrated proteomics identified "glyco-niche" signalings as a regulator of the maintenance and differentiation of cancer stem cells

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Introduction and Objectives

Cancer stem cell (CSC) has been proposed as a target for cancer curative treatment, and the suppression of self-renewal or differentiation is supposed to be an effective approach for targeting the CSCs. To clarify the molecular mechanism of CSC maintenance/differentiation, we have isolated glioma stem/initiating cells (GSCs) having the potential to differentiate into malignant gliomas, and subjected to DNA microarray/iTRAQ/2D-DIGE based integrated-transcriptome/proteome analysis. Using unique GSC-iPEACH integrated database, we tried to identify the regulatory networks associated to the maintenance and differentiation of GSC.

Methods

To identify the molecular targets related to the maintenance/differentiation of GSC, we established 9 clones from patient's gliomas having the potential to differentiate into glioblastomas. Protein and mRNA, extracted from GSC clones cultured in the stemness-sustaining or differentiation-inducible mediumand, were subjected to iTRAQ (8Plex), 2D-DIGE(PI4-7, 3-11) and DNA array (Affimetrix U133.2) based integrated proteomics. All of the data was combined by a unique data mining tool called iPEACH, and used for GO and knowledge-based network analyses such as KeyMolnet and KEGG to extract novel candidate signal networks regulated during the malignant processes in GSC.

Results and Discussion

GO and network analyses using GSC-iPEACH database with 8,471 proteins and 21,857 mRNAs revealed that the networks including the cell adhesion molecules, such as integrin subfamilies and ECMs, and RAS-MAPK-ERK/PI3K-AKT signalings were significantly upregulated, and specific proteoglycans(GAGs) synthesis and metabolic pathway (such as glycolysis and hexosamine biosynthesis) enzymes as well as stem cell markers were obviously downregulated during GSC differentiation. Among them, we focused GAGs such as chondroitin sulfate proteoglycan (CSPG) family and their synthetic enzymes downregulated during serum-induced GSC differentiation. Interestingly, GSC differentiation was significantly induced by chondroitinaseABC (chABC), GAG degradation enzyme, via the upregulation of ERK and AKT signaling pathway which was inhibited by RGD integrin inhibitors. We also found that CSPG interacts with integrin aV under the differentiation condition, suggesting that CSPG-GAG is a key regulator of GSC maintenace/differentiation via the regulation of integrin signals, and this so called "glyco-niche" signaling is a potential clinical target against malignant gliomas.

Conclusion

Functional integrated proteomics for the first time demonstrate that the GSCs

WP07-01

induce/secrete glycoproteins and their receptors, to regulate GSC stemness and differentiation processes via developing specific ”glyco-niche” signalings, being a potential clinical target against malignant gliomas. Hirayama M. Kobayashi D. et al. Mol Cell Proteomics 2013 12(5):1377, Niibori,N.A. et al. PLOS ONE 2013 8(5):e59558

Keywords: integrated proteomics, iTRAQ, DNA array, Cancer Stem Cells, Glycosaminoglycans, Niche

Proteomic analysis of low folate-induced onco spheroid formations in human colonic adenocarcinoma cells

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Introduction and Objectives

Aims of the study were to investigate proteomic analysis of low folate-induced onco spheroid formations in colonic adenocarcinoma cells.

Methods

Using SW480 cell lines as a model were divided into two groups cultured with the control medium (C) and low folate medium (LF) for 4 days. After collected and suspended the cells in the spheroid formation medium, respectively, seeded 10,000 cells in ultra-low attachment 6 well plate for 4 days to observe the cell forms. SW480 cells (control or LF) grown on the culture dish were scraped in a lysis buffer containing 4% SDS and protease inhibitor cocktails. After acetone precipitation, total proteins were trypsin digested followed by dimethyl labeling at the peptide level. The labeled peptides were combined and analyzed by nanoLC-MS/MS. The peptides were separated by MonoCap C18 High Resolution Ultra 2000 (0.1 x 2000 mm) column and analyzed by Thermo LTQ-Orbitrap XL mass spectrometer. Peptide identification and quantification were performed by MaxQuant packages. Bio-informatics analyses were performed by Perseus and GproX program.

Results and Discussion

The data found that there were obviously onco spheroid formation cells in low folate (LF) treated group. The scatter plot of the ratio with LF and control group normalized was normal distribution. Overall proteomic analysis data observed regulated proteins related to extracellular matrix organization, cell adhesion, protein catabolic process, response to ionizing radiation, and TOR signaling. The data found that there were 14, 18, 11 25, 15, 3 proteins respectively related to mitochondria, membrane, signaling pathway, transcription factor, transport and heat shock. Among these proteins, DYNLL1, DYNLL2, related to cell division, were over 32 fold-changes in LF group. PPIF was over two fold-changes in LF group and highly involved in mitochondrial metabolism and apoptosis. STMN2 was under 8 fold-changes in LF group and related with cell cycle progress. CD9 was under 4 fold-changes in LF group and modulated cell adhesion and migration. ICAM1 related to cell adhesion and was over 4 fold-changes in LF group. RPL36A, under 4 fold-changes in LF group, catalyzed protein synthesis and involved in cellular protein metabolic process. EBP was over 4 fold-changes in LF group and related to cholesterol metabolic process. PFDN4 was over 8 fold-changes in LF group and related to cellular protein metabolic process and chaperone binding. HSP90AB4P was over 4 fold-changes in LF group and involved in stabilizing proteins against hear stress and aiding in protein degradation.

Conclusion

WP07-02

Our findings suggest that folate malnutrition elicited onco spheroid formations and some metabolic-related protein expression.

Keywords: low folate, onco spheroid formations, proteomic analysis

WP07-03

A Study on Effects of sRAGE Secreting Human UCB-MSC in Parkinson's disease Model

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Introduction and Objectives

Parkinson's disease (PD) is progressive degenerative disease of the nervous system and its underlining mechanisms for neuronal death are poorly understood. This study was investigated to identify the mechanism of neuronal death in PD and the effect of soluble receptor for AGE (sRAGE) secreting human umbilical cord blood derived mesenchymal stem cells (hUCB-MSCs) on neuronal cell death and recovery of behavior in PD animal models.The sRAGE secreting UCB-MSC was generated by Zinc Finger Nuclease (ZFN) gene editing method. These cells were transplanted into Corpus Striatum of rotenone induced PD animal models then experiments were mainly performed to determine the neuronal cell death and recovery of movement.

Methods

Generation of PD mouse model- To establish suitable PD model, 30 mg/kg of rotenone (Sigma-Aldrich) which is suspended in 0.5 % CMC was treated orally once in a day for 2 months. Generation of soluble RAGE (sRAGE) secreting UCB-MSC- To generate sRAGE secreting UCB-MSC, transfection was done with mRNA Zinc Finger Nuclease (Sigma-Aldrich) (twice voltage of 1000 purse width 30)Stereotaxic surgery- Injection was performed with unilateral administration of drugs into the right CS (anterior and posterior 0.4, medial and lateral 1.8, dorsal and ventral from Bregma 3.5 mm)

Results and Discussion

Since sRAGE is protein, sRAGE itself has half-life so that it would not be the best therapy to cure Parkinson's disease. To overcome this problem, we have generated sRAGE secreting UCB-MSC.sRAGE secretion level from transfected UCB-MSC was strong in first passage and slowly decreased by transferring the cells. Cell implantation was performed by stereotaxic surgery to PD animal models. Their behavioral ability was examined by rotarod and pole test. These test showed significant improvement of movement from sRAGE secreting UCB-MSC group comparing to PD groups. Also histological analysis shows the protection effect against cell death in corpus striatum in sRAGE secreting UCB-MSC.To check mechanisms behind this protection we have observed their protein expression levels. MAPK and their phosphorylation form was measured. Results showed that the main pathway leading neuronal cells to death was assumed as p38, Erk1/2 and JNK in MAPK pathway.Therefore, the blocking the AGE-RAGE binding by sRAGE or sRAGE secreting UCB-MSC showed significant therapeutic potential and it was assumed that sRAGE from sRAGE secreting UCB-MSC play a key role to protect neuronal cells against apoptosis.

Conclusion

In conclusion, AGE-RAGE dependent cell death in CS contributes to neurodegeneration of PD when the chronic condition was continued. sRAGE has protective effect against

neuronal cell death by inhibiting AGE-RAGE binding. Therefore, sRAGE secreting UCB-MSC was assumed as one of the great therapeutic approaches to cure PD.

Keywords: Parkinson's disease, Corpus striatum, sRAGE secreting UCB-MSC, Microglia, AGE-albumin

Comprehensive N-glycoproteomics of neural stem cell differentiation

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Introduction and Objectives

Human neural stem cells (hNSCs) have the ability to self-renew and to differentiate into neurons and glia cells. Recent advance in stem cell research has provided insights into the development of hNSC therapies for neurological diseases. However, the progress is hampered by lack of well-defined markers for identification and isolation of neural lineage cells. During neuronal differentiation, membrane-associated proteins play key roles in signal transduction, cell adhesion and recognition, and their functions are often regulated by post-translational modifications such as glycosylation. In order to discover specific markers for neural lineage cells, hNSCs and their differentiated cells have been subjected to glycoform-focused membrane proteomics using the in-house software named Integrated GlycoProteome Analyzer (I-GPA) [Park et al., Scientific Reports 2016].

Methods

The immortalized hNSC line ReNcell VM (Millipore) was used for this study. After isolation of membrane fractions from undifferentiated and 3-week differentiated cells by Na2CO3 treatment combined with ultracentrifugation, the proteins were cleaved by Lys-C and trypsin. Glycopeptides were enriched with hydrophilic interaction chromatography (HILIC) and then analyzed by nano LC-MS/MS (Orbitrap Elite). Identification and quantification of N-glycopeptides and glycoforms were performed with the I-GPA software platform.

Results and Discussion

From the undifferentiated and differentiated cell membrane fractions, a total of 169 N-glycoforms of 80 glycopeptides derived from 62 glycoproteins were identified with FDR<=1%. Quantitative MS analysis reveal that 54 glycoforms of 24 glycoproteins and 58 glycoforms of 20 glycoproteins are abundant in undifferentiated and differentiated hNPCs, respectively. Many of these proteins are involved in neuronal cell adhesion, migration, and differentiation.

Conclusion

We identified and quantified N-glycosylated membrane proteins in the course of neuronal differentiation. The comprehensive glycoproteomic dataset presented here provides valuable information for understanding of neuronal differentiation process and development of potential neural cell lineage markers.

Keywords: Neural stem cell, Differentiation, N-glycoprotein, N-glycopeptide, Glycoform

WP07-05

Proteomics identifies cell surface marker for isolation of cardiac progenitors for cell-based therapy in cardiac infarction

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Introduction and Objectives

The LIM-homeodomain transcription factor ISL1 marks a Secondary Heart Field (SHF) progenitor population that makes a substantial contribution to the developing heart, giving rise to most cells in the right ventricle, pacemaker cells, both atria, the outflow tract and also specific regions of the left ventricle. Human ISL1+ cardiac progenitors can be derived from human Pluripotent Stem Cells (hPSCs) but their characterization has been limited due to the inefficiency of the differentiation protocols and lack of a proper reporter or surface marker based purification system.

Methods

To facilitate characterization and further purification of hPSC-ISL1+ progenitors, we established a hESC line expressing hygromycin resistance gene under control of the ISL1 promoter. We then purified ISL1+ cells derived from human Embryonic Stem Cells (hESCs) using hygromycin selection. To further characterize the hESC derived ISL1+ progenitors and identify surface markers to facilitate their isolation, we did a global proteome analysis using a shotgun proteomics approach to compare the ISL1+ enriched population with unenriched age matched differentiated cells. Looking for potential surface markers, we focused on the membrane proteins that are upregulated in the enriched population for purification of the ISL1+ progenitors.

Results and Discussion

Immunofluorescence staining on sorted populations showed that ALCAM labels the hESC-derived ISL1+ progenitors more specifically than other candidate proteins. We showed that purified hESC-derived ISL1+ progenitors are multipotent and differentiate into endothelial and smooth muscle cells as well as cardiomyocyte precursors. Genome wide transcript analysis of ALCAM+, ALCAM- and the unsorted age matched differentiated cells (ALCAM-/+) revealed that proliferation associated genes are significantly enriched in ALCAM+ purified cells suggesting that they are in a progenitor state and may have better regenerative potential in cardiac infarction models. To assess the ability of ALCAM+ purified progenitors to improve cardiac function in vivo, we used a LAD ligation model of cardiac infarction in rats.Transplantation of ALCAM+ progenitors enhanced tissue recovery in a rat model and improves angitogenesis through activation of Akt-MAPK signaling.

Conclusion

Taken together, our data provide a strategy to obtain large numbers of highly pure human ISL1+ multipotent cardiac progenitors from hPSCs which can be further used for translational applications in cardiac repair as well as developmental and disease related studies.

Keywords: Human pluripotent stem cells, LIM-homeodomain transcription factor ISL1, Cell surface markers, Cell therapy, Cardiac infarction

Integrative omics connects N-glycoproteome-wide alterations with pathways and regulatory events in human induced pluripotent stem cells

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Introduction and Objectives

Somatic cell reprograming technology has been introduced to generate embryonic stem cell (ESC)-like cells, known as induced pluripotent stem cells (iPSCs). Since that time, iPS cell-based therapy has become one of the major interests for clinical investigators as they avoid the use of human embryos. However, somatic cell reprograming causes genetic and epigenetic alterations in iPSCs that result in increased risk of neoplasms. These molecular level alterations potentially affect the functional characteristics, such as self-renew and differentiation potential (i.e., pluripotency), of the iPSCs, which in turn affect the basic research and clinical use of iPSCs. Therefore, to gain insights into cell reprograming and induced pluripotency, molecular level differences ranging from genomes to proteomes, but not N-glycoproteomes, have been assessed between iPSCs and ESCs.

Methods

N-glycopeptides were enriched from multiple cell lines (five iPSCs, two ESCs, two somatic cells (SCs)) by using hydrazide chemistry approach and were analyzed by LTQ-Orbitrap mass spec (MS). MS raw files were processed by MaxQuant and multiplexed label-free quantitation was performed using site-specific N-glycosylation events. The quantitative N-glycoproteomics results were integrated with proteomics and transcriptomics profiles of the same cell lines, as well as with functional network analysis to explore the biological significance of the study.

Results and Discussion

Our multiplexed quantitative N-glycoproteomics strategy identified altered N-glycoproteins, which significantly regulate cell adhesion processes, in iPSCs relative to ESCs. The N-glycoproteomics alterations identified in iPSCs were validated by integrative proteomics analysis. Further, network analysis of the altered N-glycoproteins revealed their significant functional interactions with known PluriNet (pluripotency-associated network) proteins. We found that these functional interactions regulate various pathways including focal adhesion and PI3K-Akt signaling. Further, integrative transcriptomics analysis revealed that imperfectly reprogramed subunits of the oligosaccharyltransferase (OST) and dolichol-phosphate-mannose synthase (DPM) complexes are potential candidate regulatory events for the altered N-glycoproteins levels. Together, our study reports not only the N-glycoproteome-wide alterations in iPSCs but also their upstream regulatory events and downstream functional roles.

Conclusion

WP07-06

Our study suggests imperfect reprograming of the protein complexes linked with N-glycosylation process may result in N-glycoproteins alterations that affect induced pluripotency through their functional protein interactions. These results provide a basis for future studies on improving the reprograming efficiency and induced pluripotency of iPSCs in the context of post-translational protein N-glycosylation.

Keywords: Pluripotent stem cells, multiplexed quantitative N-glycoproteomics, integrative omics, functional protein interactions, pluripotency, reprograming

WO08-01

Gene expression and proteomic analysis of cognitive dysfunction in people with remitted Major Depression

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Introduction and Objectives

Cognitive impairments are observed in a substantial proportion of patients suffering from Major Depressive Disorder (MDD), significantly impacting on patients' psychosocial functioning and quality of life.

Methods

We utilized whole-blood transcriptomic data from remitted MDD patients for weighted gene coexpression network analysis (WGCNA) to detect modules associated with residual cognitive dysfunction. Using SWATH-MS, we followed up our findings on the protein level in patient plasma.

Results and Discussion

We identified16 transcriptomic modules. One module was significantly correlated with poor versus better cognitive performance, containing ribosomal genes and modulators of B cell biology. On the plasma protein level, SWATH-MS detected 43% of module gene products, and group differences in network hub proteins were confirmed.

Conclusion

The experimental workflow may represent an effective approach to blood biomarker discovery for psychiatric phenotypes. Enduring cognitive impairments affecting a subgroup of MDD patients may be driven by distinct molecular processes.

Keywords: Major Depression, cognitive impairment, WGCNA, SWATH-MS

WO08-02

Proteomic dissection of AMPA receptor complexes identifies FRRS1l as a determinant for receptor biogenesis mutated in severe intellectual disability

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Introduction and Objectives

AMPA-type glutamate receptors (AMPARs) are key players in excitatory neurotransmission in the brain. Using a comprehensive functional proteomics approach we previously showed that native AMPARs are macromolecular complexes assembled from a pool of 34 proteins in a cell-type specific and state-dependent manner. However, most of these proteins lack functional annotation and/or have not been implicated in AMPAR physiology.

Methods

Reverse AP-MS analysis and stepwise depletion of complex populations followed by LC-MS/MS analysis (Orbitrap Elite) and label-free quantification identified distinct AMPAR complexes. These were reconstituted in heterologous cells and characterized by surface biotinylation, affinity purification, immunocytochemistry and electrophysiology. Subcellular localization in neurons was verified by electron microscopy. Stereotactic injection of viruses inducing targeted knockdown or overexpression of AMPAR subunits were used to study their impact on excitatory synaptic transmission in rat brain slices using electrophysiology. Genetic analyses of patients suffering from non-syndromic intellectual disability (whole-exome sequencing followed by appropriate filtering) revealed homozygous mutations of FRRS1l segregating with the disorder and showing loss of function in heterologous experiments.

Results and Discussion

We identified AMPAR assemblies containing FRRS1l together with Carnitine O-palmitoyltransferase 1 (CPT1C) and Phosphatidylinositide phosphatase SAC1 that are restricted to the endoplasmic reticulum (ER) and lack the core-subunits typical of AMPARs in the plasma membrane. FRRS1l is retained in the ER through direct interaction with CPT1C but promotes assembly and/or synaptic trafficking of mature AMPARs. Virus-directed deletion or overexpression of FRRS1l in adult rat neurons altered the number of surface AMPARs in individual synapses resulting in increased or decreased amplitudes of the excitatory postsynaptic currents (EPSC) without affecting their time courses. Furthermore, bi-allelic loss-of-function mutations in the human FRRS1l gene were found to cause severe intellectual disability, cognitive impairment, speech delay and epileptic activity. Our results provide insight into the early biogenesis of AMPARs and demonstrate its impact on synaptic transmission and brain function.

WO08-02

Conclusion

• FRRS1l-containing AMPARs in the ER differ from receptors at the plasma membrane and represent an early stage of receptor biogenesis. • FRRS1l is a positive and specific determinant for maturation and/or synaptic trafficking of AMPARs. • Loss-of-function mutations in FRRS1l (and thereby impaired biogenesis of AMPARs) are causative for severe human intellectual disability • Both, reverse AP-MS as well as stepwise biochemical depletion analysis are useful proteomic approaches for functional assignment and deconvolution of complex interactomes.

Keywords: AMPA receptorsReceptor biogenesisExcitatory neurotransmissionIntellectual disabilityDisease mechanismFunctional proteomics

WO08-03

Autoantibody response in meningioma patients reveals aberrations in signalling pathways

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Introduction and Objectives

Meningiomas are intracranial tumours, which constitute upto 20% of all intracranial neoplasms and 35.5% of all central nervous system (CNS) tumours. During the course of manifestation, malignancies are known to evoke immune responses countering tumour associated antigens (TAAs) in the form of autoantibodies. Autoantibodies generated in response to such self-epitopes in the course of establishment of a disease can serve as early, minimally invasive diagnostic markers.

Methods

We screened sera from 15 healthy controls, 10 grade I and 5 grade II meningioma patients for autoantibody responses using human proteome arrays harbouring ~17,000 full length recombinant human proteins. Limma from R-Package was utilized to access the dysregulated proteins and were subjected to enrichment analysis using FunRich. Interaction networks was studied using StringDB.

Results and Discussion

On analysis of the dysregulated proteins emerging from this autoantibody screening, 489 & 104 proteins were found to be significantly alteredin grades I and II of meningioma respectively with $p<0.05$, log fold change>0.5 when compared to healthy controls. Pathway analysis of these dysregulated proteins, in each grade showed enrichment of signalling pathways like RAF/MAP kinase cascade, EGFR signalling, Osteopontin-mediated events, Signalling by NGF, Signalling to RAS in grade I and RAC1 signalling in grade II. Autoantibody targets like IGHG4, CRYM, EFCAB2, STAT6, HDAC7A and CCNB1 were dysregulated across both grades when compared with healthy controls. Interesting, proteins like GSTP1, SELENBP1, FABP5,TPD52L2, CRYM, APOE, RTN4,HSPA2 etc. which were earlier reported to be highly upregulated in meningioma tissue based proteomics, showed an aggravated autoimmune response, emphasising the clinical relevance of these targets. When compared with open source transcriptomic data, targets like HSPA2 show significant correlations with the trends found from our tissue proteomic and autoantibody data.

Conclusion

This study provides us a comprehensive overview of meningioma autoantibody screening which can now be used to validate some of the crucial pathways to study meningioma manifestation and targets which can aid in minimally invasive diagnosis.

Keywords: Meningioma, Autoantibody, Autoimmunity, tumour heterogeneity, biomarkers, protein microarrays

WO08-04

Proteomic analysis of motor neurons from induced pluripotent stem cells: ALS

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Introduction and Objectives

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative condition in which loss of upper and lower motor neurons occurs due to cell death which ultimately leads to impaired muscle function and eventually mortality. Ideally the molecular profile of affected motor neurons would be interrogated in vivo, but this is challenging and precludes experiments to deduce cause vs effect. Induced pluripotent stem cell (iPSC) derived motor neurons (iMNs) allow for the interrogation of the discrete molecular effects of specific genetic perturbations within the context of human genomic backgrounds upon which ALS is manifested. The goal of this study is to identify disease specific molecular signatures characteristic of known disease phenotypes including neuronal cell death in an iPSC-derived motor neuron model of ALS.

Methods

Pathways at the molecular level were investigated by employing cell-wide proteomic analysis to determine the effects of single genetic perturbations (C9) that drives the ALS disease phenotype. We have generated iPSCs from healthy human patients as well as those with ALS and differentiated them into motor neuron precursors (n=4 cell lines/group, duplicate). By first constructing an iPSC and iMN sample specific DDA peptide assay library, we used Data Independent Acquisition Mass Spectrometry (DIA-MS, also referred to as SWATH) to quantitate 3934 proteins across the iMN samples, using FDR cutoff of 1% for peptide and protein level.

Results and Discussion

Our quantified proteome included key neuronal markers, such as MAP2, DCX and Snap25 and were enriched in protein classes include vesicle coat proteins, SNARE proteins, and non-motor microtubule binding proteins. Future studies include fingerprinting the molecular pathology of the disease at earlier stages in culture by comparing this set of iMN data to its iPSC counterpart in an effort to tease out any pathways that are affected by cell maturation and development, as well as reveal disease specific pathways that can then be associated with single genetic perturbations. We also plan on comparing the ALS-iMN data to SMA-iMN (early onset motor neuron disease) data in order to look at convergent and divergent pathways leading to disease phenotypes.

Conclusion

DIA-MS allows quantitation of 3934 proteins in iMNs across all samples, when run against an iMN specific DDA library. High confidence in quantitated data allows for statistical and network analysis of proteins that change between control and disease.

Keywords: ALS, Neurodegenerative Disease, SWATH, DIA

WO08-05

Analysis of the O-GlcNAcomic profiling of brain tissue in Alzheimer's disease

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Introduction and Objectives

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease. AD accounts for more than 70% dementia patients in clinics and the population has reached more than 23 million worldwide in the elderly (age>65). However, currently there is no satisfied therapy, which has driven interest in seeking novel therapeutic targets. Previous studies indicate that impaired glucose metabolism in brain is a contributing factor to AD and imply that it may lead to decrease brain O-GlcNAc levels which is a common post-translational modification. Several proteins that are linked to neurodegenerative disorders are O-GlcN Acylated, such as Tau and the amyloid precursor protein. In this study, we used O-GlcNA come to identify new aberrant O-GlcNAcylated proteins in AD.

Methods

O-GlcNAcomeEnrichment of O-GlcNAc proteins from human frontal cortex tissue lysates was performed by following the instructions of the Click-iT® O-GlcNAc Enzymatic Labeling System and Click-iT® Protein Enrichment Kit (Invitrogen). Before O-GlcNAc enzymatic labeling, PNGase F was used to cleave terminal GlcNAc residues of N-glycans to minimize false positives. Then, O-GlcNAc was labeled with GalNAz by Gal-T1 (Y289L) catalysis, following by incubation with alkyne agarose resin. After trypsin digestion on resin, the peptides of O-GlcNAcylated protein were released and analyzed by LTQFT Ultra (Linear quadrupole ion trap Fourier transform ion cyclotron resonance) mass spectrometer (Thermo Electron) equipped with a nanoelectrospry ion source (New Objective, Inc.). Raw MS files obtained from LTQFT Ultra were analyzed using MaxQuant. MaxQuant-based label-free quantitation between tissues was performed using normalized protein intensities.

Results and Discussion

In this study, we confirmed that O-GlcNAcylation was markedly decreased in AD frontal cortexes compared with the control tissues by dot-blots with antibody RL2 to O-GlcNAc. To identify aberrant O-GlcNAc-modified proteins expressed in AD, we compared the O-G lcNAcomic profiles between AD and control tissues by using a chemoenzymatic approach to enrich O-GlcNAcylated proteins in tissues, and analyzed by LTQFT Ultra mass spectrometer. Until now, we have analyzed the extracts of frontal cortexes from five AD and four control cases. A total of 470 O-GlcNAcylated proteins were identified. Of these, 244 O-GlcNAcylated proteins were down-regulated at least a two-fold difference in intensity and only 7 O-GlcNAcylated proteins were up-regulated at least 1.5-fold difference in intensity compared with the control specimens.

Conclusion

We found new aberrant O-GlcNAcylated proteins in AD. Further, we will focus on some proteins and elucidate their pathological roles in AD and the function of protein O-GlcNAc.

WO08-05

Keywords: O-GlcNAc, O-GlcNAcome, Alzheimer's disease

WP08-01

Proteomics biomarkers from human cerebral cortex for molecular diagnostic and prognostic management of epilepsy patients

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Introduction and Objectives

Epilepsy syndromes are characterized as highly degree of heterogeneous complex disease entities with varied pathologies, biochemical and clinical sequel. Currently it is difficult to predict treatment response or surgical outcome due to lack of accurate blood or tissue markers. Therefore; there is unmet need to identify definitive epilepsy diagnostic protein-biomarkers. We have analyzed brain tissue biopsies and peripheral blood samples from the same individual epileptic subjects towards discovery of disease-specific diagnostic/prognostic biomarkers by expression proteomics using quantitative label-free tandem mass spectrometry (LC/MS/MS).

Methods

Fifty six samples have been analyzed by expression proteomics using quantitative label-free tandem mass spectrometry (LC/MS/MS). Samples were classified into several sub tumor groups including Ganglioglioma Grade 1, Oligodendroglioma Grade 1, Oligoastrocytoma Grade3 with Early Anaplastic Transformation, Mixed Oligoastrocytoma Grade 2, High-Grade, Glioblastoma Grade 4, Anaplastic Oligodendroglioma Grade 3, among epilepsy monitoring unit (EMU) patients. Tumor types among patients not in EMU includes Ganglioglioma, Gliosis, Chronic Inflammation /Rasmussen Encephalitis, Neuronal Loss and Gliosis.

Results and Discussion

Altogether we have identified 1633 unique protein species by LC/MS/MS analysis. Expression profiles of 215 significantly differentially expressed proteins classified the samples based on their distinct clinical and pathological characteristics. Among the identified proteins with significant altered expression among the samples includes GFAP-glial fibrillary acidic protein, MAG-myelin associated glycoprotein, NAMPT-nicotinamide phosphoribosyl transferase, NCDN-neurochondrin, NCOA3-nuclear receptor coactivator 3, OMG-oligodendrocyte myelin glycoprotein, OPALIN-oligodendrocytic myelin paranodal and inner loop protein, PRDX6-peroxiredoxin 6, TMEM206-transmembrane protein 206. Others are NCDN-neurochondrin, DCTN1-dynactin 1, DNAH9-dynein, axonemal, heavy chain 9, CPNE4-copine IV, and TNRC18-trinucleotide repeat containing 18. The annotation of some of these proteins functions as chaperone, hydrolase, oxidoreductase, enzyme modulator, transferase while others acts as ligase, nucleic acid binding and kinase. Of further interest is that approximately 5% of the identified proteins in the brain tissue were among some of the identified proteins in plasma giving the likelihood of discovery of surrogate protein biomarkers that could be easily measured in peripheral blood.

Conclusion

WP08-01

Results from this study will lead to identification of disease related or tissue-specific proteins that could be potentially useful for objective classification and management of epilepsy patients once these proteins are validated in large sample cohorts.

Keywords: Epilepsy, Expression Proteomics, Biomarkers, Molecular-Diagnosis, Cerebral-Cortex,

WP08-02

Full-length TDP-43 Forms Toxic Amyloid Oligomers in Frontotemporal Lobar Dementia-TDP Patients and Disturbs Amyloid-β Fibrillization

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Introduction and Objectives

TDP-43 proteinopathy consist of several neurodegenerative diseases including frontotemporal lobar dementia (FTLD), amyotrophic lateral sclerosis (ALS), and Alzheimre's disease (AD). It is characterized by inclusion bodies formed by polyubiquitinated and hyperphosphorylated full-length and truncated TDP-43. The structural properties of TDP-43 aggregates and their relationship to the pathogenesis are still ambiguous.

Methods

Here, we employed various biophysical and biochemical methods to characterize structure and funciton of TDP-43.

Results and Discussion

We first demonstrated that the recombinant full-length human TDP-43 forms structurally stable, spherical oligomers with a diameter around 50 nm that share common epitopes with amyloid oligomers. The TDP-43 oligomers are stable, have exposed hydrophobic surfaces, exhibit reduced DNA binding capability, and are neurotoxic in vitro and in vivo. Moreover, TDP-43 oligomers are capable of cross-seeding Alzheimer's amyloid-β to form amyloid oligomers, showing the inter-convertability between the amyloid species. Using a conformational antibody against TDP-43 oligomers, we demonstrated such oligomers are present in the forebrain of transgenic TDP-43 mice and hippocampus and cortex of FTLD-TDP patients. We further studied the effect and interaction of TDP-43 to Aβ fibrillization. We found TDP-43 oligomers affect Aβ at the early stage but not at the fibril stage. Aβ oligomers generated in the presence of TDP-43 are cytotoxic.

Conclusion

Our results suggest that TDP-43 oligomers aside from filamentous aggregates reside in TDP-43 pathogenesis and TDP-43 oligomers may also play an important role in Alzheimer's disease.

Keywords: TDP-43, Amyloid-β, Amyloid Oligomers, Fibrillization, Frontotemporal Lobar Dementia, amyotrophic lateral sclerosis, Alzheimer's Disease

WP08-03

SCN phosphoproteomic analysis reveals GRK2 as an important modulator of neuronal structure and cytoskeleton organization

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Introduction and Objectives

Suprachiasmatic nucleus (SCN) serves as the master circadian pacemaker to generate self-sustained ~24h rhythms to the external environment by daily resetting in response to light. The clock neurons in the SCN communicate with each other via the activation of G protein-coupled receptors (GPCRs) by their extracellular ligands. In our previous study, GRK2, unlike other GPCR kinases, has been shown to possess a non-canonical function whereby it physically associates with PER proteins and promotes the phosphorylation of PER2. However, the global proteomic effects of GRK2 modulation on the murine SCN in the response to light are not completely understood.

Methods

In this study, we performed a SILAC-based phosphoproteomic approach to analyze the SCN phosphoproteome of wild-type and conditional knock-out of grk2 (grk2 cKO) mice in the presence and absence of light stimulation (15-min light pulse at CT 15).

Results and Discussion

Of the 1287 accurately quantified phosphorylation sites corresponding to 599 proteins, 133 phosphorylation sites were significantly altered between grk2 cKO and wt SCN phosphoproteome under light stimulation, compared to 132 phosphorylation sites altered in the absence of light stimulation. Bioinformatics analysis of the light-inducible phopshoproteome reveals their diverse distribution in different canonical pathways most notably, neuron projection development, and regulation of cytoskeleton organization. Additionally, 30 light-stimulated SCN phosphopeptides contained the GRK2 consensus substrate motifs. This finding will facilitate a more integrative understanding of GRK2 function on the SCN clock.

Conclusion

This finding will facilitate a more integrative understanding of GRK2 function on the SCN clock.

Keywords: SCN, Phosphoproteomic, GRK2

Quantitative proteomics of the cytosolic fraction from orbitofrontal cortex of patients with schizophrenia

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Introduction and Objectives

Schizophrenia (SCZ) is a complex neuropsychiatric disorder characterized by three principal symptoms: positive (hallucinations, disordered thoughts and speech), negative (anhedonia, social withdrawal) and cognitive (difficulty in attention, working memory). Following the goal set by the Chromosome-centric Human Proteome Project Organization to characterize disease-associated proteins, we used iTRAQ technique to analyze the cytosolic fraction of brain tissue from SCZ patients for better understanding of the pathophysiology process of this disease.

Methods

Cytosolic fraction of post-mortem brain tissues was prepared from the orbitofrontal cortex region collected from SCZ patients (n=11) and controls (n=8) following Cox and Emili protocol. Extracted proteins were digested and iTRAQ labeled; three reporter groups were used for schizophrenia samples and the fourth for a pool of all controls. Peptide mix were separated by RP-nLC coupled to a LTQ Orbitrap Velos; spectra were analyzed in Proteome Discoverer 2.0 against the NeXtProt (FDR <1%).

Results and Discussion

MS analyses identified 1654 groups of proteins; 677 protein groups of them consistently quantified, considering at least 3 unique peptides. The most interesting proteins with increased abundance were: 1. 14-3-3 gamma (n=7), this specifically isoform has the highest affinity with the enzyme tyrosine hydroxylase, first step of dopamine synthesis; promoting their activation. This protein has no significant quantitative changes in the nuclear fraction. 2. MAPK3/ERK1 (n=5) linked to cell proliferation, differentiation and long-term synaptic changes and behavior. ERK1 could not be consistently quantified in nuclear fraction. The most important proteins with decreased abundance were: 1.MARCKS-related protein (n=11) involved in synaptic morphology and plasticity. Its abnormal expression is associated to cognitive deficits. Correlating these data with those obtained in previous analysis of the nuclear fraction (where MARCKS was increased in all patients) it is proposed that MARCKS in nucleus is associated with PPI2 regulation. Rats treated with haloperidol do not show

is critical for embryonic neurogenesis; knockout mice revealed reduced cortical thickness and brain volume. In nuclear fraction, the abundance of this protein is increased but the biological significance and function in this subcellular fraction is still to be fully elucidated. 3. 14-3-3 protein zeta (n=5), up-regulated in the nuclear fraction (n=12), is involved in the prevention of apoptotic events by controlling the nuclear and cytoplasmic distribution of signaling molecules.

Conclusion

In conclusion, deregulation of proteins associated with dopamine synthesis, neurogenesis and synaptic plasticity contributes to the understanding of the pathophysiological process in SCZ.

Keywords: SchizophreniaQuantitative ProteomicsCytosol proteinsOrbitofrontal region Brain

WP08-05

CSF analysis for protein biomarkers identificatin in patients with CNS lymphoma

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Introduction and Objectives

Early diagnosis and treatment of CNS lymphoma attenuates disease progression and neurological deterioration. Then, new approaches for early detection based in identification of biomarkers are requiered, particularly for CNS lymphoma which cytologic examination is less than 50% sensitive than other cancers.

Methods

In this study, we report a strategy for differential proteomic analysis that also includes large-scale identification of more than 500 proteins to identify the major CSF proteins which distinguish B-cell CNS lymphoma from bening conditions.

Results and Discussion

Then, in order to generate and easy translation into the clinic, for the multiplex detection of these proteins, a kit based on color-coded bead suspension array has been designed and developed for the determination of tumoral infiltration in central nervous systems (leptomeningela metastasis(LM)).

Conclusion

Here, it will be presented a validation data set in a cohort of >100 samples which allow the early diagnosis of CNS lymphoma

Keywords: CNS lymphoma, functional proteomics, leptomeningeal metastasis,

WP08-06

Proteomic analysis of vitamin D-regulated proteins in primary cultured OLGs

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Introduction and Objectives

Oligodendrocytes (OLGs)Oligodendrocytes (OLGs) the only myelin-forming cells, generate myelin sheaths that surround axons and thus play a pivotal role in the proper neural functioning, while OLG damage leads to demyelination and neurological functional deficit. Our objective of this research is to investigate changes in expression of proteins in OLGs before and after stimuliated by vitamin D.

Methods

Primary cultured OLGs were obtained from 1-2 days newborn rats, total protein exstraction was performed after stimulated with (+VD group) or without (C group) 1, 25-(OH)2D3 for 24 hours. To find differential expression proteins between this two groups, 2-dimensional fluorescence difference in-gel electrophoresis (2D-DIGE) was applied, and the differential expression protein spots were selected by the DeCyder software. The peptides mass fingerprint (PMF) was got by the matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) and searched in SWISSPROT database to identify each proteins. Furthermore, the changes on protein were confirmed with western blot.

Results and Discussion

Primary cultured OLGs was established successfully. According to the MALDI-TOF MS results, there were more than 15 proteins, which were more than 1.5-fold difference in expression levels between the +VD group and C group. The majority of the VD-induced proteins were involved in generation of precursor metabolites and energy, oxidation of reduction, regulation of apoptosis, and glycolysis. Changes of proteins such as IGF2, gelsolin, IGFBP7 etc. suggest that 1,25-(OH)2D3 may have association with cell growth, proliferation and apoptosis.

Conclusion

Together, we find a number of novel vitamin D-regulated proteins that may contribute to a better understanding of the reported beneficial effects of vitamin D on OLGs, this could help us to discover the function of vitamin D in disease with demyelination and neurological functional deficit, such as multiple sclerosis.

Keywords: 1,25-dihydroxyvitamin D3; proteomics;Oligodendrocytes (OLGs)

WP08-07

PROTEOMIC INVESTIGATION OF GLIOBLASTOMA MULTIFORME ON THE BASIS OF SVZ INVOLVEMENT

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Introduction and Objectives

Gliomas are tumors of the brain and are subdivided into 4 grades as per the WHO classification. Glioblastomas (GBMs) comprise grade 4 of these deadly tumors and stand out for their extremely aggressive nature with the patients having a dismal survival period. The subventricular zone of the brain is a site rich in neural stem cells. Tumors identified in contact or close proximity to this region (SVZ+) of the brain are characterised by an increased aggressiveness over tumors that are away from this region (SVZ-) evident from the low median survival time in the former as compared to the high median survival time in the latter. A recent study aimed at identifying the molecular basis behind increased aggressiveness of SVZ involved tumors. However, our study aims at understanding the differences between proteomes of SVZ+ and SVZ- tumors with reference to proteomes of normal peri-tumoral tissues, and co-relating the findings with survival time of patients.

Methods

Protein extraction from the serum and tissues was performed using TCA-Acetone method and Trizol method, respectively. Following extraction of proteins form the serum, the samples were subjected to analysis using classical 2DE, 2D-DIGE and iTRAQ methods, while proteins extracted from tissues were analysed using 2D-DIGE and iTRAQ methods. The protein pellets dissolved in rehydration buffer after extraction using TCA-Acetone method and Trizol method were subjected to buffer exchange to dissolve them in TEAB buffer, thereby making them LC-MS compatible. The complexity of the protein samples was reduced by subjecting the in-solution digested peptides to off-gel fractionation prior to LC-MS. The proteins obtained after LC-MS were subjected to pathway analysis using DAVID 6.7 software.

Results and Discussion

Serum proteomic analysis revealed significant alterations of various acute phase proteins and lipid carrying proteins, while tissue proteomic analysis revealed significant alterations in cytoskeletal, lipid binding, chaperone and cell cycle regulating proteins, already established to be associated with disease pathobiology.

Conclusion

The proteins identified in the current study provide a molecular evidence for the aggressive nature of SVZ+ GBM tumors and shorter survival period of SVZ+ GBM patients. These proteins need to be further explored for their role in glioma cell lines and animal models to authenticate their role in increased aggression of SVZ+ tumors over SVZ- tumors.

WP08-07

Keywords: Glioblastomas, Subventricular zone, TCA-Acetone method, Trizol method, iTRAQ LC-MS

WP08-08

An innovative HPLC quantitative method coupled with proteomics analysis to investigate hydrogen sulphide forms in cerebrospinal fluid

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Introduction and Objectives

By introducing hydrogen sulphide (H2S) as an endogenously generated neuromodulator, a large body of data about its role in the central nervous system (CNS) has been accumulated. However, the function of H2S in the brain is not yet clear. It seems to be a "double-faced Janus" molecule with opposite effects: at low concentrations neuromodulator and neuroprotectant, at higher concentrations proinflammatory agent involved in neurological disorders. Three enzymes are responsible for H2S synthesis: cystathionine beta-synthase mainly localised in astrocytes and microglial cells; cystathionine gamma-lyase mostly expressed in periferic system, but also found in microglial cells, spinal cord and cerebellar granule neurons; 3-mercaptopyruvate sulfotransferase localised in neurons. In addition to free H2S that moves in a biologically active form, H2S can be also incorporated and stored into proteins as bound sulfur sulfane. Bound H2S can ultimately participate in sulfhydration, a physiological process involved in many signaling pathways inside the CNS. To date, a method for H2S quantification in the cerebrospinal fluid (CSF), which is a key biofluid for the study of neurological disorders, is still missing. Therefore, the aim of this study is to unravel free and bound forms of H2S in CSF through the development of a new analytical validated method for their quantification.

Methods

120 CSF with different pathological conditions have been analyzed exploring different H2 S forms (total, free and bound proteins). For H2S measurement, specific protocols and tests have been developed by the use of an HPLC system with an amperometric detector (Thermo Fisher Scientific). Moreover, a deeper proteomics analysis has been performed to investigate CSF profiling focusing on H2S target proteins.

Results and Discussion

We introduced a new protocol of sample preparation and an analytical method for H2S measurement. The application of different chemical switch protocols allowed the quantification of total, free and protein bound H2S levels. The method has been validated for linearity, sensitivity, robustness, precision, accuracy and repeatability. For each CSF sample total, free and bound H2S fractions have been investigated. The distribution of H2 S levels seemed change according to diseases status. A proteomics analysis of CSF with highest and lowest free and bound H2S levels highlighted a different expression of

proteins with reactive cysteine (e.g. transthyretin) among these groups.

Conclusion

H2S is actively involved in brain functions and different processes can contribute to its production. Understanding the H2S biosynthesis and unveiling the molecular bases of the H2S metabolic impairment observed could be helpful to explore the potential role of H2S in several neurodegenerative conditions and consequently to modulate synthesis and/or actions of this molecule.

Keywords: brain, CSF, H2S, HPLC, mass spectrometry, neurological disorders, sulfhydration

Chronic low-dose rate ionising radiation affects the hippocampal phosphoproteome in an ApoE$^{-/-}$-Alzheimer mouse model

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Introduction and Objectives

Cognitive impairment is an adverse side effect after radiation exposure. Accruing data indicate that such radiation-induced consequences resemble pathologies of neurodegenerative diseases such as Alzheimer´s. The aim of this study was to elucidate the effect on murine hippocampus of chronic low-dose-rate radiation exposure (1 mGy/day or 20 mGy/day) given over 300 days with cumulative doses of 0.3 Gy and 6.0 Gy, respectively. ApoE deficient mutant C57Bl/6 mouse was used as an Alzheimer´s model.

Methods

Global expression levels of unmodified proteins, phosphoproteins and N-linked sialylated glycoproteins were quantified using mass spectrometry. The proteomics data were validated using synaptic plasticity-targeted transcriptomics, immunoblotting, ELISA and immunohistochemistry.

Results and Discussion

Conclusion

This study shows that several molecular targets induced by chronic low-dose-rate radiation overlap with those of Alzheimer´s pathology. Therefore, we suggest that chronic low-dose-rate ionising radiation may function as a contributing risk factor to Alzheimer´s.

Keywords: Synaptic plasticity, hippocampus, dendritic spine, CREB, synapse, proteomics, phosphoproteomics, sialylation, glycoproteomics

WP08-10

Proteomic analysis reveals metabolic alteration of substantia nigra in schizophrenic patients

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Introduction and Objectives

Schizophrenia (SZ) is a most complex multifactorial neuropsychiatric disorder considered several aspects from clinical to molecular basis. Myriad role of aberrant protein expression in schizophrenia and recent efforts in proteomic and bioinformatics based studies demonstrate that how these modulated proteins expression works in physiological and pathological condition. Present work focused on to find out the pathological protein expression variation by proteome analysis of substantia nigra (SN) with age-matched controls.

Methods

Present study entails, differential proteomic analysis of autopsied schizophrenic brain substantia nigra, cortex, and hippocampus (n=7) by using two dimensional electrophoresis (2DE) followed by identification through nano LC-MS/MS. Differential expression was validated by western blot, while In Silico analysis was applied for functional interactive role in metabolism.

Results and Discussion

Using Nano LC-MS/MS analysis 1443 proteins were identified, in which 26 spots of substantia nigra, were found to be significantly differentially expressed($p<0.05$). The identified proteins were enriched in several metabolic processes like energy metabolism (ATP5H, GAPDH, MDHC and PGAM1) cytoskeleton and transportation (STXB1, NSF, DEST, VPS29, DPYL3), cell cycle (SKP1), redox dysregulation and apoptosis (HAGH, GSTT1, GSTM3, GPX4, MYDGF and PRDX1) ubiquitination and protein metabolism (UBE2V2, TCPB, PPIA CNDP2, and DHPR). One of significant finding, through network analysis of ingenuity pathway analysis, indicates that schizophrenia associated pathophysiological molecular factors showed a strong interaction with tumor proteins 53 (TP53) and NFkB complex along with glutathione and hydrogen peroxide metabolism that are creating hubs with all the proteins identified with altered expression.

Conclusion

Ingenuity pathway analysis of proteins further elucidate merge network of all the identified proteins with their interacting partners providing an evidence of complexity of disease mechanism depicting the overlap of multiple pathways, rather a single pathway, responsible for producing the deleterious effect. Identification of these differentially expressed proteins is making an understanding of the pathogenesis of this complex disease and also will be helpful to understand disease progression and functional alterations associated with Schizophrenia. In addition, molecular markers found in this study may contribute to the identification of new potential biomarkers and also be used as a drug targets.

Keywords: Proteomics, Substantia nigra, Nano LC-MS/MS, Schizophrenia

Mapping of Gangliosides from Nine Regions of Mouse Brain by Negative Ion Mode Nano LC/MS

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Introduction and Objectives

Gangliosides, a subtype of glycosphingolipids containing sialic acid, are abundant in neural tissue. They play an important role in neurophysiology including brain development, nerve formation and transmission. In addition, the brain gangliosides are known to be uniquely distributed in different regions of brain tissues. Therefore, the regional profiling of gangliosides in the brain will be useful as the informative data for the brain functional study about each region. In this study, we have optimized the negative ion detection mode nano LC/MS system for the analysis of ganglioside from brain tissue. Gangliosides obtained from different anatomic regions of mouse brain were profiled and structural elucidated by nano LC/MS/MS. We built the map of regional brain ganglioside and created the organ specific mouse brain database for leading a better understanding on the brain functions of the specific brain region.

Methods

Nine different parts of the brain of five mice was obtained at the Institute for Basic Science. Brain tissues were grinded by sonication. Homogenized brain samples were mixed with chloroform/methanol/water (4:8:3, v/v/v) and centrifuged, and the aqueous upper layer containing gangliosides was collected. The pellet was additionally washed with chloroform/methanol (1:1, v/v) for extracting remnant lipids. The extracted lipids were partitioned again with chloroform/methanol (2:1, v/v) to selectively collect gangliosides. Gangliosides were further purified and enriched by C18 solid-phase extraction and they were profiled by negative nano LC/MS using a C18 microfluidic chip.

Results and Discussion

A whole mouse brain was anatomically dissected for efficient analysis of regional gangliosides into the nine regions. We extracted the gangliosides in each region after identically adjusting protein quantity of all brain tissues for more accurate analysis of gangliosides in different brain areas. The extracted gangliosides in nine mouse brain regions were profiled by negative nano LC/MS. GD1, GT1, GQ1, and GM1 with d36:1 and d38:1 ceramide portions were observed as major gangliosides in all brain regions but showing the difference in relative abundance in the different brain areas. Furthermore, the statistical analysis was performed by Pearson correlation analysis to investigate similarity of gangliosides of each region. The prefrontal cortex that has least abundance of GT1 (d36:1) show the lowest similarity with the other regions.

Conclusion

We established an analytical platform for qualitative and quantitative analysis of gangliosides from mouse brain tissue by nano LC/MS. Furthermore, we analyzed the gangliosides in nine different areas of the mouse brain using the developed method to build up the regional glycome map and library from mouse brain. These results would be

WP08-11

useful as the informative data for the glycome study related the physiological and pathological brain functions of the specific brain region as well as whole mouse brain.

Keywords: Brain, Ganglioside, Negative ion mode, Nano LC/MS

Chronic exposure to low dose methylmercury induces various proteome changes in different regions of rat's brain

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Introduction and Objectives

Methylmercury (MeHg) is an environmental pollutant as well as a potent neurotoxin commonly found in seafood. MeHg accumulates in the human body through seafood consumption. As the MeHg load increases to a pathological level, paresthesia and numbness of the skin are the earliest observable syndromes of intoxication in human. As the load keeps increasing, other syndromes like dystaxia, dysarthria and visual impairment will be seen. The sequential occurrence of pathological syndromes indicates that different regions of the brain respond differently to different doses of MeHg. Meanwhile, potential health impacts on people accumulated subtoxic dose of MeHg chronically through fish consumption are not clear. In our pervious study, we have observed dramatic changes in proteome of somatosensory cortex in rats exposed to subtoxic dose of MeHg (40 µg/ kg bodyweight/ day) for 12 weeks. In brief, we have reported that proteins related to glycolysis, ATP production, neurotransmission, and protein synthesis were down-regulated, resulting in a metabolic deficit in the somatosensory cortex without showing any observable abnormality phenotypically. Recently, we have investigated effects of chronic exposure of subtoxic doses of MeHg on the other regions of the rat brain, including motor cortex, visual cortex and cerebellum.

Methods

Motor cortex, visual cortex, and cerebellum were isolated from rats dosed with 40 µg MeHg/ kg bodyweight/ day for 12 weeks. Proteins were labeled with isotope coded protein labeling (ICPL), then fractionated by SDS-PAGE, finally analyzed by using nano-C18 column coupled with electrospray ionization ion trap mass spectrometer. Proteomic data is analyzed by gene ontology and pathway analysis.

Results and Discussion

In contrary to results obtained from the somatosensory cortex, we found that a number of proteins involved in synaptic transmission and glycolysis (like synapsin and aldolase) were up-regulated in the motor cortex, visual cortex and the cerebellum. Moreover, dihydropyrimidinase-related proteins (that involve in axon growth) were up-regulated only in motor cortex and cerebellum. Only a few proteins like neurofilament polypeptides were down-regulated in all regions of the rat brain.

Conclusion

The current study proved that the same subtoxic dose of MeHg induced different proteomic changes in different regions of the rat brain. This may provide an insight into why different symptoms appear with respect to different MeHg loads experienced in human.

Keywords: Methylmercury, motor cortex, visual cortex, somatosensory cortex, cerebellum

WP08-13

Differential Proteome Changes in the Brains of Arctic Ringed Seal Induced by Bioaccumulation of Methylmercury

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Introduction and Objectives

Acute exposure to high level of mercury is known to have serious detrimental effects on health. Sulphur containing bacteria in our water bodies (e.g. rivers and the sea) can convert inorganic mercury to organic mercury, mostly methylmercury (MeHg). MeHg is known to bioaccumulate in the food chain. Hence, top-level consumers are exposed to low levels of MeHg chronically and could eventually bioaccumulate MeHg to high levels. Arctic Ringed-seals are one of the top predicators in the Arctic circle. They fed on fishes only and they are also one of the major traditional diet of Inuit. These seals can bioaccumulate large amount of MeHg in their bodies and consumption of these seals could have a negative impact on health of the Inuit. On the other hand, these seals provided a good animal model as they are mammals and they bioaccumulate MeHg through their diet. Therefore, understanding the biochemical effects of the accumulated MeHg on the ringed seals could provide insights to the negative health impacts on the Inuit and other human.

Methods

The ringed-seal frontal lobe samples were kindly donated by our collaborators. Quantification of total Hg content was done by using mercury analyzer. Label-free quantitative proteomic technique was done by using nano-C18 column coupled with TripleTOF 6600 system and SWATH acquisition 2.0 from Sciex. Proteomic data is analyzed by gene ontology and pathway analysis.

Results and Discussion

As a first step, proteomic technologies were used to study proteome changes in the frontal lobes of Arctic Ringed Seals that have elevated mercury level in the brain tissues (n=3, average 1.64 Hg mg/kg dry weight). Three frontal lobes samples of low mercury contents (n=3, average = 0.39 Hg mg/kg dry weight) were used as control. With standard preparatory procedures and the provision of a Triple-TOF (6600), we were able to identify 988 proteins in the frontal lobes of these seals. Thirty proteins were found to be differentially expressed. Among these proteins, some are related to responses to oxidative stress, development of the neural system and DNA/RNA controls. They were found to be down-regulated in high Hg samples. On the other hand, some

other enzymes belonged to the same category were found to be up-regulated in the high Hg samples.

Conclusion

Whether some differential expression are consequences or remedial actions by the body is yet unknown. Nonetheless, as judged from the identities of these enzymes found, Hg induced damages to the frontal lobes are multi-pronged in essence. Specially on the negative neurological impact on the biomolecular level observed, Hg may induce damages on the cellular signaling system and regular control of the oxidative pathways.

Keywords: Methylmercury, ringed seal, frontal lobe

WP08-14

A peptidomic profile of transgenic rat model for tatuopathy: LC-MALDI/MS analysis of cerebrospinal fluid

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Introduction and Objectives

The diagnosis of Alzheimer's disease (AD), the most common cause of progressive brain dementia in older adults, is based on presence of clinical features and pathological changes such as formation of senile plaques and neurofibrillary tangles. There is an ongoing intensive search for biochemical diagnostic markers over the last decade, to support the clinical diagnosis.

Methods

In present study we used SHR72 transgenic rat model for tauopathy expressing truncated tau protein (aa 151–391/4R) and analysed the peptidomic profile of cerebrospinal fluid (CSF) by liquid chromatography – MALDI mass spectrometry (LC-MALDI TOF/TOF).

Results and Discussion

Out of more than 300 peptides, we identified a total of 140 proteins. Among them six proteins differed statistically significant in CSF from transgenic rats. The amount of the following four proteins in CSF of transgenic rats was higher: chromogranin-A (p=0.0497); insulin-like growth factor II (p=0.0497); neurofilament light polypeptide (p=0.0077) and reticulon-4 (p=0.0285). The amount of the following two proteins in CSF of transgenic rats was lower: ectonucleotide pyrophosphatase (p=0.0196) and gelsolin (p=0.0403). Interestingly all of the identified proteins were previously linked to AD and other tauopathies, indicating the value of transgenic animals in biomarker research.

Conclusion

In conclusion, our results showed that LC-MALDI TOF/TOF analysis of CSF peptidome is a valuable tool for investigation of potential biomarkers in transgenic animal models of neurodegenerations. Proper investigation of peptidome changes in a course of neurodegenerative processes seen in our transgenic animals could in the future serve as an important base for human biomarker studies.This work was supported by Axon Neuroscience and competitive academic grants VEGA 2/0159/15, APVV-14-0547 and structural funds 26240220008, 26240220046.

Keywords: Tauopathy, LC-MALDI MS, peptidomics, cerebrospinal fluid, rat model, tau protein

The mechanism research on GSN remission of MS/EAE: A proteome analysis

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Introduction and Objectives

In our previous study, we observed an obviously change on the concentration of gelsolin in EAE rats. The objective of this research is to investigate changes in expression of proteins and protein isotypes with or without gelsolin(GSN) overexpression in experimental allergic encephalomyelitis(EAE) rats and analyze how GSN affects the process of EAE.

Methods

The spinal cord samples were obtained from 10 EAE rats with (EAE+GSN group) or without (EAE group) injecting GSN overexpression lentivirus into ventricle. To find more differential expression proteins, we extracted total protein in spinal cord followed by running a SDS-PAGE for separating the proteins, then liquid chromatograph-mass spectrometer(LC-MS) were taken to measure differential proteins between the two groups. Furthermore, the changes on protein and mRNA level were confirmed with western blot and real-time quantitative PCR, nuclear factor kB (NF-kB)-dependent luciferase reporter gene assay was used to detect the effect of GSN on the regulation of TNFα-induced NF-kB activation.

Results and Discussion

The onset of EAE was delayed 2 days in EAE+GSN group compared with EAE group, the score of the disease was also reduced obviously. According to the LC-MS results, there were more than 20 proteins, which were more than 1.5-fold difference in expression levels between the EAE+GSN group and EAE group. Levels of GSN, microtubule-associated protein-2, MPO, NF-kB etc. were significantly changed, which were confirmed by western blot and RT-PCR. Detection of NF-Kb-dependent luciferase reporter gene assay proved that over-expressing GSN could inhibit TNFα-induced NF-kB activation.

Conclusion

Increasing the GSN protein level in the rat can apparently remit the EAE process, this result indicates that GSN might play an important part in human MS disease, this may provide a new idea for the treatment of MS. The remission of EAE process by GSN, might through regulating of TNFα-induced NF-kB activation.

Keywords: EAE/MS;Gelsolin;Proteomics

WP08-16

Human platelet lysate (HPL) proteome analysis by nanoLC-MS/MS mass spectrometry: new insights to understand HPL neuroprotective properties

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Introduction and Objectives

Human platelet lysates (HPL) constitute an abundant source of growth factors and other bioactive molecules and have an essential physiological and therapeutic role in wound healing and tissue repair. Interestingly, recently HPL was shown to exert demonstrable in vitro and in vivo effects on neuron survival suggesting that HPLs-based therapies could be a powerful novel strategy to prevent neuronal loss in neurodegenerative diseases.In the present work, we attempted to determine, using proteomics, the complete proteome profile of an HPL product that has been proven to exert neuroprotective effects in cellular and animal models of Parkinson's disease and amyotrophic lateral sclerosis. A better knowledge of the proteome composition of HPLs can provide powerful information to develop more efficient HPL preparations for therapeutic use.

Methods

HPL proteins were first separated by gel electrophoresis before nano-LC-MS/MS mass spectrometric analysis, or were directly submitted to a mass spectrometry-based shotgun proteomics method.

Results and Discussion

By using multiple proteomics approaches, hundreds of proteins were unambiguously identified including many proteins implicated in the mechanism of neuroprotection.

Conclusion

On the basis of these results, it is considered possible to include in the HPL preparation process, a judicious enrichment step to increase the amount of neuroprotective proteins in HPL samples. The same strategy can be highly effective to develop specialized HPL preparations for dedicated applications in the diversified fields of regenerative medicine.

Keywords: Human platelet lysates; neurodegenerative diseases; shotgun proteomics

Estrogen partially reverses proteomic changes induced by excessive stretching of brain endothelial cells

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Introduction and Objectives

Almost one in seven (i.e., 1B) people globally suffer hypertension (high blood pressure) that is a significant predetermining factor in the development of cardiovascular disorders like arteriosclerosis, heart failure, aneurysm and stroke. Aneurysms especially have a significantly higher rate of incidence in Australasia (4/1000), often resulting in debilitating strokes or even mortality. Interestingly the incidence of stroke and aneurysm has been found to be significantly lower in pre-menopausal women than men, with some evidence that estrogen may be protective. It has been proposed that the overstretching of cerebellar endothelial cells may lead to structural weaknesses in cellular junctions and that estrogen protects cells against this. To date, no rational molecular mechanisms for such a proposal have been tabled.

Methods

Human cerebral microvascular cells (HCMEC) were cultured on silicone cubes and stretched at either 5% (normal) or 20% (pathological) for a period of 2 or 18hrs in the presence or absence of 1nM estrogen (17β-estradiol). The cells were then lysed, proteins purified by acetone precipitation and using iTRAQ and label free techniques quantified with tryptic peptides being fractionated by SCX followed by ESI MS/MS on an ABSciex TripleTOF 5600. The data was statistically analysed and significantly differentially-expressed proteins further analysed by Ingenuity Pathway Analysis (IPA) and gene ontology (PloGO).

Results and Discussion

For the first time, using precision proteomics, we were able to demonstrate that pathological stretching of endothelial cells induces the activation of “detrimental” pathways (e.g., inflammation, and apoptosis) and we demonstrate that estrogen exposure at physiological levels is able to reverse some of these effects. Furthermore, we were able to demonstrate that normal stretching of cells leads to a significant upregulation of structural, cell adhesion and proliferation pathway proteins having a level of protective effect on the vasculature.

Conclusion

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This investigation sheds light on the molecular mechanisms of the cerebrovascular protective actions of estrogen on HCMEC exposed to pathological levels of cyclic stretch. The study recapitulates and extends findings in in vivo models and sets the ground for more detailed studies with potential therapeutic and diagnostic implications.

Keywords: Strokes, Aneurysms, brain proteomics, hypertension

WP08-18

A glimpse into the proteomic profile of Rasmussen's Encephalitis

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Introduction and Objectives

Rasmussen's encephalitis (RE) is rare, chronic inflammatory neurological disorder typically affecting only one hemisphere of the brain. This disease occurs mostly in young children and is usually characterised by cortical inflammation, progressive neurological dysfunction, intractable drug resistant seizures and paralysis on one side of the body. Surgical procedures such as hemispherectomy are used in the later stages of RE to reduce the frequency of seizures, however it is not curative and most individuals are left with neurological sequelae. In the case of RE several biomarkers have been previously reported based on insights from singular or a limited number of marker experiments. Here we report a novel investigative approach using unbiased proteomics in RE brain tissue.

Methods

Ethical approval for this study was obtained from a broader study approved by the institutional review boards of the University of Cape Town and the Red Cross children's hospital. The tissue was obtained with informed consent from a RE diagnosis confirmed case, undergoing left hemispheric peri-insular hemispherotomy. Tissues were homogenised and subject to filter aided sample preparation and digestion with trypsin protease (FASP). Reverse phase liquid chromatography coupled to high resolution mass spectroscopy was the analytical technique performed. MaxQuant with the Andromeda search engine was used to identify and quantify peptides and protein groups from human and viral databases. Protein pathway analysis and protein list enrichment analysis was performed using List2Networks and Enrichr respectively.

Results and Discussion

Here we report a novel investigative approach using unbiased proteomics in RE brain tissue samples and show a snapshot view of 1272 proteins using mass spectrometry based discovery proteomics performed on a high-resolution LC-MS system (UPLC - QExactive). The relative abundance of markers seen in this study suggests a dual innate and acquired immune response as well as excitatory neurotransmission. Active signalling processes were present, as evidenced by peptides associated with phosphorylation and synaptic plasticity. Certain destructive processes noted were cell death, cytotoxicity and halting of neurogenesis. Furthermore, we also explore the dataset for the presence of viral component peptides and the dataset suggest Epstein-Barr and Human Adenovirus peptides are likely present and could contribute to pathogenesis of RE.

Conclusion

All of this evidence suggests that in late stage RE a large immune response with vast

WP08-18

cell death and excitotoxicity occurs at the protein level. These findings correlate with the clinical presentation of RE. Kinase markers identified in this study could provide an avenue for further validation and possible candidate biomarkers for novel treatment.

Keywords: Brain proteomics, Neurological disorders, Encephalitis, Neuroinflammation

Toxicoproteomics highlights the role of vimentin in neurotoxicity induced by fipronil

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Introduction and Objectives

Fipronil is a phenylpyrazole insecticide that is commonly used to control agricultural, veterinary and household pests. Even though fipronil is more selective in its potency towards insects than humans, the increasing use still poses a health risk.

Methods

We elucidated the toxic mechanisms of fipronil on SH-SY5Y neuroblastoma cells using proteomics and validated the findings using Western blots, immunofluorescence staining and functional assays.

Results and Discussion

Fipronil induced alterations in proteins involved in oxidative stress, ER stress and unfolded protein response, neuronal structure and neurite projection, and transcription and translation. Not only did the expression of vimentin increase, different isoform patterns were observed, indicating alterations in post-translational modifications. Neurite outgrowths of SH-SY5Y cells were impaired and vimentin was localized at the neurite outgrowth, possibly to attempt in repairing the damage. However at high concentrations of fipronil, vimentin was found to be in less defined fibrils, its disassembly disrupted, and dense surrounding vacuole-like structures. Our results indicate that vimentin plays a critical role in neuroprotection as well as neurodegeneration induced by fipronil in SH-SY5Y cells.

Conclusion

These findings suggest that vimentin has the potential to be a diagnostic marker and/or therapeutic target for fipronil exposure and that fipronil is indeed a risk factor for neurodegenerative diseases, which necessitates recommendations for its proper handling and management to reduce the impact on human health.

Keywords: fipronil, neurotoxicity, toxicoproteomics, vimentin, neurite outgrowth

WP08-20

Amyloid Beta Activated Human Microglial Cells Through ER-Resident Proteins

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Abstract

Microglial activation in the central nervous system is a key event in the neuroinflammation that accompanies neurodegenerative diseases such as Alzheimer's disease (AD). Among cytokines involved in microglial activation, amyloid β (Aβ) peptide is known to be a key molecule in the induction of diverse inflammatory products, which may lead to chronic inflammation in AD. However, proteomic studies of microglia in AD are limited due to lack of proper cell or animal model systems. In this study, we performed a proteomic analysis of Aβ-stimulated human microglial cells using SILAC (stable isotope labeling with amino acids in cell culture) combined with LC-MS/MS. Results showed that expression of 60 proteins was up- or down-regulated by 1.5 fold or greater. Among these, ER-resident proteins such as SERPINH1, PDIA6, PDIA3, and PPIB were revealed to be key molecular biomarkers of human microglial activation, by validation of the proteomic results by immunostaining, PCR, ELISA, and western blot. Taken together, our data suggests that ER proteins play an essential role in human microglial activation by Aβ and may be important molecular therapeutic targets for treatment of AD.

WP08-21

Differential protein expression in the cortex of mice exposed to the antipsychotic drugs haloperidol and clozapine

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Introduction and Objectives

Psychotic disorders are very serious and costly mental disorders. Throughout the years the understanding of the genetic basis of these illnesses has improved considerably but there is only rudimentary knowledge of its pathophysiology, mostly due to inconsistent results and confounding factors.In the case of schizophrenia, several explanations for the origin of the disorder have emerged, such as the dopaminergic, glutamatergic or serotoninergic hypotheses. Having this in mind, 2 antipsychotics were selected for this project: haloperidol and clozapine, first and second-generation antipsychotics, respectively. Haloperidol is believed to act essentially in D2 receptors, and clozapine, along with softer action in D2 receptors, also has affinity for 5HT and other receptors. Haloperidol is effective against positive symptoms, and clozapine has shown effectiveness in positive and negative symptoms.The main goal of this project was to investigate the differential protein expression in the rodent cortex following chronic exposure to commonly prescribed psychotropic medication, with the objective of distinguishing between pharmacological and disease-related changes and correlating expression profiles with drug effects.

Methods

Two different quantitative mass spectrometry proteomics approaches (label-free SWATH quantification and stable isotope labelling (iTRAQ)) were used to investigate the differential protein expression in the rodent cortex, following chronic exposure to the 2 antipsychotic drugs: haloperidol and clozapine.In order to increase the proteome coverage, a subcellular fractionation was performed, using an ultracentrifugation step, giving rise to two distinct fractions, membrane-enriched and soluble protein fractions.

Results and Discussion

In total, over 2000 proteins were quantified, where about 60% of the quantified proteins in the membrane-enriched fraction are annotated as “membrane”.As expected, the majority of the quantified proteins in each drug injected mice maintained a stable expression when compared with the control mice, nonetheless some statistically meaningful differences were observed. Using a principal component analysis with these significantly altered proteins it was possible to distinguish between the control and medicated groups. Moreover, when gene ontology analysis was performed for these proteins, the majority

are related with metabolic processes, especially with the primary metabolism (glycolysis and oxidative phosphorylation) suggesting a metabolic shift caused by the two drugs. Also some proteins involved in synapse and synaptic vesicle cycle show altered expression, as well as proteins involved in calcium signalling pathways.

Conclusion

In summary, this study can give clues about the mechanism of action of long-term antipsychotic exposure and depict possible target molecular pathways for the investigation of psychotic disorders' physiology and treatment.

Keywords: Psychotropic Drugs; SWATH; iTRAQ;

WP08-22

PROTEOMIC ANALYSIS OF MATURATION DEPENDENT ADVERSE EFFECTS OF PARAQUAT EXPOSURE IN 3D RAT BRAIN CELL CULTURES

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Introduction and Objectives

Exposure to environmental toxicants during vulnerable windows of brain development is suspected to raise the prevalence for neurological dysfunctions at later stages in life. The mechanisms rendering the developing central nervous system more susceptible to insult are not fully understood. We aimed at investigating the underlying neurotoxic mechanisms for these maturation dependent differences in susceptibility to the herbicide paraquat (PQ).

Methods

To compare effects of PQ-exposure between immature and mature brain cells we chose the 3D rat brain cell culture model. This in vitro system consists of all brain cell types, which undergo extensive differentiation into a histotypic tissue. In order to understand acute- versus adaptive toxic mechanisms in response to PQ at the two different brain cell maturation stages, samples were collected after a single exposure (24 hours) and after a 10-day repeated exposure in the sub-micromolar range. Specific differences in adversity of PQ-treatment were evaluated by assessing changes in morphology and gene expression. To elucidate differences in toxic mechanisms, acute versus adaptive, at the two stages an untargeted proteomic profiling approach was adapted. This was obtained using Tandem Mass Tag (TMT) labelling, analyzed by LC-MS/MS, and quantification was performed via Easyprot and Isobar platforms.

Results and Discussion

PQ-induced adverse effects on glutamatergic-, GABAergic- and dopaminergic neurons were more pronounced in immature cultures as compared to mature. Furthermore, immature cultures also displayed stronger astrogliosis and an expression pattern of microglial activation markers different to mature cultures. Proteomic analysis revealed a PQ-dependent shift in regulation of proteins involved in processes like reactive oxygen species formation, mitochondrial dysfunction, and protein homeostasis.

Conclusion

Our results show that immature brain cell cultures are more susceptible to PQ-induced insult, and that cellular response differs to mature brain cell cultures, indicating that PQ could possibly be considered as a developmental neurotoxicant. Verification of proteins of interest together with further pathway analysis on the proteomic profiles could identify signatures specific to exposure duration (acute versus sub-chronic) and to the maturation stage.

Keywords: Neurotoxicity, Paraquat, neuroinflamation

WP08-23

Beta-amyloid induces pathology-related patterns of tau hyperphosphorylation at synaptic terminals via CDK5 activation

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Introduction and Objectives

Methods

We characterized the synaptic phosphoproteome of APP/PS1 mice (amyloid model) by label-free quantitative mass spectrometry, covering about 4000 phosphopeptides.

Results and Discussion

We found that amyloid pathology induced hyperphosphorylation on a small subset of synaptic proteins, with tau being one of the primary targets. Proteins associated with cytoskeletons and involved in calmodulin signaling pathways were particularly affected. Tau hyperphosphorylation occurred at proline-directed sites (S199, S202, S396, S404) and non-proline directed sites (S400, S416). The proline-directed sites were prominently associated with the pathological features of tau found in AD subjects, likely promoted by conserved proline-directed kinases (PDK). Global analysis of hyperphosphorylation motifs suggested cyclin-dependent kinase 5 (CDK5) as the major synaptic PDK involved in amyloid-induced tauopathy, while casein kinase II activity was also upregulated.

Conclusion

Keywords: Abeta, tau, CDK5, synapse, Alzheimer disease, hyperphopsphorylation

WP08-24

Investigation the roles of TDP-43 oligomers in neurodegenerative diseases

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Introduction and Objectives

The TAR DNA binding protein 43 (TDP-43) resides mainly in nucleus and plays important roles in the neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) and frontotemporal lobar dementia (FTLD). These neuropathology are characterized by deposition of TDP-43-positive cytoplasmic inclusions. In our previous research, we found that the recombinant full-length TDP-43 can form stable oligomers that are neurotoxic in vitro and in vivo. Such oligomers are also detected in brains of transgenic TDP-43 mice and FTLD-TDP patients. TDP-43 has been reported to be secreted and transmitted between cells. We will test whether exogenous TDP-43 oligomers are prone to induce cellular stress.

Methods

We employ our recombinant TDP-43 as the model to resemble the latter transmission events and exam the cellular localization of these oligomers.

Results and Discussion

Our preliminary data show that TDP-43 oligomers are endocytosed and are partially localized in lysosomes within 3 hours after treatment. After 48 hours, the oligomers are present in both cytoplasm and perinuclear regions in the HEK293, NSC-34 cells, and iPS–derived motor neurons. We further demonstrated that 67% of TDP-43 oligomers are accumulated in the cytoplasm while 33% of that are accumulated in the perinuclear regions in HEK293 cell, and the perinuclear oligomers are embedded in the nuclear membrane and closed to the nuclear pore complex using a super-resolution microscope.

Conclusion

Future directions will be focused on whether oligomeric TDP-43 can be secreted, how the endogenous TDP-43 oligomers can be formed, and what are the subsequent consequences and binding partners.

Keywords: TDP-43, oligomer, nuclear pore complex

WP08-25

Chronic Exposure to Low Doses of Methylmercury Induced Proteome Changes on Polar Bear Brains

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Introduction and Objectives

The bioaccumulation of methylmercury (MeHg) in marine food chain is well known. However, the effects of chronic exposure to low doses (CELDs) of MeHg on top predators are still unclear. Polar bear (Ursus maritimus) is the apex predator in the Arctic region. Because their main sources of food are marine fishes, they are good models to evaluate the effects of CELDs of MeHg in mammals. In this study, the protein changes of polar bear brain samples were studied to investigate the effects of CELDs of MeHg on top predators.

Methods

The effects of chronic MeHg exposure in different brain regions (cerebellum, occipital and frontal lobes) of polar bears were studied using shotgun proteomic approach with isobaric tag for relative and absolute quantitation (iTRAQ) technique. Polar bear brain samples were collected and different regions of the brain including cerebellum, occipital and frontal lobes were dissected. The samples were divided into two sample groups according to the total mercury (tHg) levels of the brain samples. Proteins were extracted, followed by protein digestion and iTRAQ labeling steps. After tryptic digestion, the labeled peptides were analyzed using liquid chromatography coupled with quadrupole time-of-flight mass spectrometer (LC/Q-TOF MS). Protein changes of the brain samples with high/low tHg exposures were revealed by comparing the ion peak intensity ratios of their iTRAQ reporter ions.

Results and Discussion

It was found that most of the differential proteins were down-regulated in the brain sample with high mercury exposure. The down-regulated proteins were involved in different biological processes including carbon metabolism (i.e., glycolysis and ATP production), synaptic transportation (i.e., neurotransmission, translation and protein folding) and cytoskeleton regulation (i.e., actin and tubulin dynamics, calcium hemostasis and signal transduction).

Conclusion

These results showed that the CELDs of MeHg induced significant changes on the cellular functions of the brain which may led to neurological deficits in the polar bears.

Keywords: Chronic methylmercury toxicity, brain proteomics, quantitative proteomics, iTRAQ

WP08-26

Imaging Mass Spectrometry (IMS) for the proteomic studies of Experimental Autoimmune Encephalomyelitis (EAE) Mouse

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Introduction and Objectives

Experimental autoimmune encephalomyelitis (EAE) is the most commonly used experimental model for the human inflammatory demyelinating disease, multiple sclerosis (MS). EAE is a complex condition in which a variety of pathological mechanisms leads to an approximation of the key pathological features of MS: inflammation, demyelination, axonal loss and gliosis. In this study, we applied matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS) for EAE mice brain and spinal cord. We will perform a detailed analysis of the different clinical scores of the brain and spinal cord from EAE mice and will detect marker proteins to promote basic molecular mechanism of the diseases.

Methods

Brains and spinal cords from EAE mice were resected and snap-frozen in liquid nitrogen. Ten µm sections were cut and transferred to Indium Tin Oxide (ITO) coated glass slides. For Protein ID (Bruker Daltonics K.K.) experiments, trypsin and sinapinic acid as a matrix were sprayed using ImagePrep device (Bruker Daltonics K.K.). LC-MS/MS analysis was performed to the peptides extract from tissue samples. The MALDI-IMS data was obtained and analyzed using the FlexControl 3.0, FlexImaging 3.0 and the ClinProTools 2.2 software (Bruker Daltonics K.K.).

Results and Discussion

Earlier work has shown that marked periventricular accumulation of Dendritic Cells (DCs) and myelin-specific T cells during EAE disease onset prior to accumulation in the spinal cord, indicating that the choroid plexus-CSF axis is a CNS entry portal. Here we systematically visualized the distribution of proteins and peptides from CNS of the mice during EAE development. Using multivariate analysis, area specific proteomic pattern was delineated in wild type mice and aberrant distribution was noticed in EAE mice. Furthermore, we have succeeded in identification of proteins responsible for the diseased areas of spinal cords and or brains. We have tried to validate and compare MALDI-IMS based and immunohistochemistry-based figures for candidate peptides or proteins.

WP08-26

Conclusion

We have identified several proteins in which localization were perturbed with EAE pathogenesis. These results highlight MALDI-IMS as a versatile approach to elucidate molecular mechanisms of EAE and MS.

Keywords: Multiple Sclerosis, EAE, MALDI-IMS, brain, spinal cord

WO01-01

Proteome-wide drug dose-response of prostate cancer cell lines exposed to androgen receptor antagonists by microflow-LC SWATH MS analysis

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Introduction and Objectives

Many proteomic studies rely on the steady-state analysis of tissues or cell lines to identify markers of diseases or predict drug treatment outcome. In many instances, however, proteome changes upon drug exposure may be better suited to clarify in-cell drug action, identify off-targets or reveal mechanisms involved in drug resistance. Complete drug dose-response curves are however rarely carried out on a proteome-wide scale due to the prohibitively long acquisition times usually required by bottom-up proteomic approaches using liquid chromatography coupled to mass spectrometry. A method for fast and robust measurement of protein changes in a large scale would be instrumental to enable proteome-wide drug screening.

Methods

The microflow LC-MS data was acquired on a nanoLC™ 425 system coupled to a TripleTOF® 6600 system (SCIEX). Various microflow LC gradient setups were compared for data independent acquisition (DIA; SWATH® acquisition, 64 variable Q1 windows) strategies using 5ug of HEK human cell lysate. For the drug response experiment, the LNCaP prostate cancer cell line (sensitive to the androgen receptor antagonist Enzalutamide) and LNCaP-abl (resistant to Enzalutamide) were treated each with 10 different concentrations of drug and harvested at 5 different time points in duplicate, resulting in a 200 sample set. The peptide samples of LNCaP and LNCaP-abl were prepared by pressure cycling technology-assisted cell lysis and trypsin digestion and analyzed by microflow-LC SWATH.

Results and Discussion

Microflow-LC SWATH MS showed minimal attrition in peptide and protein identifications between 2 hours and 30 min gradients indicating that in DIA mode, even for the shortest run times, signals of multiple co-eluting peptides are still recorded with sufficient specificity to allow unambiguous peptide identification. The 45 min gradient setup enables exactly a one-hour run time injection-to-injection. The microflow-LC SWATH analysis of the LNCaP prostate cancer cell lines resulted in the identification of approximately 5'000 proteins consistently across all the time series and drug treatment conditions. The time-dimension of the dataset allows assessment of the cellular accessibility of the drug and the kinetics of drug action on the proteome scale. The drug concentration dimension allows the assessment of the level of sensitivity / reactivity of the proteins to the various drug concentrations.

Conclusion

Microflow liquid chromatography coupled to data independent acquisition enables rapid

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and robust proteome-wide dose-response drug profiling. The dose-response curves of several proteins showed significant IC50 difference between the sensitive LNCaP and the resistant LNCaP-abl cell lines, pinpointing potential cellular pathways involved in the drug resistance mechanisms of LNCaP-abl.

Keywords: Microflow LC, SWATH Acquisition, DIA

Profiling changes in the phosphoproteome of hematopoietic cells in response to a novel class of anti-oncogenic compounds.

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Introduction and Objectives

A set of recently developed small molecules based on sphingolipid structures impair nutrient transport pathways offering a novel and effective strategy to kill cancer cells by starving them to death (Chen et al., ACS Chem. Biol. 2015). Compound SH-BC-893 blocks nutrient access by: 1) disrupting intracellular trafficking of transporters for glucose and amino acids and 2) blocking lysosomal fusion reactions that produce cytoplasmic vacuolation. Both of these effects of SH-BC-893 on intracellular trafficking occur secondary to protein phosphatase 2A (PP2A) activation. The structurally related PP2A agonist C2-ceramide also down-regulates nutrient transporters but does not produce vacuolation. This result suggests that SH-BC-893 targets two distinct PP2A complexes, one that is also activated by ceramide and regulates transporter trafficking, and a second complex that is uniquely activated by SH-BC-893 that blocks lysosomal fusion reactions. To understand the mechanism by which PP2A activation affects these events, we profiled the temporal changes of protein phosphorylation in murine hematopoietic cells (FL5.12) following incubation with the PP2A agonist SH-BC-893, the specific PP2A inhibitor LB100, or C2-ceramide.

Methods

Dynamic phosphoproteomic experiments were performed using metabolic labeling (SILAC) over a period of 60 min with a time resolution of 5 min. Phosphopeptides were extracted using TiO2 media and fractionated on strong cation exchange stage tips prior to LC-MS/MS analysis on an Orbitrap Fusion. LC-MS/MS data were analyzed using Maxquant and results were processed in R (www.r-project.org).

Results and Discussion

Mass spectrometry-based phosphoproteomics quantified more than 14,000 phosphorylation sites, of which 647 sites on 460 proteins were dynamically regulated. The comparison of dynamic phosphorylation sites regulated by LB-100 and SH-BC-893 enabled the identification of 116 sites that showed opposite kinetic trends, thus representing putative PP2A substrates. In particular, these analyses identified known substrates such as KSR1, RPS6KB1, PRKCE and PRKAR1A, and facilitated the identification of unexpected substrates of PP2A including ARFGEF2, AGAP2, DOCK2, and CD2AP. Comparison of treatment with C2-ceramide or SH-BC-893 revealed 123 regulated phosphosites common to both stimulations such as GEFs (ARHGEF2), GTPases (AGAP2) and actin regulators (FNBP1 and FMNL1) that could regulate intracellular trafficking in response to changes in PP2A activity. Interestingly, our data also identified proteins involved in vesicular trafficking and endocytosis that are dynamically

regulated only upon treatment with SH-BC-893 and thus these putative PP2A substrates may regulate lysosomal fusion reactions.

Conclusion

Temporal profiling of protein phosphorylation in FL5.12 cells treated with different compounds known to affect PP2A activity enabled the correlation of cell signaling events with cellular phenotypes to rationalize their specific mechanism of action.

Keywords: Phosphoproteomics, Drug Discovery

WO01-03

Cancer proteomics towards precision medicine by molecular targeting drug

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Introduction and Objectives

Cancer is a genetically and clinically diverse disease, and the best optimized treatments for individual cancer patients have long been desired. The better clinical outcome based on the stratified treatments harnessing the molecular backgrounds of cancer are the goal of precision medicine. This goal is now realistic owing to the molecular targeting drugs. Cancer proteomics can provide valuable information which cannot be available otherwise. Many lines of evidence suggested that cancer proteomics has a potential to contribute to the clinical practice.

Methods

To explore the mechanisms of inherent resistant against molecular targeting drug and identified the innovative seeds for precision medicine, we investigated four layers of proteome. Using a set of sarcoma cell lines which showed the different response to the treatments with tyrosine kinase inhibitors, we investigated, 1) the global protein expression level by mass spectrometry, 2) the expression of all tyrosine kinases by antibody-based proteomics, 3) the activity of tyrosine kinases using global in-vitro kinase assay system, and 4) the amount of secreted proteins by antibody-conjugated beads technology. Moreover, we combined the proteome data with transcriptome one, using our original mRNA expression database, which includes the DNA microarray data of more than 1000 sarcoma patients.

Results and Discussion

We found the proteome signature for resistance to the treatments with tyrosine kinase inhibitors. We also found the overexpression of six tyrosine kinases associated with the drug resistance. Interestingly, the overexpressed tyrosine kinases didn't always provide apparent advantages to sarcoma cells, because the gene-silencing affected the cell growth for a limited type of tyrosine kinases. Moreover, the amount of tyrosine kinases didn't always correlate with their activity level, probably explaining the discordance of expression level and functional significances. The antibody-based technology identified the unique proteins which were highly secreted from the resistance sarcoma cells. Some of those proteins were more secreted after the treatments with tyrosine kinases. Those proteins are the candidates to predict and monitor the effects of treatments. Finally, we validated our results using the database of global mRNA expression database. We initially confirmed the concordance of protein and mRNA expression of the candidate genes, and the expression of mRNA was examined across the different types of sarcomas. The expression levels of candidate genes were diverse among the patients' samples, probably reflecting the different response to the treatments with tyrosine kinase inhibitors.

WO01-03

Conclusion

By investigating the four layers of proteome and combined the proteome data with transcriptome one, we approached to the molecular mechanisms of drug resistance. Our results strongly suggested the possible utility of cancer proteomics for the precision medicine using molecular targeting drugs.

Keywords: Cancer proteomicsPrecision medicine

WO01-04

Drug Development by Linking Pathophysiology in Cancer to Proteomics

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Introduction and Objectives

The Objective is to develop an in depth understanding of Protein Function in Disease, and Drug mechanisms.

Many of the modern approaches for studying disease compare steady state functions, such as repair, growth, and regulated gene expression within the various biological compartments organized by specialized function, be it mitochondria or blood vessels. The assignment of protein identities, which are linked to key biological mechanisms, which are associated with disease processes and disease progressions are an important area of this work. Today, the technology available for studying proteome expression and resolving exact protein and peptide identities in complex mixtures of biological samples allows global protein expression within cells, fluids, and tissue to be approached with confidence. The establishment of key representative reference proteome systems representing the dynamic changes in protein expression during disease will be vital to the interpretation of changes observed in specific samplings of disease states and specific cells obtained from these samples. Global Healthcare is under pressure to meet the demands from patients to reach an improved efficiency in Cancer diseases. Societies have difficulties in managing an increasing burden of healthcare costs. New drug characterization assays are central in providing evidence to the specificity and selectivity of drugs. Targeted drug development requires mode of drug action mechanistic studies in order to get approval from the FDA.

Methods

Protein deep-mining is performed by utilizing Orbitrap technology, linked to pathology grading of patient tissues, by using both manual histological evaluation, as well as automated machine learning technologies.

Results and Discussion

Examples will be given in drug development studies where protein expression patterns, and drug localization have been generated from compartments within tissue sections as well as clinical studies directed to mode-of-drug-action and disease

new thinking considering the diagnosis and treatment of disease, including “Precision Medicine”.

Conclusion

Drug efficacy and patient safety are key concerns in modern drug development where mandatory requirements on drug mechanisms are further developed. Disease presentation and clinical sample collection are key strategic resources that need to be invested in order to understand disease mechanisms and to design the next generation diagnostics and treatments for Cancer patients. Elucidating disease changes related to Proteoform status and drug interactions are key areas where modern drug industry will need further breakthrough.

Keywords: Pathology, protein function, disease correlation, cancer

WP09-01

A Study on the Intestinal Absorption and Function sofbioactive Peptide

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Introduction and Objectives

Producing peptides derived from milk has become a hot research area for its potential health benefits.To date, many research results have demonstrated that the milk-derived peptides containing fragments “Leu-Pro-Leu-Pro (LPLP)”, such as HPLPLP,HLPLPL, LHLPLP and so on, have various biological activities. In addition to the existing results, our team has observed that LPLP from probiotic fermented milk cannot be broken down by the gastrointestinal digestion method in the simulated experiments. According to these results, we speculate that LPLP acts as the core fragment in theses bioactive peptides. In previous studies, peptides with amino “Pro” play a vital role in immunomodulatory which is closely related to anti-aging function. Accordingly, this study focused on the absorbability and potential function of LPLP. The absorbability of LPLP was investigated using Caco-2 cell models and the potential functions were investigatedby D-galaging animal model.

Methods

This experiment is mainly divided into two parts. In the first part, the area of absorption peaks at each concentration is determined by high performance liquid chromatography (HPLC), and the standard curve of different concentrations corresponding to the absorption peak area of LPLP is drawn. After the successful establishment of the Caco-2 model, the apparent permeability (Papp) values of LPLP is calculated according to the standard curve calculated. In the second part, the anti-oxidation and potential anti-aging function of LPLP is examined in term of the organizational structure of the spleen, antioxidant biochemical indicator, and immune factors in D-gal aging animalmodel.

Results and Discussion

Firstly, the apparent permeability (Papp) values showed that LPLP underwent bidirectional transport, with slightly higher flux rates in the apical-to-basolateral direction (1.25 10-5 cm/s, AP→BL) than in the basolateral-to-apical direction (3.26 10-6 cm/s, BL→AP), indicating relatively high cellular-permeability of LPLP (Papp >1 × 10−5 cm/s). Then, the observing of spleen HE slice showed that the white pulp and red pulp structure is blurred of the spleens in the model group mice, suggesting a certain degree of atrophy of spleen. Meanwhile, the atrophy of spleen in administrated

group mice is lighter than that in model group,which was resemble that of blank group mice. These results indicated the anti-oxidation function of LPLP. The cytokines of D-gal aging animal model showed that LPLP could effectively alleviate the secretion of IL-6 (from 168.01pg/mL to 66.51pg/mL) and TNF-α (from 4.34 to2.08), but the concentration of IL-2 and IL-10 were not significantly changed according to statistical test. Furthermore, the increase of SOD and T-AOC, and the decrease of MDA in D-gal aging mice indices showed that LPLP had a protection effect on liver and kidney,indicating the anti-aging function of LPLP.

Conclusions

Our experiments indicate that LPLP was likely to be directly absorbed by small intestine epithelium mucosa in the intact, and from the part of the organizational structure,biochemical indicators,and the immunity,LPLP has aprotective effect in D-gal agingmice. However, additional transport model in vivo and natural aging animal model researches are needed to confirm these results in order to suggest the use of peptide LPLP as functional factor for medical purposes.

Keywords: Peptide,LPLP,Intestinal Absorption,Functions,Anti-oxidation

Phamacoproteomics reveals the pan-Aurora kinase inhibitor tozasertib as a potential therapeutic drug for Mycn-amplified neuroblastoma

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Introduction and Objectives

Neuroblastoma is the most common extracranial solid tumor of childhood. Clinical outcome remains poor in patients with high-risk neuroblastoma; therefore, novel treatment approaches are required. Repositioning existing drugs for new indications could deliver the productivity increases and reduce development risk because they have well-known safety and pharmacokinetic profiles. Our objective is to discover new therapeutic indications for neuroblastoma and to elucidate the molecular mechanism of the drugs.

Methods

We have been developing an expression-based similarity framework to compare a large number of drug perturbation profiles in Library of Integrated Network-based Cellular Signatures (LINCS), a project as the second-generation Connectivity Map initiated by National Institute of Health (NIH). Furthermore, we used MTS assay to measure the cell proliferation rate in Mycn-amplified neuroblastoma after treatment with drugs. An animal model of transgenic mice or xenograft mouse with SK-N-DZ cells was treated with 100mg/kg/day by intraperitoneal injection for five consecutive days. The molecular mechanism of the drug (tozasertib) on the tumor tissues of transgenic mice and xenograft mode was analyzed by iTRAQ labeling and LC-MS/MS.

Results and Discussion

Conclusion

This study indicates the potential therapeutic value and the molecular mechanism of tozasertib in high-risk Mycn-amplified neuroblastoma.

Keywords: High-risk; Mycn-amplified; neuroblastoma; tozasertib; drug repurposing; proteomics; iTRAQ.

WP09-03

The Discovery and Potential Application of Bioactive Peptides from Fermented Milk and Probiotics

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Introduction and Objectives

Up to now, many research results indicated that one single or few bioactive peptide fragments from fermented dairy products have low biological activities and limited functions. Such conclusion is not comprehensive and lack of systematic comparison. The aim of this research project in the past 10 years was using the Lactobacillus delbrueckii SSP. Bulgaricus LB340 to ferment skim milk (12%, v/v) under the fixed fermentation conditions relatively, and applying UPLC-Q-TOF-MS-MS to scan peptides totally intracellular and extracellular that is in the fermented milk, as well as investigating the amount and type of bioactive peptides from fermented milk and probiotic with their potential application value.

Methods

For preliminary study, quantity of intracellular and extracellular peptide mass spectrum trials were conducted. Firstlly, we applied our own computer program to compare mass spectrum data and known amino acid sequence of milk protein. Then we used Biolynx based on the secondary mass spectrogram to find peptides derived from milk protein. Secondly, using the same analyzing method to find peptides derived from the protein of Lactobacillus delbrueckii. Finally, by conducting in vitro and vivo bioactive test to the intracellular and extracellular peptides.

Results and Discussion

First, we obtianed possible peptide sequences for each mass spectrum data between 200Da and 2000Da, which was the target peptide range in this research project, then we used Biolynx based on the secondary mass spectrogram, and found 119 peptides derived from milk protein. Second, using the same analyzing method, we found more than 300 peptides derived from the protein of Lactobacillus delbrueckii, which was obtained based on the results of genetic analysis. The comparision result showed the analysis results of intracellular and extracellular peptides,which indicaed some peptides share a same segment or the same amino acid sequence. Finally, the current available results of bioactive test in vitro and vivo showed the intracellular and extracellular peptides with a same segment have the good biological activities, such as antioxidant, scavenging free radicals, immunoregulatory and anti-aging functions and can be applied as a new peptide drugs.

Conclusion

The bioactive peptide mass spectrum database from fermented milk and Lactobacillus delbrueckii is like a gold mine, there are many bioactive peptides related to human health waiting for us to find. So far, we have found 119 peptides derived from milk protein as

extracellular peptides and more than 300 peptides inside the cell of Lactobacillus delbrueckii as intracellular peptides. Some intracellular and extracellular peptides have a same segment or the same amino acid sequence and very good biological activities like antioxidant, scavenging free radicals, immunoregulatory and anti-aging function. Therefore, those intracellular and extracellular peptides have high application value as a potential new peptide drug.

Keywords: Bioactive Peptide; Fermented milk; Probiotic

Corosolic Acid, the Active Principle of Actinide chinensis, Inhibits Hepatocellular Carcinoma by Targeting the VEGFR2/Src/FAK Pathway

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Introduction and Objectives

Hepatocellular carcinoma (HCC) is one of the most commonly occurring cancer and the most common cause of death from cancer., and the inhibition of VEGFR2 activity has been proposed as an important strategy for the clinical treatment of HCC. In this study, we identified corosolic acid (CA), isolated from the root of Actinidia chinensis , as possessing an anti-cancer effect on HCC cells.

Methods

We used in vitro model to assess the effects of CA on HCC cell migration/invasion and study the mechanisms underlying. The molecular docking was used to examine the interaction between CA and VEGFR2. Tumor xenograft analysis was performed to evaluate the efficacy of CA in vivo. The synergistic analysis was performed to study the synergism between CA and the multi-kinase inhibitor Sorafenib.

Results and Discussion

We found that CA inhibits VEGFR2 kinase activity by directly interacting with the ATP binding pocket. CA down-regulates the VEGFR2/Src/FAK/cdc42 axis, subsequently decreasing F-actin formation and migratory activity in vitro. In a xenograft mouse model, CA exhibited an effective dose (5 mg/kg/day) on tumor growth. We further demonstrated that CA has a synergistic effect with sorafenib within a wide range of concentrations.

Conclusion

This research elucidates the effects and molecular mechanism for CA on HCC cells and suggests that CA could be a therapeutic or adjuvant strategy for patients with aggressive HCC.

Keywords: hepatocellular carcinoma, corosolic acid, AKT, mTOR, VEGFR2, mouse xenograft model

Deciphering the molecular insights of phytoagent deoxyelephantopin against triple negative breast cancer using LC-MS based quantitative proteomics

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Introduction and Objectives

Breast cancer is one of the frequently diagnosed and leading causes of cancer death in women. Especially, the triple negative breast cancer (ER-/PR-/HER2-, TNBC) is a clinically challenging breast cancer subgroup due to lack of efficient targeted therapeutics. Recently, the medicinal plant-derived phytocompounds as chemotherapeutic or chemopreventive agents for treatment or prevention of human cancers have attracted great attentions and investigations. In our previous study, deoxyelephantopin (DET), a major germacranolide sesquiterpene lactone isolated from a traditional medicinal herb Elephantopus scaber L., has shown a markedly suppressive effect on ER(+) TS/A breast cancer by inhibiting cancer cell motility and metastasis in vitro and in vivo; however, less cytotoxic activity was found in human TNBC cell line, MDA-MB-231. We thus designed and created a semi-synthesized DET derivative (DETD) which showed a better activity than DET on MDA-MB-231 cell proliferation.

Methods

In this study, we aim to decipher the molecular insights of DET and DETD against TNBC using LC-MS based quantitative proteomics. We have designed a DET-near infrared fluorescent compound conjugate (DET-NIR) to study the direct protein target(s) in the cancer cells.

Results and Discussion

Of noted, direct binding assay couple with proteomics analysis, we observed that DET-NIR bound with nuclear exosomal proteins of mouse TNBC 4T1 cells. Furthermore, immunofluorescence cell staining of DET-NIR treated MDA-MB-231 cells found that DET-NIR and exosomes were colocalization. Both DET and DETD compounds can also significantly enhance the release of exosomes from MDA-MB-231 cells into media compared to the vehicle control. Further, quantitative proteomics investigation of the exosomes released after compound treatment showed that DET and DETD responsive exosomal proteins were participated in regulation of several signaling pathways, e,g., EIF2, mTOR, and CDK5 cascades, and inhibition of angiogenesis.

Conclusion

Together, this study provides a novel mechanistic insight of DET and DETD against

WP09-05

TNBC activity, suggesting the potential therapeutic candidates by modulation of specific exosome proteins in cancer cells.

Keywords: breast cancer, deoxyelephantopin, exosomes

Identification of Novel HLA-A2 Restricted Phosphopeptide for Cancer Vaccine Development

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Introduction and Objectives

Immunogenic MHC class I-bound peptides have been considered as potential target for cancer immunotherapeutic vaccine development.

Methods

In this study, we used W6/32 Ab-immobilized column to isolate HLA 02:01-peptide complex from lung cancer cell, and identify the peptide sequence by MS. To increase the peptide identity, TiO2 column was applied to enrich the phosphopeptide from extracted peptide pool. Both the flow through and elution fractions were collected for MS analysis.

Results and Discussion

The results indicated that there are 63 MHC-bound peptides identified from the immunoprecipiated MHC complex, and twelve of them are selected as potential candidates since they are derived from tumor-associated antigens. It is noteworthy that phosphor group of MS-identified phosphopeptide seems to enhance the binding affinity with HLA*0201 based and effectively stimulated cytotoxic T lymphocyte (CTL) response in mice. Moreover, such phosphopeptide can inhibit the tumor growth in HLA transgenic mice as well as known CTL epitope.

Conclusion

These findings demonstrated that MS-based immunoproteomics is an effective analysis platform to screen out the potential epitope, which facilitate the development of therapeutic vaccine against cancer disease

Keywords: MHC class I, phopsphopeptide, immunoproteomics, vaccine, cancer

WP09-07

Cellular Thermal Shift Assay (CETSA) enables identification of drug targets of natural products

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Introduction and Objectives

Drug target and off-target identification still remains a big challenge in the pharmaceutical field, especially inside a cellular environment. Cellular Thermal Shift Assay (CETSA) has been recent proposed for this purpose. CETSA is based on the idea that a drug-protein interaction may stabilize or destabilize a protein. Up on thermal treatment and precipitation, the proteins still left in solution can then be quantified. This is used as a proxy to surrogate the drug effect. Natural products and secondary metabolites represent a huge under-exploited resource for drug development. We plan to apply the CETSA approach to elucidate the mechanism of action for some of these compounds, taking advantage of the sensitivity and throughput of the former. The platform will allow us to systematically identify drug-protein interactions, to learn about novel drug targets and off-targets, and help repurposing drugs.

Methods

Cancer cell lines were cultured according to standard protocols. Cell viability assays with different drugs were performed to learn the IC50. Relatively high dose of drugs (1× - 100×) were administered to cultured cells for a defined amount of time and heated to a range of temperatures. Cells were then lysed by freeze-thaw cycles and the lysates were clarified by ultracentrifugation. The proteins in the supernatant were then digested with trypsin, labeled with TMT reagents, fractionated and analyzed by LC-MS/MS. The resulting data were then searched with MaxQuant and melting curves for individual proteins were generated and compared to control.

Results and Discussion

From hydroxide-selective anion exchange (hSAX) fractioning and TMT-10plex labeling, over 7,000 proteins could be consistently quantified across all CETSA experiments. The fractionation partially alleviates the TMT ratio compression problem and alternative higher resolution fractionation may further improve this. MS3 based approaches are also quite effective in relieving ratio compression. Compared to the same experiments performed on cell extracts, the protein melting temperatures in cellular environment were lower, presumably because of much higher protein concentration in cells. Cases of false negative also exist, when the drug binding only affects a small part of a big protein, or a minor conformational isomer.

Conclusion

CETSA combined with MS readout is a powerful tool to interrogate drug-protein interactions. The high throughput, sensitivity and comprehensiveness afforded by multiplexing MS enables an unbiased drug target and off-target identification. Not needing

to immobilize or tag drugs in some manner or another, and the ability to work with cells make this approach also highly biologically relevant.

Keywords: CETSA, Chemical proteomics, drug target identification, cancer, TMT

WO06-01

Target Identification in Living Cells via Mass Spectrometry-based Chemical Proteomics

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Introduction and Objectives

Target identification is important but challenging in drug discovery. The usual practice involves affinity chromatography, where the drug compound is used as ligands to probe cell lysates, and integration with quantitative SILAC-based mass spectrometry (MS) strategy enables target identification on a proteome-wide scale. However, such affinity-based methods are limited by the necessity to modify each drug compound individually but without loss of bioactivity resulting from addition of functional moieties. Differences in stability between drug-bound and free proteins form the basis of new strategies – a protein complexed to a ligand is generally more resistant against proteolysis, oxidation, or detergent- and heat-induced unfolding. Assays such as drug affinity responsive target stability (DARTS), stability of proteins from rates of oxidation (SPROX) and cellular thermal shift assay (CETSA) are used to detect such differences. The above strategies share a common drawback; they are all in vitro assays limited to analysis of soluble protein targets, thus often resulting in misleading target identification. Therefore, chemical proteomics, which rely on small molecules that can covalently bind their intracellular protein targets in vivo, offers an alternative method for target identification in a living cell.In addition to discussing the different approaches to drug target identification, I will share several applications of chemical proteomics for target identification of fluorescent probes.

Methods

Highly sensitive and selective small molecule fluorescent probes: CDy1, CDy2, CDy5, were developed for imaging pluripotent stem cell, smooth muscle differentiation, and cell division in neurosphere respectively. For target investigation of these probes in the living cell, chemical-affinity probes were synthesized with a thiol-reactive chloroacetyl group to enable covalent binding to target proteins in vivo. For in vitro comparison, CDy2 probe was immobilized on affinity matrix for traditional affinity competition assay. Probes-bound proteins were isolated and separated by gel electrophoresis and identified by MS.

Results and Discussion

The in vivo targets were identified as mitochondrial aldehyde dehydrogenase for CDy1 and CDy2, and acid ceramidase for CDy5. However, CDy2 targeted tubulin in vitro, thus demonstrating the importance of using an in vivo approach for the identification of a biological target. Intriguingly, CDy5 was subsequently found to be a derivative of CDy1 and both probes bound the same or two different target proteins depending on the cell type, thus implying that a small molecule drug may bind different protein targets in different cells or under different cellular microenvironment.

Conclusion

WO06-01

Our results demonstrated the complexities of chemical drug targets identification and the potential for off-target side effects, both of which could contribute to chemical drug development failure.

Keywords: Target identification, imaging probe, living cells, chemical proteomics

WO06-02

Comprehensive mass spectrometry analysis identifies a novel therapeutic target in the Wnt signaling pathway

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Introduction and Objectives

The great majority of colorectal cancers carry somatic mutations in one of two genes involved in the canonical Wnt/β-catenin signaling pathway: the adenomatous polyposis coli (APC) and β-catenin (CTNNB1) genes. Either type of genetic alteration results in accumulation of β-catenin and mimics the activation of Wnt signaling. Numerous attempts have therefore been made to develop therapeutics targeting the Wnt/β-catenin pathway. In colorectal cancer, however, due to the genetic inactivation of APC, only the molecules downstream of APC can be considered as therapeutic targets. We have therefore been searching for druggable target molecules downstream of APC, especially in the nucleus.

Methods

Proteins of two colorectal cancer cell lines, HCT-116 and DLD1, immunoprecipitated with anti-TCF-4 antibody were analyzed directly by nano-flow liquid chromatography (LC) and tandem mass spectrometry (MS/MS). The redundant LC-MS/MS data were compiled using in-house software.

Results and Discussion

Seventy proteins were found to be constantly immunoprecipitated with anti-TCF-4 antibody in four independent experiments (two experiments using HCT-116 and two experiments using DLD1). These proteins included poly(ADP-ribose) synthetase/polymerase (PARP-1), thyroid-lupus autoantigen p70 (Ku70), DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 (DDX5), desmoplakin, heterogeneous nuclear ribonucleoproteins (hnRNPs) A2/B1 and M, and Traf2- and Nck-interacting kinase (TNIK). TNIK phosphorylated the conserved serine 154 residue of TCF4. Small interfering RNA (siRNA) targeting TNIK inhibited the proliferation of colorectal cancer cells and the growth of tumors produced by injecting colorectal cancer cells subcutaneously into immunodeficient mice. The growth inhibition was abolished by restoring the catalytic domain of TNIK, thus confirming that its enzyme activity is essential for the maintenance of colorectal cancer growth. Several therapeutics targeting various molecular components of the Wnt signaling pathway, including porcupine, frizzled receptors and co-receptor, tankyrases, and cAMP response element binding protein (CREB)-binding protein (CBP), have been developed, and some of those are currently being evaluated in early-phase clinical trials (Sawa et al., Expert Opin Ther Targets., 20:419, 2016). TNIK regulates Wnt signaling in the most

downstream part of the pathway, and its inhibition is expected to block the signal even in colorectal cancer cells with APC gene mutation (Masuda et al., Pharm. Ther., in press). We discuss a TNIK inhibitor under preclinical development (Masuda et al., Nature Commun., in revision).

Conclusion

TNIK was essential for the activity of Wnt signaling and the proliferation of colorectal cancer cells. Several ATP-competing kinase inhibitors have been applied to cancer treatment and have shown significant activity. TNIK is considered to be a feasible target of pharmacological intervention for manipulation of the aberrant Wnt signaling pathway in colorectal cancer.

Keywords: Wnt signaling, colorectal cancer

WO06-03

The Phosphoproteome of the NCI-60 Cell Line Panel Reveals Markers of Drug Sensitivity

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Introduction and Objectives

The identification of molecular markers of drug treatment response remains a major challenge. Many cancer cells are driven by de-regulated kinase pathways which have proven to be effective therapeutic targets. To investigate the hypothesis that the signaling/activation status of a cell allows for the prediction of drug sensitivity or resistance, we determined the global phosphoproteomes and proteomes of the NCI 60 cancer cell line panel by quantitative mass spectrometry. The NCI-60 panel consists of 60 tumor cell lines from nine cancer types and has been extensively characterized on the molecular and pharmacological level.

Methods

We used reproducible and comprehensive Fe-IMAC chromatography to purify phosphopeptides out of complex digests and high pH reversed-phase fractionation in a tip format to increase the analytical depth of the phosphoproteome and the proteome. A (phospho)peptide spike-in was employed as an efficient means for quality control. Elastic Net and outlier analysis are applied to relate (phospho)proteome and drug-response profiles.

Results and Discussion

The data obtained covers >70,000 phosphopeptides (~25,000 phosphopeptides per cell line) and >12,000 proteins (~8,500 proteins per cell line) representing most of the major signaling pathways in cancer cells. New kinase-substrate relationship were discovered by integrating the phospho-proteomics and kinome data using correlation and RLQ analysis. The analysis of phosphopeptide abundance and drug response for more than 600 cancer drugs revealed known and novel markers of drug efficacy, some of which were not apparent from proteomic or transcriptomic data, underscoring the irreplaceable role of phospho-proteomics in understanding drug response. In addition, multi-omics data integration increases predictive power and enables a systems level view on mechanisms of drug sensitivity and resistance. We implemented a shiny application to facilitate the data sharing and visualization.

Conclusion

Quantitative phosphoproteomic analysis of a large number of cell lines provides a unique perspective on biomarkers of pharmacological response. We used existing and new

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computational methods to extract biological knowledge from the large-scale multi-dimensional data.

Keywords: phosphorylation, cancer, phosphoproteomics, drug response, multiple omics, omics data analysis, NCI-60

WO06-04

Targeted proteomic profiling of enzymes that activate the prodrug PR-104A in human leukaemias

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Introduction and Objectives

The bioreductive prodrug PR-104A, developed in the University of Auckland, is activated by reductases that are selectively active in cancer cells. In most cases, this selectivity is due to hypoxia, which permits PR-104A reduction by a class of reductases that are silent in well-oxygenated tissues. The best studied of these hypoxic reductases is NADPH: cytochrome P450 oxidoreductase (POR). However, PR-104A is also activated by an aerobic reductase, aldo-keto reductase 1C3 (AKR1C3). High expression of AKR1C3, and/or hypoxia, may underlie the recently-reported activity of PR-104A in a phase I/II clinical trial in refractory/relapsed acute leukaemias (Konopleva et al., Haematologica 2015). However, this activity is highly variable between patients. Thus further clinical is critically dependent on quantitation of predictive biomarkers in individual leukaemias. In this study, we aim to evaluate whether expression of AKR1C3 and POR can predict aerobic and hypoxia PR-104A metabolism in leukaemia cell lines and primary patient samples.

Methods

Protein expression of POR and AKR1C3 were determined in a panel of 5 cell lines (HCT116, its AKR1C3 and POR-overexpressing transductants, TF1 and REH) by targeted proteomics using an Agilent 6460 triple quadrupole mass spectrometer. Expression of 7 housekeeping protein candidates (ACTB, TUBB5, B2M, TBP, PPIA, HPRT1, RPL13A, RPLP0, UBC and GAPDH) was evaluated in cells grown under different O2 concentrations for 72 hr. Functional assay for AKR1C3 in cells was carried out using the fluorogenic probe coumberone (a substrate for all AKR1C isoforms) in conjunction with a specific inhibitor of AKR1C3, SN34037. Metabolism of PR-104A under oxic and anoxic conditions was evaluated by quantifying intracellular reduced metabolites by LC-MS/MS.

Results and Discussion

Proteotypic peptides were selected for POR (NPFLAAVTTNR and FAVFGLGNK), AKR1C3 (AIDGLDR and ALEVTK) and the whole AKR1C family (TPALIALR and LAIEAGFR) after method optimisation and validation. Expression of the housekeeping proteins in leukaemia cell lines TF1 and REH was not significantly affected by hypoxia (1 and 0.2% O2 vs 20%). Anoxic metabolism of PR-104A was strongly inhibited by a flavoenzyme inhibitor diphenyliodonium in all cell lines, while SN34037 inhibited oxic metabolism only in high AKR1C3 cell lines (HCT116/AKR1C3 and TF1). Preliminary results also showed that AKR1C3 expression was consistent with the functional enzyme assay and positively correlated with PR-104A metabolism under oxia, while POR expression correlated with PR-104A metabolism under anoxia.

WO06-04

Conclusion

Expression of AKR1C3 and POR proteins seems to predict PR-104A metabolism. However, this needs to be validated on a wider leukaemia panel and with patient samples.

Keywords: Targeted proteomics, AKR1C3, Reductases, Hypoxia-activated prodrug, PR-104, Leukaemia

WO06-05

Identification of TIFA as a novel therapeutic target in acute myeloid leukemia

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Introduction and Objectives

Aurora A-dependent NF-κB signaling portends poor prognosis of cancers including acute myeloid leukemia (AML). Our previous study demonstrated that phosphorylation-dependent oligomerization of TIFA protein triggers the activation of NF-κB.

Methods

In vitro interplays between TIFA and Aurora A were determined. Effects of TIFA silencing on Aurora A-dependent NF-κB signaling factors and the enhancement of chemosensitivity were next analyzed in PBMCs from AML patients and normal donors. The correlation between the bone marrow TIFA expression and prognostic features in AML patients was then established. In vitro chemosensitivities, secretions of leukemic cytokines, and the in vivo clearance of myeloblasts under dominant-negative inhibition of TIFA were also investigated.

Results and Discussion

We identified that Aurora A is an essential kinase for the Threonine 9 phosphorylation of TIFA, and that TIFA functionally mediates the Aurora A-dependent NF-κB survival pathway in AML. Overexpression of TIFA occurs concurrently with Aurora A and NF-κB signaling factors in PBMCs from de novo AML patients in contrast to healthy individuals, and statistically correlates with the poor prognosis group of patients. Silencing of TIFA specifically attenuates leukemic cell growth and enhances chemosensitivity of AML cells through downregulation of NF-κB-dependent anti-apoptotic events. In addition, molecular targeting of TIFA perturbs leukemic cytokine secretion, significantly lowers IC50 of chemo drug treatment in AML cells, and efficacizes clearance of myeloblasts in the bone marrow of xenograft-recipient mice through improved chemotoxicity.

Conclusion

These results demonstrate a supporting role of TIFA in the leukemogenesis of AML and affirm its therapeutic potential in the treatment of AML.

Keywords: AML; NF-κB; Aurora A; TIFA; therapeutic target

WP10-01

Drug Target Identification in Multiple Myeloma by Chemical Proteomics

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Introduction and Objectives

In Multiple myeloma (MM), abnormal B lymphocytes accumulate in the bone marrow, leading to osteolytic bone destruction, impaired hematopoiesis and progressive renal failure. MM is largely incurable due to the development of resistance against effective therapeutic agents and most patients succumb to their disease.A promising treatment target is the heat shock response (HSR), which is a highly conserved rescue mechanism and it is important for multiple myeloma cells to survive drug treatment. Key protein of this salvage mechanism is the heat shock factor 1 (HSF1). It regulates the expression of heat shock proteins like HSP72 which are crucial for the folding of oncogenic client proteins. Inhibition of the HSR results in reduced cell survival and seems to be a groundbreaking strategy for new therapies in multiple myeloma.Here we are aiming at identifying potential molecular targets of α-acyl aminocarboxamides using affinity-based chemoproteomics.

Methods

Streptavidin magnet beads, biotinylated α-acyl aminocarboxamide and protein lysates from MM cells were used for the drug target enrichment. Inhibitor and control beads were prepared by incubating the streptavidin magnet beads with the biotinylated inhibitor or DMSO. Affinity purification of cell lysates from INA6 (MM model) cells with the prepared beads was done for 3h or 24h at 4 °C. Captured proteins were eluted and separated with SDS-PAGE. Each lane was cut into 15 bands, digested with trypsin and analyzed by nanoLC-MS/MS on an Orbitrap Fusion or Velos (Thermo) using a HCD TOP5 method. MS data from 5 biological replicates were processed with MaxQuant. To calculate the ratio between control and enrichment we used the MaxQuant LFQ.

Results and Discussion

In this study we used several α-acyl aminocarboxamides, which caused reduced levels of HSP72 in multiple myeloma cells after triggering the HSR with the HSP90 inhibitor AUY922. We optimized the enrichment workflow to identify 88 proteins which bind exclusively to the inhibitor beads. Among the enriched proteins we found the DNA-dependent protein kinase (DNA-PK) which is a known interaction partner of

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HFS1. In addition, we also enriched the structurally related kinases ATM, ATR and mTOR which belong to the phosphoinositide 3-kinase related kinases (PIKKs). DNA-PK, ATM and ATR are involved in the DNA Damage Response (DDR) where they also interact with each other.Furthermore, we analyzed our drug targets for domain similarities. We could identify 13 proteins which contain the Armadillo Domain (AD). The AD is a right-handed super helix which is important for protein-protein interaction. Among these proteins are the PIKK's, phosphatases, transport proteins, kinase activators and proteins of the DDR. On the basis of these results we suggest, that the α-acyl aminocarboxamides bind to the AD and prevent protein-protein interaction.

Conclusion

Chemical proteomics is a promising tool to identify drug targets from new inhibitors by using MaxQuant LFQ.

Keywords: Chemical Proteomics, Multiple Myeloma, Drug targets

WP10-02

Matrix Assisted Laser Desorption Ionization-Mass Spectrometry Imaging based analysis of distribution of anti-tumor agents on tissue

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Introduction and Objectives

In previous study, we characterized the effect of APZ and CUR in tumor cells. APZ is a promising anti-tumor agent to induce defective autophagy by binding protein targets related to lysosome function. CUR is an anti-tumor natural product to inhibit angiogenesis by binding APN (Aminopeptidase N). To validate APZ and CUR binding to protein targets in vivo, MALDI-MSI (Matrix Assisted Laser Desorption Ionization-Mass Spectrometry Imaging) is applied as one of the target validation methods.

Methods

In vivo tumor model and treatments The mouse colon adenocarcinoma cell line U87MG was cultured in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C, 5% CO2 in a humidified atmosphere. Balb/C mice from our colony were inoculated subcutaneously with $5x10^6$ U87MG cells. Treatment of APZ and CUR began 2 weeks after tumor cell injection and was performed 10 times during 20 days at a dose of 5 and 10 mg/kg respectively. Three hours after the last treatment, Tumors, livers and kidneys were removed and snap frozen by submerging the tissues into liquid nitrogen Detection of the compound and analysis of drug distribution A MALDI LTQ Orbitrap XL mass spectrometer was used both for compound characterization, drug detection in tissue. For compound characterization, drug was dissolved in DMSO 1mg/mL stock concentration. As matrix 7.5 mg/mL α-cyano-4-hydroxycinnamic acid was dissolved in 50% acetonitrile (ACN) at hypergrade for LC-MS and 50% water, containing 0.1% trifluoroacetic acid. For tissue drug imaging, 10-µm frozen sections were cut using a cryotome and placed on glass slide. Tissue sections were sampled in the 150−800 Da mass range in positive mode with 50 µm raster size. Analysis of the spectra was implemented with Xcalibur v 2.0.7. software.

Results and Discussion

We made an U87MG xenograft mouse model and injected the mouse with APZ (5 mg/kg) and CUR (10 mg/kg) 10 times during 20 days. In this period, APZ and CUR

reduced tumor size without toxic effects. After 3 hours from last injection, U87MG xenograft mouse was sacrificed and tumor, liver and kidney were obtained. Tissue sections were prepared by microtome and attached it onto glass slides. CHCA (α-Cyano-4-hydroxycinnamic acid), as a matrix, was sprayed onto tissue section. The distribution of compound in the tissue was measured by MALDI-MSI. The interaction of protein targets with the compound on the tissue by IHC (immunohistochemistry) will be presented.

Conclusion

To the best of our knowledge, this study provides the first direct evidence that APZ and CUR is localized on the targeted proteins, in the U87MG tumor. Moreover, the presented results are the first demonstrating that MALDI-MSI is a versatile and simple method to examine drug pharmacokinetic attributes, such as ADME. Hence, the current study warrants further investigations to define the precise and optimal role of MALDI-MSI in elucidating the mechanisms of drug action and for validating transport to sites of intended effect.

Keywords: MALDI-MSI, CUR, APZ, Xenograft

Comparative Proteomic Analysis Reveals the Molecular Mechanisms of Mandibular Glands in Two Lines of Honeybees (Apis mellifera ligustica)

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Introduction and Objectives

The mandibular glands (MGs) of honeybee workers are vital for secretions of larval nutrition and pheromones for a colony. An understanding of the molecular mechanisms of MGs, however, is still lacking.

Methods

We employed a mass-spectrometry-based approach to characterize the proteome during the MG development in the Italian bees (ITB) and the high royal jelly producing bees (RJB, a line bred for increased royal jelly production) at different ages.

Results and Discussion

More than 4000 proteins were identified in the MGs of the two honeybee lines over three time-points. We found that >2000 proteins were shared during MG development and they were enriched to the functional classes and pathways of metabolizing protein, organic substance, nucleic acid, small molecule, fatty acid, carbohydrate, energy and substance transport. This indicates the centrality of the shared proteins in regulating MG development and supporting the basic configuration and fundamental functions in honeybees. However, the different aged MGs show that each unique proteome signatures fit with each distinct physiology. For the newly emerged bees, the enriched functional groups and pathways related to protein synthesis, cytoskeleton, development and morphogenesis by the uniquely and highly expressed proteins, signify their roles for initializing the development of the young MGs. At the nurse stage, specifically expressed proteins were enriched to substance transport and the enhanced expression of proteins were associated with fatty acid synthesis and transport, illustrating that the proteome at this stage functions in order to prime high secretory activity of the MGs, thus providing fatty acid nutrition for the brood. For the forager bees, the uniquely expressed proteins were enriched to lipid metabolism and the elevated expressed proteins were implicated in small molecule and carbohydrate metabolisms, indicating their essential roles in biosynthesis of 2-heptanone (alarm pheromone) to enhance the efficiency of bees defending the colony and foraging by acting as a short-term scent marker to prevent the probing of flowers that have recently been foraged. Due to selection for increased RJ output, the enhanced biosynthesis of fatty acids and minimized degradative activity in nurse RJB bees suggests that it may adapt a strategy to increase synthesis of 10-hydroxy-2-decenoic acid (10-HDA), a major component of royal jelly, to maintain a rational portion of the fatty acids alongside enhanced royal jelly production.

Conclusion

Our first comprehensive proteome data garner novel mechanistic insights into

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understanding the development and functionality of honeybee MGs, and offer a trustworthy basis for future studies of honeybees and other insects.

Keywords: Proteome, Mandibular glands, Honeybee workers, 10-HDA concentration

Anti-cancer Gold(III) Porphyrins Target Mitochondrial Chaperone Hsp60

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Introduction and Objectives

The distinct structural properties and reactivity conferred by metal-ligand coordination offer appealing possibilities in the design of anti-cancer therapeutics based on metal coordination chemistry. However, identification of the direct molecular targets accounting for the anti-cancer action of metal complexes has always been a formidable challenge as most of the anti-cancer metal complexes are unstable toward ligand exchange reactions or biological reduction under physiological conditions. In recent years, a number of gold(III) complexes supported by strong donor ligands have been reported to possess good stability and display potent anti-cancer activities, examples of which are a series of anti-cancer gold(III)-porphyrins. Among them, gold(III) meso-tetraphenylporphyrin (gold-1a) is notable for its high stability in biological milieus, potent in vitro and in vivo anti-cancer activities. The outstanding stability of gold-1a under various biological conditions makes it possible to probe the molecular targets by using chemical biology approaches such as affinity-based proteome profiling.

Methods

In this study, affinity-based proteome profiling was used to isolate the protein binding partner of gold-1a. A trifunctional clickable photo-affinity probe of gold-1a was synthesized by appending a clickable tag (alkyne) and a photo-affinity tag (benzophenone) onto one of the meso-phenyl rings of the porphyrin ligand of gold-1a. Extensive chemical biology approaches employing cellular thermal shift, saturation-transfer difference NMR, protein fluorescence quenching and protein chaperone assays were used to examine the identified target.

Results and Discussion

The affinity-based proteome profiling together with various supporting biochemical and cellular experiments revealed heat shock protein 60 (Hsp60) to be an important molecular target of gold-1a. Hsp60 is an essential chaperone for mitochondrial protein transport and folding, playing important roles in apoptosis regulation and tumor maintenance, and up-regulated in primary human cancerous tumors. Gold(III)-porphyrin complexes display high binding activities to Hsp60 and strong inhibitory effects on the chaperone activity of Hsp60. Structure-activity studies with a panel of non-porphyrin gold(III) complexes and other metalloporphyrins revealed that Hsp60 inhibition is specifically dependent on both gold(III) ion and porphyrin ligand.

Conclusion

The present study features the identification of a direct molecular target of stable anti-cancer metal complexes by the combination of a photo-affinity and click chemistry approach. The mitochondrial chaperone Hsp60 as a direct molecular target of gold(III)-porphyrins was supported by multiple, independent assays. This finding broadens

WP10-04

the scope of investigating the mechanisms of action of bioactive metal complexes other than cisplatin and its derivatives that target DNA.

Keywords: chemical proteomics; gold(III) porphyrins; molecular target; Hsp60

WP10-05

A novel platform for target identification of a natural small molecule and its target protein interaction on tissues using MALDI-MSI analysis

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Introduction and Objectives

The objective of our research is to discover new bioactive small molecules with cell-based screening and mode of action study. Furthermore, we performed the target validation for demonstrating the drug-target interaction *in vivo* level using MALDI-MSI (MALDI-mass spectrometry imaging) and IF (immunofluorescence) staining. This approach will provide new information on elucidating the pharmacokinetic and pharmacodynamic properties of bioactive small molecule and its target protein on xenografted tumor tissues.

Methods

Endothelial cell-based screening assay with 300 natural plants library, viability assay (chemotoxicity test), *in vitro* angiogenesis assay (tube formation assay, chemo-invasion assay), *in vivo* CAM(chicken chorioallantoic membrane) assay, Western blotting (HIF-1α level, various phospho-RTK, VR receptor, ERK, Akt), hVEGF ELISA, tumor cytokine invasion assay, DARTS (Drug Affinity Responsive Target Stability) assay, MALDI-MSI (Thermo Fisher Scientific, MALDI LTQ Orbitrap XL), IF(Immuno-Fluorescence) staining

Results and Discussion

In previous studies, a natural small molecule, YCG185, was identified as an anti-angiogenic agent *in vitro* and *in vivo*. A natural small molecule, YCG185, was characterized as an angiogenesis inhibitor *in vitro* and *in vivo* with no toxicity. Additionally, YCG185 significantly decreased the expression levels of HIF-1α and its target gene, VEGF. Moreover, YCG185 significantly suppressed not only the phosphorylation of VR receptor but also its downstream signaling, ERK and Akt and directly interacted with VR receptors in DARTS assay. Furthermore, to explore the mode of actions of YCG185, we focused on elucidating the pharmacokinetic and pharmacodynamic properties with identifying distributions of YCG185 and its target protein on xenografted tumor tissues using MALDI-MSI (MALDI-Mass Spectrometry Imaging) analysis and IF (Immuno-Fluorescence) staining. From the comparison of

the results, the MALDI-MSI of YCG185 is highly correlated with IF of VR receptors on xenografted tissues, suggesting that YCG185 interacts with its target protein *in vivo*.

Conclusion

In conclusion, this study provides a novel platform for understanding the pharmacokinetic properties of natural small molecules with label-free and novel insights into deciphering interaction of a drug and its targets even on tissues.

Keywords: Natural small molecules, anti-angiogenesis, DARTS, MALDI-MSI (MALDI-Mass Spectrometry Imaging), IF (Immuno-Fluorescence) staining

GLOBAL DATA STANDARDIZATION ALGORITHM FOR APPLIED METABOLOMICS

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Introduction and Objectives

In omics sciences, thousands of substances can be detected in a single assay. This capacity motivates the development of omics tests, which is currently a very promising option for improving laboratory diagnostics. However, the simultaneous measurement of a huge number of substances leads to omics data often representing concentrations only in conditional units, while laboratory diagnostics generally require actual concentrations. To convert omics data to actual concentrations, calibration curves need to be generated for each substance, and this process represents a significant challenge due to the number of substances that are present in the omics data. To overcome this limitation, a standardization algorithm was developed and tested on mass spectrometric metabolomics data.

Methods

In order to develop the standardization algorithm, the characteristics of the biosamples required to provide a background for a universal and stable internal standards were determined and an algorithm that converts omics data into a unified scale was developed.

Results and Discussion

It was observed that biosamples always have a set of stable internal standards, which are independent of the limit of detection (LOD) of the measurement method. The appropriate usage of these newly discovered internal standards provides a background for the global standardization of metabolomics data by the developed algorithm. This algorithm was named SantaOmics (Standardization algorithm for nonlinearly transformed arrays in Omics).

Conclusion

The results showed that the omics data could be converted into a unified scale that could substitute actual concentration measurements, thus making the omics data directly comparable with each other as well as with reference data presented in the same scale. Therefore, the developed algorithm sufficiently facilitates the usage of omics data in laboratory diagnostics.

Keywords: metabolomics, mass spectrometry, standardization, blood plasma, laboratory diagnostics

MO04-02

Systemic proteomic and metabolomic analyses identify crucial roles of the polyol pathway in tumorigenesis

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Introduction and Objectives

Metabolic reprogramming is acknowledged as one of the prominent hallmarks of cancer. The earliest discovery of an altered metabolic phenotype in tumor cells centered on glucose metabolism. In addition to alterations in the glycolytic, pentose phosphate, and tricarboxylic acid pathways, there could be additional pathways by which cancer cells evade normal cellular glucose metabolism and generate precursors to support their increased growth and proliferation. The polyol pathway is a bypass pathway for glucose metabolism, and fructose production that cells can use to evade the regulatory steps of glycolysis. In this study, we explore the role(s) of the polyol pathway in glucose metabolism and tumorigenesis using a systems biology approach.

Methods

Model systems consisting of various cancer cell lines and corresponding immortalized non-cancerous cell lines were screened for an active polyol pathway. Cancer cell lines with an activated poyol pathway relative to the immortalized counterpart were employed for gene editing, and toxicity studies. Alterations in tumor-associated phenotypes, cellular proteome, and metabolome, after defined perturbations, were systematically examined using molecular assays, and untargeted and targeted MS methods. Comprehensive quantitative proteome maps were generated for samples using DIA/SWATH MS, while the global cellular metabolome was quantitatively examined with GC-MS and LC-MS/MS

Results and Discussion

Cellular abundance of the two enzymes involved in the polyol pathway, sorbitol dehydrogenase (SORD) and aldose reductase (AKR1B1), were significantly altered in colon cancer cells compared to the immortalized colon cell line. Furthermore, SORD expression was distinctly regulated in androgen-sensitive prostate cells. In cancer cells, preliminary data implicates the polyol pathway in the generation of three-carbon precursor metabolites to fuel metabolic processes downstream and/or connected to glycolysis. A systematic study of the cellular proteome and metabolome after perturbation with primary carbon sources and therapeutic metabolic inhibitors are ongoing using high-throughput MS methods. The resulting data would elucidate network and signaling events connecting the polyol pathway to cellular carbon utilization.

Conclusion

The prevalence of an altered polyol pathway in some cancers represents an evolved solution to some constraints during tumorigenesis. Unraveling the molecular mechanisms behind such an occurrence could provide precise therapeutic strategies targeting new aspects of metabolic reprogramming in neoplastic tumors.

Keywords: Cancer, proteome, metabolome

Urine proteomics for evaluation of taking nano-mist sauna effects on the health

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Introduction and Objectives

Sauna bathing has been said benefitial for health such as relaxing or detoxing thought improvement of the metabolism. However, sauna bathing at high temperature may be harmful for health as well, and may induce heart problems as an example. Nano-mist sauna was developed to lower the temperature but to maintain the humidity with ionized nano-size mist. In this study, we performed a comprehensive proteomics analysis of urine from volunteers before and after taking the nano-mist sauna bathing to examine its benefits for health.

Methods

Human urine samples were obtained from 20 volunteers before/after nano-mist sauna bathing, once a day for 2 weeks. Another 20 volunteers took also sauna bathing without nano-mist spray as a control. The urine proteins were collected by a precipitation method and digested by trypsin. Peptides were purified with C18 spin columns and peptides were analyzed by Q-Exactive mass spectrometry. Proteins and peptides were identified by Proteome Discoverer search engines (v2.1, peptide FDR> 0.1%, protein FDR>1%).

Results and Discussion

The current analysis showed identification of 1542 and 1763 urine protein group before and after the nano-mist sauna bathing, respectively and 1644 and 1865 urine protein group before and after the sauna bathing without nano-mist. Interestingly, some of proteins were increased/decreased more than 10 times different. Furthermore, some of oxidative-stress marker proteins were identified and found that nano-mist sauna effected to increase the expression. Pathway analysis of the increased (>2.0) proteins after the nano-mist sauna bathing demonstrated significant enhancement of “Lysome pathway”, suggesting the stimulation of the pathway by nano-mist.

Conclusion

We performed comprehensive proteomic analysis of urine collected before and after nano-mist sauna bathing for 2 weeks to examine its effects on health. Our data suggested that the nano-mist sauna bathing improve the human metabolism and especially to enhance the lysosome pathway.

Keywords: health, proteome, biomarker, urine

MO04-04

Mass spectrometry-based proteomic and metabolic analysis of different cell lines after perturbation of cellular cholesterol regulation

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Introduction and Objectives

Understanding the context-dependent cellular response to drugs is an important step towards predicting differential drug response in organs or individuals. Cellular cholesterol and lipid regulation is a complex biological process underlying several complex diseases such as cardiovascular disease or cancer. The expression of proteins in cholesterol and lipid regulation are determined by the transcription factors SREBP and LXR. How specific genetic or pharmacologic perturbations affect the proteome through these pathways has not been systematically analyzed. We employed mass spectrometry-based proteomics and logic-based ordinary differential equations (ODE) modelling to quantitatively characterize the proteomic response downstream of the SREBP and LXR pathways and generate a mechanistic model.

Methods

More than 3'000 cellular protein and 1'000 metabolite levels were quantified within the total cell lysate of four human cell lines after treatment with 14 different pharmacological conditions. In addition, the proteomic response to knockdown by 12 different siRNAs was assessed. The samples were acquired by SWATH-MS on a Sciex TripleTOF 5600 and the data was analyzed using targeted data extraction with the OpenSWATH software and the combined human assay library. Logic-based ODE modeling was performed using the CNORode R package.

Results and Discussion

We experienced very high reproducibility across biological replicates highlighting the ability of using SWATH-MS for large systems biology projects measuring over 200 samples. Widespread regulation to the drug perturbations was observed as 282 metabolites and 367 proteins significantly changed their levels in at least 2 of the 33 treated conditions. For 126 (34%) of the regulated proteins this is the first report that these proteins are regulated in relation with cholesterol regulation. Differences in both basal abundance level and changes in abundance upon treatment was observed for different proteins and metabolites among the different cell lines making this a good model system to analyze context-dependent regulation mechanisms. Different cell line-specific models describing the regulation of the SREBP and LXR pathway were generated and could be used to identify the likely root cause for the different phenotypes within this complex regulatory network.

Conclusion

MO04-04

Our work represents a prototypical mass spectrometry-based approach to describe a complex biological process of high clinical relevance and the generated models can be used to simulate and predict the response to perturbations that were not previously tested. Differences in the cellular response were not only observed due to differences in the intracellular drug concentrations, but especially due to the different intracellular response mechanisms. This shows that context-dependent regulation mechanisms are important and need to be taken into account when studying complex biological or pathological processes.

Keywords: Data-independent acquisition, DIA, SWATH-MS, cholesterol, modeling

MO04-05

imCorrect: New UHRMS Signal Handling Approach for More Accurate Elemental Composition Determination

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Introduction and Objectives

The field of metabolomics has become increasingly important in the functional genomics. Until now, the large scale identification of metabolites is still challenging. The key challenging step for the metabolite identification is on the determination of accurate elemental composition. An accurate elemental composition determination can largely reduce the number of possible chemical structures need for further validation. The specificity for the determination of metabolite elemental composition is based on the mass and isotopic ratio accuracy. To date, the current development of ultra-high resolution mass spectrometry (UHRMS) can facilitate the separation and determination of accurate molecular weight of the metabolites. Unfortunately, most of UHRMS cannot obtain accurate isotopic pattern because of the ion-decay effects during the mass measurement.

Methods

In consideration of the effects of noise level, signal stability, ion-decay effects and mass error, a new signal processing software was developed. There are three major functions in the software. First, the adaptive background subtraction function can improve the noise removal in different m/z range. Second, the use of two-dimensional spectra smoothing can improve signal stability of the MS signal. Third, the isotopic pattern error cause by ion-decay effect and mass error can be corrected using polyfit regression.

Results and Discussion

In this study, a sample with PEG standard was analyzed by LC-OT-MS (Orbitrap Elite operated) in 240,000 resolution. With conventional approach, the average relative isotopic abundance (RIA) error determined by Orbitrap directly was ~5%. With noise removal, spectra smoothing and RIA correction by imCorrect, the RIA error can be reduced to ~1%. Furthermore, we analyzed a Jurkat cell lysate with a mixture doped of several lipid standards. With the aids of imCorrect processing, with sub-ppm mass accuracy and ~1% RIA error, all the doped standards their elemental composition can be correctly identified and with high confidence and specify.

Conclusion

With the use of imCorrect, the possible elemental composition can achieve for each compound were significantly reduced. This reduction can reduce to effort for unknown metabolite identification and highlight the application of UHRMS in metabolomics application.

Keywords: Metabolomics, Elemental Composition Determination

WP11-01

Investigations Into the Effect of Eish Oil on Enzymes of Lipid Metabolism: A Proteomics Approach

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Introduction and Objectives

Fish consumption has been associated with several health benefits as demonstrated by several epidemiological studies worldwide. The positive effects are chiefly thought to be due to the predominance of omega-3 polyunsaturated fatty acids (n-3 PUFA) in fish though fish protein is also partly responsible. Investigations have shown that n-3 PUFA improves symptoms of lifestyle diseases like diabetes, heart disease, obesity etc. Though several possible mechanisms of action of n-3 PUFA have been put forth, there is a considerable lacuna in the understanding of the proteins and metabolic pathways that are influenced by these fatty acids.

Methods

We used RNA expression studies for determining the level of expression of four genes in liver of wistar strain rats namely acetyl co a carboxylase (ACC) A & B, fatty acid synthase (FAS) and steroyl Co A desaturase-1 (SCD-1) in response to feeding of four different diets namely groundnut oil (control), coconut oil, fish oil at 10 and 20 %. Using western blotting technique we determined the levels of the enzymes expressed by the mRNA in the liver namely ACC, FAS and SCD-1. Proteomic analysis was also carried out on liver protein using 2-D electrophoresis and mass spectrometric analysis to pick proteins of lipid metabolism that are either up-regulated or down-regulated.

Results and Discussion

The findings demonstrated that the levels of mRNA for ACC and FAS decreased and that of SCD-1 increased in groups of rats fed fish oil. Similarly fish oil feeding resulted in a decrease in the intensity of bands for ACC and FAS and an increase for SCD-1 in as determined by western blot analysis of protein levels in rat livers. It was also interesting to note that the changes in the mRNA expression and the protein levels were dose dependent

Conclusion

Our study employed RNA and proteomic approaches and our results establish that fish oil feeding caused significant alterations in proteins of lipid metabolic pathways that are likely to have profound effects on serum and liver lipid content. These proteins are potential targets to develop therapeutic strategies against lipid-related disorders.

Keywords: Acetyl Co A carboxylase, fatty acid synthase, steroyl Co A destaurase, RT-PCR, western blotting, proteomics

Study the regulation of bioactive compounds and metabolome of W. chinensis under different plant hormone and stress treatment

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Introduction and Objectives

Wedelia chinensis is a very common ingredient of herbal tea in Taiwan. In previous research, the principal bioactive phytocompounds, wedelolectone (Wed), luteolin (Lut), and apigenin (Api) in the ethanol extract of Wedelia chinensis were demonstrated, showing that they can synergistically inhibit AR-positive prostate cancer cell growth and the water extracts have anti-colitis function. Unfortunately, these phytochemicals are present in low abundance within Wedelia chinensis plant, approximately 0.16 to 0.39% of the dry weight of the plant. Since these compounds are secondary metabolites, they exist and evolve as a part of a plant defense system for addressing different abiotic or biotic stresses. Therefore, to facilitate Wedelia chinensis plant to synthesize bioactive compounds at high levels, it is important to investigate how these bioactive or contributing phytocompounds can be actively synthesized under different growth conditions and environmental stresses.

Methods

In order to establish an efficient and reliable platform to evaluate the quality of bioactive secondary metabolites in Wedelia chinensis, a high sensitivity, resolving power and the wide quantitation dynamic range, analytical platform based on the UHPLC-HRMS was adapted in a high throughput analysis manner. This system can largely reduce the efforts on the sample preparation for the complex plant metabolomics analysis.

Results and Discussion

The plant batches grown in soil yield high level, and they also showed some infectious phenotype. We hypothesize that the compounds may involve in a defense responses of biotic stress. In order to evaluate and enhance the Wed level by activating possible plant defense responses, we treated the plant with a biotic defense hormone and monitored the metabolite level changes in a time course experimental with hormone treatment. We tested the levels of compounds under different stress conditions, and have found that the treatment with defense hormone SA can apparently upregulate the expression of Wed higher level, as compared to that in plants under untreated control condition. The result indicates that compound Wed is involved in the activation of systemic acquired resistance (SAR) for biotic stresses.

Conclusion

This platform can optimize the Wedelia chinensis extraction protocol for high medicinal activity and storage stability. In addition, more novel compounds with high anti-tumor, anti-colitis and anti-inflammatory activity in Wedelia chinensis may be activated by the specific growth or stress conditions. This analytical system is capable to elucidate preliminary structure information for helping the identification of novel metabolites. The setup of this platform will also facilitate the development of other botanical drug from a

WP11-02

single or defined plant raw material.

Keywords: Secondary Metabolite, Metabolomics, Ultra High Performance Chromatography Mass Spectrometry, Plant Hormone, Plant Stress

WP11-03

Monitoring phosphatidylcholine and sphingomyelin for concentration normalization in cellular lipidomics studies using FIA-ESI-MS/MS

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Introduction and Objectives

Cellular lipidomics studies have been a favored approach for various biomedical research areas. To provide fair comparison for the studying cells, it is essential to do concentration normalization before lipidomics analysis. This study proposed a cellular lipidomics normalization method by measuring phosphatidylcholine (PC) and sphingomyelin (SM) amounts in cell extracts.

Methods

The flow injection analysis-electrospray ionization-tandem mass spectrometry (FIA-ESI-MS/MS) with precursor ion scan (PIS) of m/z 184 method was used to measure total PC and SM amounts in cell extracts. Cell extracts from reference cells were used to generate the calibration curve between the dilution factor (DF) and the reciprocal of peak area of total ion current from the PIS of 184 method. The calibration curve can be used to estimate the relative cell numbers in the unknown test samples. The test sample is then diluted accordingly before the lipidomic analysis.

Results and Discussion

Concentration normalization can be achieved though adjusting total PC and SM amounts in cell extract by PIS of m/z 184 analysis. Good linearity could be observed between the cell extract dilution factor and the reciprocal of the peak area of the total ion current in the PIS of 184 within the dilution range of 1- to 16-fold (R2= 0.998). The intra-day and intermediate precisions were below 10%. The accuracy ranged from 93.0% to 105.6%. The performance of the new normalization method was evaluated using different numbers of HCT-116 cells. Sphingosine, ceramide (d18:1/18:0), SM (d18:1/18:0) and PC (16:1/18:0) were selected as the representative test lipid species, and the results showed that the peak areas of each lipid species obtained from different cell numbers were within a 20% variation after normalization. We also compared the proposed results to commonly used methods, and the results showed highly correlations (Pearson's correlation, R>0.90). Finally, the PC and SM normalization method was applied to study ischemia-induced neuron injury using oxygen and glucose deprivation (OGD) on primary neuronal cultured cells.

Conclusion

We established a novel concentration normalization method by measuring PC and SM for cellular lipidomic studies. The proposed normalization method provides similar results compared with the commonly used methods, and it has better accuracy at lower cell numbers. In addition, the proposed uses the same cell extract samples and MS

instrument as that typically used for lipidomics studies, which make the incorporation of this method very convenient. The limitation of this method is that we only demonstrated the effectiveness of this method for cells with similar sizes and morphologies. The successful application of the PC and SM normalization method in the OGD-induced neuron injury demonstrates its feasibility for cellular lipidomic studies.

Keywords: Normalization; cellular lipidomics; phosphatidylcholine; sphingomyelin; FIA-ESI-MS/MS; neuron; oxygen-glucose deprivation

WP11-04

Development of the absolute quantification method for amino acids

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Introduction and Objectives

Although, there are several method for absolute quantity amino acids are developed. However, most of them are based on the addition of stable isotope labeled standard amino acids to the sample. It will be too expensive and inconvenient if all of the amino acids in the sample need to be complementally labeled and quantified. We have set-up an economic and efficient method for quantitative all amino acids in the biological sample.

Methods

This method is not only can perform absolute quantification of amino acids but also feasible for the use of RP-LC column with high sensitivity and recovery. In this study, the quantities of each amino acid in plant tissue were determined using triple quadrupole LC-MS system operated in multiple reaction monitoring (MRM) mode and Q-TOF operated in MS/MS mode.

Results and Discussion

We can successfully determine the concentrations of 19 amino acids in the Arabidopsis thaliana before and after the high salinity stress. The results show the standard deviation are less than 5% with the use of triple quadrupole, indicate the whole approach is optimal in quantification.

Conclusion

In summary, this approach is convenient, sensitive and accurate for amino acids analysis.

Keywords: amino acids, absolute quantification

WP11-05

Epigenetic mechanism investigation in recessive neurometabolic disorder mice.

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Introduction and Objectives

Accumulation of L-2-hydroxyglutarate (L-2-HG) is a key feature of L-2-hydroxyglutarate aciduria (L-2-HGA), an autosomal recessive neurometabolic disorder caused by L-2-hydroxyglutarate dehydrogenase (L2HGDH) gene mutation. To study the biochemical and pathophysiological consequences of L-2-HG accumulation, we created L2hgdh knockout (KO) mice by piggyBac transposon mediated insertion mutation of L2hgdh gene.

Methods

In this study, we generated L2hgdh knockout (KO) mice, and observed an age-dependent increase of L-2-HG in the cerebrum and an increase of a subset of histone methylation in the central nervous system (CNS). L2hgdh KO mice exhibit white matter abnormalities, extensive gliosis, increased oligodendrocyte lineage cells, and microglia-mediated neuroinflammation. Moreover, L2hgdh deficiency leads to impaired adult hippocampal neurogenesis and late-onset neurodegeneration in mouse brains. We developed a sensitive and robust LC-MS method for the histone profiling in different mice organs, provide epigenetic mechanisms for disease progress.

Results and Discussion

We found that the H3K9me2 level was significantly increased, and the H3K9me0 level was accordingly reduced in the cerebrum of L2hgdh KO mice. These findings suggest that L2hgdh deficiency leads to global changes in a subset of histone methylation marks in mouse testis and brains where L-2-HG is the highest. Our data demonstrate an age-dependent accumulation of L-2-HG in the cerebrum and alterations in a subset of histone methylation in the central nervous system (CNS) of L2hgdh KO mice.

Conclusion

Our study thus offers new insights into the pathophysiology of L-2-HGA in human diseases.

Keywords:

WP11-06

IMS-DIA-MS CHARACTERISATION AND IMS-MRM QCONCAT QUANTITATION OF THE LIPIDOME AND APOLIPOPROTEIN COMPLEMENTS OF OBESITY AND DIABETES COHORTS

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Introduction and Objectives

Risk factors associated with increased possibility of developing diseases are commonly referred to as metabolic syndrome. Obesity is one such risk factor causing excess body fat to be accumulated to the extent that it adversely affects health and life expectancy. Obesity is known to initiate inflammation, which in turn can lead to type 2 diabetes. The exact mechanism as to how this occurs is however not well understood. Here, we describe a lipidomic approach to reveal molecular factors that may be involved in these biomolecular processes. The results of these experiments were complemented with absolute quantitation of the plasma Apolipoprotein complement using QconCAT concatenated signature peptides to identify multi-factorial disease associated components and pathways.

Methods

Plasma samples from three biological states of varying phenotype (control, diabetic and obese) were used with each group consisting plasma from six individuals. Extracts were LC separated and lipidomics data acquired using ion mobility assisted data independent acquisitions, whereby the collision energy was switched between a low and elevated energy state during alternate scans. Simultaneously, experimentally derived CCS values using travelling-wave ion mobility were determined. The data were processed and searched with compound databases, providing normalized quantitation results with increased selectivity afforded by the ion mobility/CCS dimension. The same samples were also tryptic digested and targeted proteomics data acquired and quantified with the same MS platform using combined ion mobility/mass selected oa-ToF MRM analysis.

Results and Conclusions

Plasma samples were treated with isopropanol and centrifuged to precipitate proteins. The lipid-containing layer was collected and diluted to adjust the water content prior to analysis. Label-free LC-MS data were acquired in positive and negative ion electrospray mode with an IMS oa-QTof platform using an ion mobility assisted data

independent analysis acquisition workflow. Unsupervised MVA of the data showed clear distinction between cohorts. OPLS-DA was used to filter for features of significant correlation and covariance prior to identification. Identifications matching criteria were as follows, collision cross section (CCS) values <5%, mass accuracy <5 ppm, ANOVA p <0.05 and fold change >2 were considered for further interrogation. Ion mobility-derived CCS measurements allowed for improved specificity with the inclusion of drift time, providing additional confidence in the identifications returned. A variety of synthetic lipid standards representing the most significant classes identified were measured to determine their CCS for reference and used to populate the in-house database providing additional identification stringency. Normalized label-free quantitation results highlighted differential expression of specific lipid classes including fatty acids, phosphatidylcholines, triglycerides and phosphatidylserines. Additional identifications were obtained by mapping putative identifications with those from independent studies and biochemical networks. The identified networks were extended and confirmed by targeted IMS-MRM experiments of the most common alleles of the apolipoprotein plasma complement. Apolipoprotein AI, CI, CII, D, and E are key constituents of LDL and HDL and related to obesity, diabetes, and cardio vascular diseases, whereas Apolipoprotein E, a multifunctional protein, is also involved in lipid metabolism by mediating the binding of lipoproteins or lipid complexes to specific cell-surface receptors. The inclusion of IMS in the targeted oaToF MRM workflow increased quantitative precision and accuracy with the results in agreement with expected changes in relation with disease and/or phenotype.

Metabolomics Profiling of Paired OSCC Using Chemical Isotope Labeling LC-MS

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Introduction and Objectives

Oral cavity squamous cell carcinomas (OSCC) remains a disappointed disease worldwide which is frequently associated with poor prognosis and mortality. Barriers in the effective clinical treatment of OSCC include the late diagnosis and the limited knowledge of molecular events governing tumor progression. A better understanding of underlying mechanism in OSCC pathogenesis may help improving diagnostic efficiency and therapeutic outcomes of OSCC.

Methods

To achieve this goal, we performed a quantitative metabolomics profiling on thirty one paired OSCC and adjacent noncancerous tissues using differential isotope labeling coupled with LC-MS analysis that targets amine- and phenol-containing metabolites. A series of four programs, IsoMS, IsoMS-Align, Zero-fill, and IsoMS-Quant, were used to process the raw LC-MS data in batch mode to generate a metabolite-intensity table that represented the expression levels of metabolites in each tissues specimen.

Results and Discussion

A total of 3552 metabolic features were detected and quantitated among 31 paired samples. Based on the metabolome profile, most OSCC could be differentiated from noncancerous tissues using principal component analysis and hierarchical clustering. The candidate metabolites involved in the tumor progression of OSCC were determined using paired two-sample t test and orthogonal projections to latent structures discriminant analysis (OPLS-DA). The identification of metabolites was obtained using M-RT search against a library of labeled standards based on the accurate mass and calibrated retention time.

Conclusion

All these results together revealed the change of metabolome in OSCC during tumor progression.

Keywords: OSCC, metabolomics, dansylation, and tumor progression

WP11-08

Quantitative Metabolome Analysis of Pleural Effusion with High-Performance Chemical Isotope-Labeling Liquid Chromatography-Mass Spectrometry

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Introduction and Objectives

Pleural effusion (PE), an abnormal accumulation of excess pleural fluid in pleural space, is usually found among patients with various lung diseases. Malignancy and pneumonia account for the two leading causes of exudative PE generation. Approximate 20%-40% of patients suffered from pneumonia are accompanied by generation of parapneumonic effusion (PPE), the PE resulted from pneumonia or other lung parenchyma infections. PPE is a growing health burden worldwide with an increasing incidence. Based on progression of inflammation, PPEs could be categorized as the uncomplicated PPE (UPPE), complicated PPE (CPPE), or empyema. The molecules in PPE are worthy of profiling to reveal possible causes and develop treatment strategy of the disease.

Methods

To this end, we aimed to profile the metabolome of 59 PPE sample (27 UPPE and 32 CPPE) using differential isotope dansylation labeling combined with liquid chromatography-Fourier transform ion cyclotron resonance mass spectrometry (LC-FTICR-MS), by which the metabolites containing amine or phenol groups could be quantitatively profiled.

Results and Discussion

The procedures of sample preparation and LC-MS settings firstly were optimized, resulting that more than 500 metabolite features could be detected in each PE sample. More importantly, the CPPE could be discriminated from UPPE based on the metabolome profiles using principle components analysis (PCA) and clustering analysis.

Conclusion

The results provide the evidence that the metabolite profiles is changed upon the PPE progression.

Keywords: Chemical isotope labeling, metabolomic profiling, liquid chromatography-mass spectrometry, parapneumonic effusion

WP11-09

Metabolomic profiling of subgrouping ischemic stroke

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Introduction and Objectives

Defining and subgrouping of ischemic stroke, especially for intracranial large artery atherosclerosis, can be a challenge in a typical outpatient setting without image data. In the present study, we aimed to identify unique metabolites of intracranial large artery atherosclerosis to facilitate subgrouping of ischemic stroke.

Methods

We measured such differences in blood samples by using nuclear magnetic resonance spectroscopy (NMR). Clinical and serum-based NMR data were collected on aged individuals (> 50yrs) meeting the criteria of intracranial large artery atherosclerosis (n= 20), extracranial large artery atherosclerosis (n=19), and normal individuals (n=23). Metaboanalyst 3.0 analysis was performed on the NMR data to identify target metabolites for separation.

Results and Discussion

Compared with age-matched normal control, eight unique serum metabolites (Score >2.5) were identified in the extracranial large artery atherosclerosis group; two unique serum metabolites (Score >2.5) were identified in the intracranial large artery atherosclerosis group. Of note, these two unique serum metabolites of intracranial large artery atherosclerosis group were not identified in the extracranial large artery atherosclerosis group.

Conclusions

This is the first report showing that metabolomic analysis of human serum samples could be a practicable way to identify intracranial large artery atherosclerosis.

Keywords

intracranial large artery atherosclerosis, metabolites, NMR, stroke,

WP11-10

Monte Carlo Simulation Reveals Reliable Molecular Formula Annotation of Metabolomic Data Obtained by Accurate Mass Spectrometry

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Introduction and Objectives

In metabolomic studies, metabolite identification from LC-MS or LC-MS/MS data is one of the major obstacles. It is commonly believed that mass accuracy of even <1 ppm is insufficient to allow reliable molecular formula annotation of a metabolite feature on the basis of an observed mass. However, validity of this common belief has not been systematically examined. This study was aimed to investigate how the mass accuracy and search tolerance as well as choice of database affected the confidence in molecular formula annotation of MS data.

Methods

By Monte Carlo Simulation, we simulated “observed masses”, which were measured by MS platforms with various measurement mass accuracy (MMA) and standard deviation of mass error (SD), for over 40,000 known human metabolites. Then the individual “observed masses” were searched against various publicly available databases at various degrees of error tolerance.

Results and Discussion

Comparing various publicly available metabolite/chemical databases, we have found that HMDB, a database focused on human metabolites, is the best database for reliable human metabolite search from accurate MS data. The correctness of search results was affected by the mass accuracy and search tolerances. The best error tolerance should be 2 to 4 times of SD values of the mass error. If the performance of the instrument accuracy has the SD of mass error = 1 ppm and MMA = 1 ppm, using an search tolerance of 3 or 4 ppm, more than 98% metabolites could be annotated with only one molecular formula, and the correctness was 99.9%. Our results indicate if one wants to obtain reliable molecular formulae for >95% metabolites (i.e., returning one molecular formula for 1 metabolite), the MS instrument should have a performance of MMA < 2 ppm and SD of mass error < 3 ppm.

Conclusion

Our results demonstrated that using accurate MS, optimized error tolerance and a database focused on human metabolites, a single molecular formula could be correctly assigned to more than 95% metabolites, suggesting that annotation of a large-scale metabolomic data obtained by accurate MS is practically feasible. This observation is opposite to the common belief that an observed accurate mass of a metabolite is insufficient for correct metabolite annotation.[*Melody M. T. Lam and Pengwei Zhang contributed equally to this study. This study was supported by Multi-Year Research Grant (MYRG) from the University of Macau (RC Reference Number: MYRG2015-00233-FHS)]

WP11-10

Keywords: Metabolite identification, Accurate mass spectrometry, Mass accuracy, Search tolerance, Database search

WP11-11

Urinary exposure marker discovery for toxicants using UPLC-LTQ-Orbitrap and three untargeted metabolomics approaches

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Introduction and Objectives

Human biomonitoring is the assessment of actual internal contamination of chemicals by measuring exposure markers, chemicals or their metabolites, in human urine, blood, serum, and other body fluids. However, the metabolism of chemicals within an organism is extremely complex. Therefore, the identification of metabolites is often difficult and laborious. Several untargeted metabolomics methods have been developed to perform objective searching/filtering of accurate-mass-based LC-MS data to facilitate metabolite identification.

Methods

Three metabolomics data processing approaches were used for chemical exposure marker discovery in urine with an LTQ-Orbitrap high-resolution mass spectrometry (HRMS) dataset; di-isononyl phthalate (DINP) was used as an example. The data processing techniques included the SMAIT, mass defect filtering (MDF), and XCMS Online.

Results and Discussion

Sixteen, 83, and 139 probable DINP metabolite signals were obtained using the SMAIT, MDF, and XCMS procedures, respectively. Fourteen probable metabolite signals mined simultaneously by the three metabolomics approaches were confirmed as DINP metabolites by structural information provided by LC-MS/MS. Among them, 13 probable metabolite signals were validated as exposure-related markers in a rat model. Six (m/z 319.155, 361.127, 373.126, 389.157, 437.112 and 443.130) of the 13 exposure-related DINP metabolite signals have not previously been reported in the literature.

Conclusion

Our data indicate that SMAIT provided an efficient method to discover effectively and systematically urinary exposure markers of toxicant. The DINP metabolism information can provide valuable information for further investigations of DINP toxicity, toxicokinetics, exposure assessment, and human health effects.

Keywords: Biomonitoring, Metabolomics, Orbitrap, signal mining algorithm with isotope tracing (SMAIT), mass defect filtering (MDF), XCMS Online

Discovery of early-stage biomarkers for diabetic nephropathy using LC/MS-based metabolomics

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Introduction and Objectives

Diabetic nephropathy, with increasing prevalence of diabetes mellitus among adult population, has remained the leading cause of end stage renal disease (ESRD). Thus, current consensus of managing diabetes mellitus have addressed the importance of early detection of diabetic nephropathy which enables early initiation of specific therapy and dietary restriction in patients with diabetic kidney disease to halt progression of renal function decline and prevent progression into later kidney failure stages. In current practice, diabetic nephropathy is defined as presence of microalbuminuria (urine albumin excretion 30-300 mg/24h) or decline of estimated glomerular filtration rate. However, many studies have shown that microalbuminuria is a poor predictor for future macroalbuminuria, chronic kidney diseases, or even future ESRD. Therefore, new non-invasive markers are needed for early diagnosis and enable intensive management of diabetic nephropathy.

Methods

To discover early-stage biomarkers for diabetic nephropathy, untargeted LC/MS-based metabolomics was performed in the urine samples from subjects of healthy, chronic kidney disease (CKD), diabetes mellitus (DM), and DM with microalbumin. Urine samples were normalized with creatinine and analyzed with LC-Q-TOF. The metabolite candidates were identified and quantified in 274 urine samples.

Results and Discussion

The results showed that the 6 metabolite biomarkers were significantly altered in DM with microalbumin group compared to DM groups.

Conclusion

These can serve as early-stage biomarkers to predict the development of nephropathy from DM patients.

Keywords: metabolite
diabetic nephropathy

Defining the aberrant molecular profiles in liver and adipose tissues of ovariectomized rat model with different n-3 fatty acid interventions

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Introduction and Objectives

Postmenopausal women have high risk of developing hepatic steatosis and adipocyte hypertrophy caused by estrogen deprivation. n-3 fatty acids have become one of the most popular supplements in Taiwan for helping lower the blood triglyceride levels. Accumulating evidences suggest that increasing n-3 fatty acid intake would regulate lipid metabolism and inflammation responses, yet the molecular and pathological mechanisms underlying different n-3 fatty acid intake on a ovarian hormone deficiency status remain unclear. Thus, we aim to elucidate the changes in proteome expression level in liver and adipose tissues of an ovarietomized (OVX) rat model feeding with fish oil and perilla oil.

Methods

24 female 8-weeks-old Sprague-Dawley rats were randomly divided into four groups: sham-operated (C), OVX control (OC), OVX-Fish oil (OF) and OVX-Perilla oil (OP) groups for 4-month treatments. The blood, liver and abdominal adipose tissues samples were collected to evaluate biochemical variables. We conducted the iTRAQ-based quantitation analysis on liver and AT proteomes. The functional analysis of differentially expressed proteins was performed by using Ingenuity Pathway Analysis software.

Results and Discussion

Compared with OC group, fish oil and perilla oil interventions would differentially regulate 243 and 196 proteins in liver, 275 and 138 proteins in AT of ovarietomized rats. In OF group, we observed down regulation of PPAR α/RXRα pathway in liver, suggesting accumulation of free fatty acid that might lead to serious fatty liver phenotype. The down regulation of insulin receptor signaling was observed in OP group, meaning the decreased level of fatty acid and cholesterol synthesis. Besides, many proteins involved in lipid metabolism were up-regulated. Taken together, the perilla oil intervention would significantly mitigate the lipid deposition in liver, which was further confirmed by immunohistochemistry staining of liver tissue. Regarding the AT, we observed the higher level of ROS and nitric oxide production in both OF and

OP groups as evidences of a chronic low-grade inflammatory state in adipocytes. In addition, compared with the C group, the ovariectomy-induced parametial fat pad weight gain could be significantly modulated by fish oil and perilla oil. The AT proteome profiles revealed elevated lipid metabolism in both OF and OP groups resulting from the activation of LXP/RXR pathway to reduce fatty acid accumulation.

Conclusion

Our study provided systematic investigation of the molecular profiles of which fish oil and perilla oil would influence the lipid metabolism and inflammation in the liver and AT proteomes of OVX rats. Based on the result, we suggested fish oil supplement would worsen hepatic steatosis in estrogen deficient rat. In the other hand, perilla oil supplement could decrease plasma AST and TG and alleviate the fatty liver and AT phenotypes as a better choice of nutrient.

Keywords: Ovariectomy; fish oil; perilla oil; liver, adipose tissue, quantitative proteomics

Metabolomics investigation of voriconazole-induced hepatotoxicity

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Introduction and Objectives

Voriconazole (VCZ) is a triazole-antifungal agent with broad spectrum, which has been widely used in invasive Aspergillosis infections. Hepatotoxicity is one of its most commonly seen adverse side effects, which might lead to treatment-failure, withdrawal, and even death. The aim of this study was to investigate in the mechanism of VCZ-induced hepatotoxicity.

Methods

We applied the targeted metabolomics approach to study VCZ-induced hepatotoxicity via liquid chromatography-triple quadrupole mass spectrometry (LC-QqQ MS). The metabolic patterns of VCZ-induced hepatotoxicity in C57BL/6 mice were first analyzed. Mice treated with three repeated dose of 40 mg/ kg by tail vein injection to induce hepatotoxicity (VCZ-induced hepatotoxicity group, n = 8) were compared with the mice without treatment (control group, n = 10). Both liver tissue and plasma were collected and analyzed to propose mechanisms associated VCZ-induced hepatotoxicity. We additionally collected plasma samples from clinical patients for validation of the proposed mechanism of VCZ-induced hepatotoxicity in human. All of the recruited patients had received VCZ treatment, and were divided into control group (n = 89) and VCZ-induced hepatotoxicity group (n = 21) by their liver function. VCZ-induced hepatotoxicity was defined by the evaluation of Naranjo scale and RUCAM scale.

Results and Discussion

Results indicated that the metabolites associated with oxidative stress had altered, and most of the altered metabolites were involved in glutathione biosynthesis, which is an important antioxidant in vivo. In addition, the ratios of glutamine and glutamate showed significantly reduction in VCZ-induced hepatotoxicity group compared to control group in both studies, suggested that glutamine might be transformed into glutamate for glutathione biosynthesis. Our results suggested that VCZ-induced hepatotoxicity was associated with oxidative stress to cause cell dysfunction, leading to the alterations in energy metabolism, urea cycle, and bile acids metabolism.

Conclusion

To our knowledge, this was the first study to apply metabolomics for the investigation of the mechanism of VCZ-induced hepatotoxicity. We proposed that VCZ-induced hepatotoxicity was associated with oxidative stress to cause cell dysfunction. Future work is required to clarify the specific molecular mechanism in oxidative stress induced by VCZ and to investigate the biomarkers for clinical practice.

Keywords: voriconazole, hepatotoxicity, metabolomics, LC-MS/MS, oxidative stress

WP11-15

Using a Targeted Metabolomic Approach to Investigate Plasma Metabolites Associated With Risk of Diabetes Mellitus

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Introduction and Objectives

The burden of diabetes mellitus is increasing worldwide. Prediction and prevention of diabetes mellitus is of central interest in the context of diabetes therapy, and yet, the mechanism of diabetes onset is not detailed understood. Metabolic profiling of human biological samples could help to improve our understanding of metabolic alterations in diabetes mellitus. While considerable attention has been paid to amino acid and fatty acid related metabolites, but the metabolites in purine and pyrimidine pathways were rarely be investigated in previous studies.

Methods

Forty-eight metabolites, mainly involved in purine and pyrimidine metabolism, were selected as our target metabolites. We used liquid chromatography - electrospray ionization - triple quadruple tandem mass spectrometry (LC-ESI-QQQ) to measure the expression of 48 metabolites in patient plasma samples. A hydrophilic interaction chromatography method was used for the positive mode analysis and a ion pair chromatography method was applied in the negative mode analysis.

Results and Discussion

A total of 236 subjects were recruited for the investigation of pre-diabetes mellitus markers. The prediction model was built to evaluate the performance of using differential metabolites for prediction of risk of diabetes mellitus. We successfully found five metabolites that showed statistically significant difference between case and control groups.

Conclusion

Through this study, the findings point out there may have a novel pathway involve in diabetes occurrence. Future study should elucidate the mechanisms of these findings.

Keywords: diabetes mellitus, targeted metabolomics, mass spectrometry

Fragmentation Analysis of Amino acids by High Resolution Tandem MS and High-Energy Collision Dissociation

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Introduction and Objectives

Amino acids (AAs) analysis in biological sample is crucial and important for disease diagnosis and newborn screening. Typical LC-MS/MS based AA analysis needs derivatization to enhance sensitivity. However, such approach is time consuming, and suffers from potential inaccuracy. Recently, AA analysis without derivatization becomes more and more popular by LC-MS/MS. Therefore, having a good knowledge of the fragmentation reactions of AAs is helpful for their quantification and identification of AAs in metabolomic profiling studies. However, previous MS/MS spectra (i.e. fragmentation spectra) of AAs were obtained using low resolution QqQ or Ion trap machine, which can only give the nominal mass of the ions, and therefore cannot annotate the fragment ions confidently. Without the correct annotation, it is not easy to elucidate the fragmentation patterns correctly. In the present study, we aim to characterize the fragmentation patterns of amino acids using high resolution tandem MS and high-energy collision dissociation (HCD).

Methods

We systematically investigated the MS/MS fragmentation reactions of 22 pure AA standards by HCD on the high resolution Q-Exactive Quadrupole Orbitrap platform operating at a resolution of 14,000 FWHM.

Results and Discussion

Facilitated by the accurate mass of fragments, we elucidated their fragmentation patterns clearly, and proposed new fragmentation mechanisms for cystine and tryptophan. Furthermore, we identified the unique fragmentation patterns for quantification and identification of different AAs using high resolution accurate mass MS without requiring good chromatographic separation. The unambiguously and comprehensive fragmentation patterns of these AAs provided useful guidance for structural elucidation of other similar metabolites and peptides as well as for selection of appropriate fragment ions for reliable quantification.

Conclusion

To the best of our knowledge, this is the first time to systematically study the fragment reactions of AAs by the high resolution MS/MS and HCD. Our data provided a valuable foundation for accurate identification and quantification of AAs in metabolomic profiling studies. [This study was supported by Multi-Year Research Grant (MYRG) from the University of Macau (RC Reference Number: MYRG2015-00233-FHS).]

WP11-16

Keywords: amino acids, fragmentation, high resolution tandem mass spectrometry, high-energy collision dissociation

WO09-01

Quantitative Proteomic Analysis of Flooding and Its Recovery in Soybean Exposed to Aluminum Oxide Nanoparticles

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Introduction and Objectives

Understanding the complex mechanisms involved in plant response to nanoparticles is indispensable in assessing the ultimate impact of nano-pollutants on environment. To investigate the role of aluminum oxide nanoparticles in mitigating the effects of flooding stress, modulation of proteome composition was analyzed using a gel-free proteomic technique.

Methods

Soybeans at early stage were exposed to flooding without or with aluminum oxide nanoparticles and/or withdraw of flooding stress. After investigation of morphology of plant, proteins extracted from roots were analyzed with gel-free/label-free proteomic technique. The proteomic results were validated with bioinformatics, transcriptional, and enzymatic techniques.

Results and Discussion

The treatment with aluminum oxide nanoparticles of 30-60 nm size of soybean improved root growth even under flooding stress. Survival percentage was improved after 4 days of flooding with 50 ppm aluminum oxide nanoparticles leading to recovery as compared to only flooding. Exposure to aluminum oxide nanoparticles elevated the capacity of reactive-oxygen species scavenging of cells by regulating the ascorbate/glutathione pathway. Hierarchical clustering analysis indicated that ribosomal proteins were increased in flooding-stressed soybeans supplemented with aluminum oxide nanoparticles. Mitochondrion was the target organelle of aluminum oxide nanoparticles under flooding stress condition. In the late and recovery stages, the differentially abundant proteins were mostly related to protein synthesis, stress, cell wall, and signaling. Among the identified stress-related proteins, S-adenosyl-L-methionine dependent methyltransferases were recovered from flooding-stressed soybeans supplemented with aluminum oxide nanoparticles.

Conclusion

Proteomic findings suggest that under flooding stress aluminum oxide nanoparticles primarily affect mitochondrial proteins, regulating membrane permeability and tricarboxylic acid cycle activity. Furthermore, high abundance of proteins involved in oxidation-reduction, stress signaling, and hormonal pathways related to growth and development might be the principal key for the optimum growth of soybean under aluminum oxide nanoparticles treatment.

Keywords: Plant, Stress, Proteomics, nanoparticle

WO09-04

Integrated extracellular matrix proteome and organ specific transcriptome decipher cell wall mediated immunity in plant

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Introduction and Objectives

Innate immunity in plant is a complex phenomenon and the exact structural and functional modification caused by pathogen-stress is poorly understood. The ultimate phenome of eukaryotic organism is highly sophisticated and is modulated by cellular cross-talks of diverse pathways. Extracellular signals perceived at cell surface trigger signaling pathways that branch to several biological responses. Regulatory behavior of cells using integrated OMICs may reveal complex architecture of living systems, including immune response.

Methods

The proteome and phosphoproteome was developed with ECM enriched fraction using combination of 1 DE, 2 DE and subsequent staining with Pro-Q Diamond, SCX, IMAC and MOAC. Proteins were identified using MALDI-TOF/TOF, ESI-MS/MS and Triple TOF MS. RNA-seq experiments were performed using libraries generated from infected and uninfected tissue, sequenced on Illumina HiSeq2000 using 100-bp paired-end chemistry. Further, interactome model was developed.

Results and Discussion

To determine the cellular circuitry that operates in cell wall mediated immunity in patho-stress, we analyzed comparative organ-specific transcriptome and ECM proteome in a grain legume, chickpea during vascular wilt caused by Fusarium oxysporum. RNA-seq analysis yielded 407.13 million reads and 64,856 transcripts. Of which 6.28% were novel while 5572 transcripts were differentially expressed. Deregulated expression of several ECM related transcripts suggests the ability of the plant to evoke cell wall mediated signaling. At protein level, we identified ~2500 proteins and 1800 phosphoproteins involved in cell wall hydration, acidification, and innate defense. Further, functional enrichment and co-expression network analysis revealed different immune-responsive transcriptional regulators. ECM associated network was constructed with specialized hubs of secondary master switches; mechanics associated modules along with those involved in innate defense.

Conclusion

Our study provides novel imminent in ECM dynamics and elucidated the poorly understood molecular processes that initiate from cell wall and culminated in circuitry of innate immunity encompassing ETI, PTI and CTI. Integrated network analyses identified

novel prognostic biomarkers for future translational research.

Keywords: Extracellular matrix, Proteomics, Integrated omics, Cell wall mediated immunity, Plant

WO09-05

Phosphoproteomic Analysis of Abscisic Acid Signaling Components in Arabidopsis seed

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Introduction and Objectives

Abscisic acid (ABA) is a plant hormone that regulates seed dormancy and germination. Previous reports have revealed the core ABA signaling pathway. There are three major components; ABA receptor (PYR/PYL), a type 2C protein phosphatase (PP2C), and SNF1-related protein kinase 2 (SnRK2). Member of the PP2C family genes, including ABA-HYPERSENSITIVE GERMINATION1 (AHG1), AtPP2CA/AHG3, and ABA-INSENSITIVE1 (ABI1) are central negative regulators and loss-of-function of these genes leads to ABA hypersensitivity. AHG1 and AHG3 have the function related to ABA response in seeds and germination. The SnRK2 became activate when they are de-repressed from their inhibition by PP2C and act as a central positive regulator in ABA signaling. Several phosphoproteins that are components of ABA signaling have been identified in the seedling of Arabidopsis by phosphoprotomic analysis. But only few ABA signaling components are identified in seeds. We tried to identify the ABA signaling components in seeds to reveal ABA signaling pathway specific to seeds.

Methods

To investigate ABA signaling components in Arabidopsis seeds, we enriched a phosphopetides from wild type and PP2C triple mutants (ahg1ahg3abi1), and peptides were analysed by LC-MS/MS (LTQ-Orbitrap). PP2C are phosphatase that inactivate SnRK2. Disruption mutations of the three ABA-related PP2Cs leads to ABA hypersensitivity. Therefore we hypothesized that phosphorylation of substrates downstream of PP2C in response in ABA should be increased in PP2C triple mutants. We performed a comparative analysis between Col wild-type seeds and PP2C triple mutant seeds to identify proteins that were differentially phosphorylated after abscisic acid treatment.

Results and Discussion

By proteomics analysis of seeds, we identified a total of 298 unique phosphopeptides derived from 268 unique phosphoproteins. Furthermore, we identified 694 phosphorylation sites in which the ratio of each phosphorylated residue, serine, threonine and tyrosine, was calculated to be 70.2%, 22.9% and 6.9%, respectively. By

extracted 20 phosphoproteins that is thought to be ABA responsive and regulated by PP2C.

Conclusion

We have succeed in identifying number of phosphproteins in Arabisopsis seeds by analyzing with LC-MS/MS. By using comparative analysis between wild-type seeds and PP2C triple mutants we were able to extract 20 phosphoproteins that is thought to be ABA responsive and regulated by PP2Cs. In order to confirm the involvement of these phosphoproteins in ABA signaling pathway, we will examine the interaction between 20 phosphoproteins and PP2C and SnRK2.

Keywords:

Phosphoproteomic analysis, phytohormone abscisic acid, Arabidopsis thaliana

WP12-01

Quantitation of tonoplast proteins simultaneously regulating inorganic phosphate(Pi) influx-efflux during varying Pi levels

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Introduction and Objectives

Early P31-NMR studies in plants grown under phosphate deficient conditions have implicated the role of vacuoles in both inorganic phosphate (Pi) storage and redistribution. However, the mechanism by which Pi is stored and translocated across vacuolar membrane or the tonoplast remains elusive.Hypothesizing that specific influx-efflux system in tonoplast simultaneously mediates the storage and redistribution of inorganic phosphate under varying Pi levels, we aim at quantifying the tonoplast proteome in plants grown under normal Pi, Pi- deficient and Pi-recovery (replenishment post deficiency) conditions.

Methods

Tonoplast integral proteins were extracted from purified vacuoles isolated from Arabidopsis mesophyll cells grown under three external Pi conditions. Following extraction, we tryptically digested and quantified the proteins using label free LC-MS/MS.

Results and Discussion

Preliminary analysis identified 656, 614 and 587 proteins in total in normal, deficient and recovery treated plants respectively. Further analysis revealed approximately 40%-70% of the total proteins to be localized in vacuoles in each of the respective treatment indicating the robustness of our MS-based proteomics pipeline. In an attempt to increase the proteome coverage further, di-methyl labeling based quantification is currently under progress.

Conclusion

Thus, we expect to identify and quantify the global tonoplast protein repertoire including importers and exporters simultaneously regulating Pi-influx-efflux during Pi deficiency.

Keywords: Inorganic phosphate, tonoplast, vacuole, di-methyl labeling, proteomics

WP12-02

The CAP Secretory Pathway is required for CAPE peptide production for the induction of SAR

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Introduction and Objectives

Systemic acquired resistance (SAR) is a plant immune response that occurs following by local pathogen infection throughout the whole plant. SAR leads to the accumulation of salicylic acid (SA) that induces PR-1 expression in uninfected tissues. PR-1 is commonly used as a marker for examination of SAR induction. However, the biological function of PR-1 is still unclear. In our recent study, the tomato PR-1 has proven to as a proprotein, which can be processed to a mature peptide SolCAPE1 using our high-throughput MS-based peptidomics approach. SolCAPE1 can induce both anti-pathogen and anti-herbivore response in tomato. The putative orthologous CAPE of Arabidopsis PR-1, AtCAPE9, has similar anti-pathogen function as SolCAPE1. Both tomato and Arabidopsis PR-1 are known to be localized in the extracellular region. Previous studies have shown that the PR-1 secretion is required for SAR and the secretion level is correlated to anti-pathogen activity. Thus, we believe that the CAP secretory pathway is required for CAPE peptide production for SAR induction.

Methods

To understand the importance of CAP secretory pathway involved in the production of AtCAPE for SAR, the several mutants of Arabidopsis including proatcape1, proatcape9 and ER-resident secretion mutants were used. Pst DC3000 were grown in water- or peptide-treated plants for several days to observe the symptoms and then the bacteria were collected from the leaves and evaluated by bacterial titers. For phytohormone quantitation, the phytohormones were separated by a HSS T3 column (Waters, Millford, MA) using gradients of 0.5-25% ACN at 0-2 min, 25-75% ACN at 2-7 minutes and 75-9. 5% ACN at 7-7.5 minutes. The mass spectrometer was operated in the negative ion mode and set to one full FT-MS scan and switched to five FT-MS product ion scans for five precursors: m/z of 137.02 (for SA), 209.12 (for JA), 322.20 (for JA-Ile), 141.05 (for d6-SA dissociated to d4-SA) and 211.13 (for H2JA). The fragmentation reactions of m/z 137.02 to 93.03 for SA, 209.12 to 59.01 for JA, 322.20 to 130.09 for JA-Ile, 141.05 to 97.06 for d6-SA and 211.13 to 59.01 for H2JA were selected for quantitation.

Results and Discussion

In contrast to wild type, those mutants showed less resistance to bacterial pathogen Pst DC3000 infection. The resistance to Pst DC3000 can be recovered by AtCAPE9 pretreatment in those mutants. The level of SA can be induced by AtCAPE9 in wild type and several mutants. The results imply that not only existence and secretion but also processing of PR-1 is important for SAR in Arabidopsis.

Conclusion

In conclusion, the secretory CAP proteins and their C-terminal CAPE peptides might play the significant roles of immune signaling in various plant species.

WP12-02

Keywords: CAPE peptide, plant immunity, SAR, PR-1, secretory pathway

Studying of Plant Novel Systemic Signaling and RNA Trafficking Proteins under Pi Deficiency Using Proteomics Approach

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Introduction and Objectives

Phosphorus (P) is one of the major elements to build-up fundamental biomolecules and functions in energy transfer as well as the regulation of enzyme activity. Unlike the animal uptake the organic forms of P, the plant can directly uptake the inorganic phosphate (Pi) from soil. However, the Pi level in soil is normally low and distrubuted unevenly. In response to the limited Pi availability in soil, plant display a high degree of adaptive responses, called phosphate starvation responses (PSR) to cope Pi deficiency. This relies on both local and systemic regulation networks to coordinate Pi homeostasis of whole plant. Previous studies have revealed that microRNAs (miRNAs) function as systemic signal in PSR and the long-distance transportation of miRNAs probably associates with the protein trafficking. However, the role of the protein in the regulation of PSR is currently unclear.

Methods

To study the protein involved in systemic regulation for plant PSR, it is hard to distinguish the protein are locally or systemically produced. In attempt to distinguish local and systemic signal, we developed a method by heterograft two kinds of vasculature to collect systemically transported proteins from phloem sap in different Pi level. The collected sap was analyzed by high throughput multiplex quantitative proteomic approach to discover the probable protein candidates for facilitating the transportation of miRNA or functioning as the signal for regulation PSR.

Results and Discussion

In this study, we have identified more than one thousand proteins which were systemic translocated, and about 70 of systemic transported proteins can be induced by Pi deficiency. Some of those identified proteins categorized as RNA-binding and tRNA synthetase proteins which are important candidates for facilitating miRNA trafficking. For the proteins not involve in the RNA binding, but systemically transported under low Pi. We suggested those protein candidates might involve in the miRNA independent regulation of PSR.

Conclusion

This study highlights the role of proteins in the systemic regulation of plant Pi deficiency and can be used to study the role of proteins in the systemic regulation of other plant physiologies.

Keywords: Phosphate Starvation Responses (PSR), Systemic Signall, RNA-binding Proteins, Proteomics

WP12-04

Proteomic analysis of "Oriental Beauty" oolong tea leaves with different degrees of leafhopper infestation

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Introduction and Objectives

Oriental Beauty, a variety of oolong tea native to Taiwan, is highly prized by connoisseurs for its unique fruity aroma and sweet taste. Oriental Beauty owes its unique quality to the tea green leafhopper (Jacobiasca formosana), an insect of the Cicadelleidae family that feeds on the tea leaves. Leaves of Oriental Beauty vary in appearance, aroma and taste depending on the degree of leafhopper infestation. However, no studies have looked at differences in the proteome in leaves of Oriental Beauty with different degrees of tea green leafhopper infestation.

Methods

In this study, we used 2DE and nanoscale LC/MS/MS (nano-LC/MS/MS) to investigate the differential expression of proteins in tea leaves with different degrees of leafhopper infestation. Those proteins may produce or alter metabolites which are actually the molecules responsible for the unique aroma and taste of Oriental Beauty Tea.

Results and Discussion

A total of 65 protein spots with at least two-fold differences in expression among leaves with low, medium and high degrees of infestation were excised from the gels and 157 proteins were identified by nano-liquid chromatography-electrospray/tandem mass spectrometry (nanoLC-ESI-MS/MS). In gene ontology analysis, it showed that the majority of those proteins participated in biosynthesis, carbohydrate metabolism, transport, response to stress, and amino acid metabolism.

Conclusion

In this study, we used gel-based proteomic techniques to investigate the differential expression of proteins in Oriental Beauty tea leaves with different degrees of leafhopper infestation. A total of 149 proteins were found have different expressions significantly. These proteins related with the appearance of leaves, defensive mechanism, signal transduction and transport activities, repair mechanism, and taste-related proteins. Our results supposed that when Oriental Beauty Tea infested by tea green leafhopper, the protein expression profiles changed, and those proteins may produce or alter metabolites which are actually the molecules responsible for the aroma and taste. In future, we will investigate the aromatic compounds of different infestational level of Oriental Beauty tea, and try to connect the interactions between proteins and aromatic compounds.

Keywords: Proteomics, Oriental Beauty tea, Green leafhopper, 2-DE.

WP12-05

Proteometabolomic study illustrates dual role of oxalic acid in anti-nutrient signaling and non-host resistance

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Introduction and Objectives

Fruit is an assimilator of metabolites, nutrients, and signaling molecules, thus considered as potential target for pathogen attack. In response to patho-stress, such as fungal invasion, plants reorganize their proteome and reconfigure their physiology in the infected organ. This remodeling is coordinated by a poorly understood signal transduction network, hormonal cascades, and metabolite reallocation. Oxalic acid (OA), an ubiquitously present metabolite plays dual role in fungal pathogenicity in a concentration dependent manner. While at higher concentration it induces programmed cell death leading to fungal invasion, low oxalate build resistance in plant. Although OA has been identified as a virulence determinant for rot disease caused by Sclerotinia sp., our understanding of how oxalate downregulation impart host immunity is limited. The aim of the study was to explore organ-based proteometabolomic alterations in the susceptibility of oxalate rich and low oxalate heterotrophic fruit to necrotrophic fungal attack.

Methods

We conducted time-series protein and metabolite profiling of Sclerotinia rolfsii invaded wild-type and E8.2-OXDC tomato (Solanum lycopersicum) fruit using 2-DE coupled LC-MS/MS and GC-MS analysis (1). Correlation network was built to identify oxalate induced disease and immunity related pathways. Furthermore, immunoblot and qRT-PCR analyses was performed to validate the omics datasets.

Results and Discussion

The differential display of Sclerotinia infected tomato fruit proteome revealed 105 patho-stress responsive proteins (PSRPs), while network analysis identified major protein hubs pointing towards the onset and context of disease signaling and metabolic pathway activations. A step further, to investigate the role of OA and the metabolic consequences of oxalate down-regulation in oxalic acid-accumulating tomato fruit, we generated transgenic tomato plants (E8.2-OXDC) expressing an oxalate degrading enzyme (FvOXDC) from the fungus Flammulina velutipes specifically in the fruit. Our data showed that ectopic expression of FvOXDC specifically degrades OA in tomato (2). To elucidate low oxalate regulated molecular mechanism imparting immunity, a comparative proteomics approach has been applied to E8.2-OXDC tomato fruit displaying fungal resistance. Mass spectrometric analyses identified 92 OXDC-responsive immunity related protein spots (ORIRPs) presumably associated with acid metabolism, defense signaling and endoplasmic reticulum stress. Metabolome study indicated differential abundance of some of the organic acids paralleling the proteomic analysis (3). Further, we interrogated the proteome data using network analysis that identified modules enriched in known and novel disease- and immunity-related prognostic proteins.

WP12-05

Conclusion

Taken together, our data demonstrate that low oxalate may act as metabolic and immunity determinant through translational reprogramming.

Keywords: Proteometabolomics, Oxalic acid, Non-host resistance, Antinutrient, Tomato fruit, Plant

Proteomic Analysis of Bee Pollen from a Natural Forest in Central Taiwan

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Introduction and Objectives

Researchers are becoming increasingly aware of the nutritional and therapeutic value of bee pollen. Bee pollen contains all of the substances required for the growth and development of plants, including amino acids, lipids (triglycerides, phospholipids), proteins, sugars, vitamins, minerals and phenolics. This study addressed the lack of proteomic analysis in this area by seeking to develop an efficient method for the extraction and separation of total protein found in bee pollen.

Methods

Bee pollen samples were first separated into three groups according to color. In order to extract high-quality of total protein from the bee pollen, trichloroacetic acid (TCA)/Acetone was then used for protein precipitation followed by SDS and the phenol-based method with minor modification. Whole proteins from the different groups of bee pollen were analyzed and detected using one-dimensional (1-D) SDS-PAGE and two-dimensional electrophoresis (2-DE) followed by Coomassie Blue staining.

Results and Discussion

By using the matrix-assisted laser desorption/ionization (MALDI) time-of-flight/time-of-flight (TOF/TOF) and liquid chromatography–tandem mass spectrometry (LC-MS/MS) method, we identified 10 distinct proteins from 4 spots and 80 bands in bee pollen. Functional category analysis indicated that these newly identified proteins are involved primarily in transport, stress responses, transcription, and metabolic pathways.

Conclusion

In conclusion, the modified protocol is based on the phenol/SDS method described previously. For comparison, proteins were extracted from bee pollen samples using the modified method described here and the original phenol/SDS method. The total protein that was obtained by modified method improved the resolution and clear separation of more bands than were described in the previous studies. This work presents preliminary results from the proteomic analysis of bee pollen.

Keywords: bee pollen; proteomic analysis

Quantitative Proteomics of Phaeodactylum tricornutum in Acidified Environment

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National Taiwan Ocean University, Taiwan

Introduction and Objectives

Carbon dioxide emission rate has been rapidly increasing since industrial revolution. The continuously rising CO2 concentration leads to global warming and ocean acidification. With seawater pH value drops, the primary producer in the ocean, the diatoms, are greatly affected. In order to investigate the effect of ocean acidification on marine ecosystem, the quantitative proteomics approach was utilized in this study. Diatom Phaeodactylum tricornutum was selected as the model organism.

Methods

Phaeodactylum tricornutum was cultured in a special designed incubator with stable temperature control, 14h/10h day/night cycle, continuous pH monitoring, and automatic CO2 adjustment system. The quantitative proteomics approach was performed using stable isotope labeling by amino acids in cell culture (SILAC) method. Either Na14NO3 or Na15NO3 were regard as the nitrogen source for Phaeodactylum tricornutum culture. After culture, Phaeodactylum tricornutum cells were collected and lysed for protein extraction. The in-gel trypsin digestion were performed for the quantitative proteomics analysis of diatom proteins.

Results and Discussion

Phaeodactylum tricornutum is one of two diatoms with genome sequenced. In the Diatom EST database, over 30,000 expressed sequence tags (ESTs) from Phaeodactylum tricornutum have already been identified. Therefore, Phaeodactylum tricornutum serves as a good model organism for understanding cellular response of primary producers in the ocean when facing environmental changes. The effects of ocean acidification on diatoms were addressed in a few studies before; however, systematic and comprehensive proteomics analysis is still obscure. Additionally, few studies were taken under a well-controlled environment which involves an automatic CO2 adjustment system to maintain a stable pH condition in diatom culture. The 15N-labeling SILAC method has been previously examined in Phaeodactylum tricornutum culture. There are no obvious biological differences in cell morphology, growth rate, and proteomics profile between 15N and 14N culture conditions. Thus, the 15N-labeling SILAC is a suitable method for quantitative proteomics analysis. Our preliminary result shows that levels of photosynthesis-related proteins were significantly altered in acidified environment. More detailed analyses are still undergoing.

Conclusion

In this work, we confirm that quantitative proteomic analysis could use 15N-label SILAC in Phaeodactylum tricornutum. Using this quantitative mothed, the diatom wouldn't change their growth rate , and protein level. CO2 controller effectively control pH value of Phaeodactylum tricornutum growth environment in small region for real time. In total, 5036 unique proteins were identified from Phaeodactylum tricornutum.2386 proteins were quantified in MassMatrix. Our result show acidic environment promote Phaeodactylum tricornutum to increase carbohydrate and oil and growth rate. In future, we will make the mechanism clear.

Keywords: Phaeodactylum tricornutum, 15N-label SILAC, proteomic

Comparative Proteomics of Chlorella sp. FC2 IITG during Nitrogen-starvation using iTRAQ and MRM based Mass Spectrometry

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Introduction and Objectives

Algae are considered as third-generation biofuel due to several rationales, however till date high volume biofuel production from algae is not economically viable. Nitrogen (N) starvation elevates gross lipid accumulations particularly triacylglycerides (TAGs) but the net production is reduced due to severe reduction in biomass. Overall, N-starvation alters carbon concentrating mechanisms to induce TAG accumulations; however the exact molecular mechanisms triggering this phenomenon are unexplored. Investigation of the protein pool regulated during N-starvation in Chlorella sp. FC2 IITG may provide insights into the molecular knowhow.

Methods

Comparative proteomic analysis of various N-starvation time-points 40 h, 88 h and 120 h and N-sufficient conditions were performed using difference in gel electrophoresis (DIGE) and 4-plex Isobaric tag for relative and absolute quantitation (iTRAQ) analysis in combination with ESI-Q-TOF mass spectrometry for three biological replicates. Validations of a few significantly regulated proteins were performed using Multiple Reaction monitoring (MRM)-based mass spectrometric analysis, and immunoassay-based approaches (Western blotting).

Results and Discussion

A total of 137 differentially expressed proteins were identified using shotgun approach, among these 66 were expressed globally during N-starvation while others served as adaptors for transition from one time-point to other. DIGE complemented the iTRAQ analysis and 7 out of 13 proteins were found in common. Sedoheptulose 1, 7-bisphosphate, malate dehydrogenase, phosphoribulose kinase, triose phosphate isomerase, superoxide dismutase and reversibly glycosylated proteins were validated using immuno- and MRM-assays. Several trends including increase in energy metabolism, protein breakdown via proteasomes, reduction in chlorophyll, altered amino acid metabolism, and enhanced fatty acid biosynthesis and elongation were observed.

Conclusion

Comprehensive knowledge of the differentially modulated protein repository during N-starvation has improved our molecular understanding of the phenomenon and may be translated to generate transgenic algae with enhanced oil amassment.

Keywords: Chlorella sp FC2, N-starvation, comparative proteomics, Multiple Reaction Monitoring

Quantitative Proteomics of Phaeodactylum tricornutum in Phosphate Limited Environment

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Introduction and Objectives

Phosphorus plays decisive roles in living organism such as being the building block of cell membrane, and the genetic information storage. In order to investigate how phosphorus availability affects cellular functions at the protein level in diatoms, a modified stable isotope labeling by amino acids in cell culture (SILAC) method coupled with mass spectrometry based quantitative proteomics approach was performed in this study. Phaeodactylum tricornutum serves as a good model organism for understanding the environmental impact to cellular responses in diatoms because of the available comprehensive genomics and proteomics information. The s

Methods

Phaeodactylum tricornutum was cultured in a temperature controlled incubator with 14h/10h light/dark cycle. In order to examine the biological effect of phosphate to diatom Phaeodactylum tricornutum through quantitative proteomics approach, sodium nitrate with stable isotope of nitrogen, Na14NO3 and Na15NO3, were utilized as the nitrogen source in culture medium. The phosphate concentrations for normal environment (NP medium) and low phosphate condition (LP medium) were 36.6 and 3 uM, respectively. After 10 days cultivation period, diatom Phaeodactylum tricornutum in 14N-NP and 15N-LP medium were collected and mixed together followed by protein extraction, SDS-PAGE separation, and in-gel trypsin digestion procedure for sample preparation. Tryptic peptides were analyzed by LC-MS/MS and the 14N/15N ratio of peptide signals were utilized for protein quantitation.

Results and Discussion

Nitrogen isotopes (14N and 15N) were used as the SILAC labeling for protein quantitation in this study. In the 10 days cultivation period, the diatoms growth curves indicated that using 15N as the nitrogen source has no effect to Phaeodactylum tricornutum growing. Additionally, the cell morphology of Phaeodactylum tricornutum showed no difference from Na14NO3 and Na15NO3 medium. Finally, the quantitative proteomics result demonstrated that protein expression levels in both 14N and 15N medium were almost identical. According to these evidences, using stable isotope of 15N as the nitrogen source in diatom culture for quantitative proteomics study is feasible. As for the effect of insufficient phosphate in environment, the quantitative proteomics experiment in diatom Phaeodactylum tricornutum is still undergoing.

Conclusion

According to protein quantitative date, many molecular function in Phaeodactylum tricornutum were down regulated in low-phosphate environment, ATP synthase, photosystem, and G-protein, for example. However, triosephosphate isomerase, glyceraldehyde 3 phosphate dehydrogenase, phospholipid scramblase, and 2

phosphoglycerate dehydratase were up-regulate. These protein can help cell obtain phosphate as much as possible. Especially phospholipid scramblase, a protein can translocate phospholipid between the two monolayers of cell membrane. We hypothesize cell may obtain phosphate from hydrolysis phospholipid.

Keywords: SILAC; Phaeodactylum tricornutum; Phosphate Limited

Proteomic Analysis to Reveal the Calcium Function on Protein Glycosylation in Endoplasmic Reticulum of Soybean under Flooding and Drought Stresses

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Introduction and Objectives

Soybean is sensitive to flooding and drought stresses, which limit its growth and production. The endoplasmic reticulum (ER) mediates protein quality and calcium content; and proteins related to protein glycosylation and signaling were responsive to both stresses. To investigate the calcium function on protein glycosylation, quantitative proteomic analysis was performed in soybean under both stresses.

Methods

Two-day-old soybeans treated without or with calcium and its inhibitors were exposed to flooding and drought stresses. Cellular proteins, ER proteins, and glycoproteins were analyzed using gel-free/label-free proteomic technique. The gene expression determination, exogenous calcium quantification, and bioinformatic annotation were further carried out to validate the proteomic data.

Results and Discussion

The ER was isolated with high purity, which was assessed using enzyme assay and immunoblot analysis. ER proteins related to protein glycosylation and signaling were responsive to both stresses. Among them, protein abundance of calnexin was decreased under both stresses. Protein disulfide isomerase like proteins and heat shock proteins were significantly decreased under flooding and drought, respectively. The number and abundance of glycoproteins were decreased under both stresses. Out of calcium related proteins, gene expressions of calmodulin binding proteins, RAB homolog1, and calcium transporting ATPase were downregulated under both stresses. Inositol 1,3, 4-trisphosphate 5/6-kinase family protein was only upregulated under flooding. Furthermore, cytosolic calcium content was increased under both stresses. In addition, ERAD-E3 ligase was upregulated under flooding; while BiP was downregulated and upregulated under flooding and drought, respectively.

Conclusion

These results suggest that reduced glycoproteins under both stresses might be due to dysfunction of protein folding through calnexin/calreticulin cycle. Additionally, to eliminate ER stress, ER-associated degradation might be activated under flooding; however, unfolded protein response might be stimulated under drought, which requires calcium release under stresses.

Keywords: plant, stress, endoplasmic reticulum, calcium, glycosylation

WP12-11

Proteomic and Transcriptomic Analyses to Reveal the Tolerance Mechanism in Soybean at Initial Flooding Stress

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Introduction and Objectives

Soybean is sensitive to flooding stress, which markedly reduces its growth and yield. Although the effects of flooding on soybean have been explored, the mechanism of tolerance to initial flooding stress remains unclear. To identify the marker proteins and genes for flooding tolerance in soybean at initial stage, proteomic and transcriptomic techniques were used.

Methods

Results and Discussion

A total of 146 proteins and 31 genes were commonly changed between mutant line and ABA-treated soybean at initial flooding stress, which were mainly related to protein synthesis and RNA regulation. The initial flooding tolerance in soybean was regulated by the commonly identified 140 proteins and 12 genes, which had same change tendency between mutant line and ABA-treated soybean. Among the commonly changed proteins, nascent polypeptide-associated complex/chaperonin 20 and glycine rich RNA binding protein/eukaryotic aspartyl protease, which are involved in protecting newly synthesized proteins and enhancing the activities of antioxidases, respectively, were increased in protein abundance and mRNA expression at initial flooding stress. Among the commonly changed genes, metalloproteinase, which is involved in promoting degradation of extracellular matrix proteins, was up-regulated in control; however, it was down-regulated in mutant line and ABA-treated soybean at initial flooding stress.

Conclusion

These results suggest that initial flooding tolerance in soybean is produced might be through protecting newly synthesized proteins and enhancing activities of antioxidases to remove active oxygen species. Furthermore, metalloproteinase might involve in inhibition of cell wall loosening and contribute to tolerance at initial flooding stress in soybean.

Keywords: plant, stress, proteomics, transcriptomics

WP12-12

Comparative phosphoproteomic analysis of dormant and after-ripened seeds of barley

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Introduction and Objectives

Barley (Hordeum vulgare) is one of major cereals in the world. Seed dormancy is known as a critical issue for maintenance of seed quality of barley, and it is dynamically changed from dormant (D) seed to after-ripened (AR) seeds. However, it is still unclear how the dormancy is regulated at intracellular signaling level. Previous studies revealed that a phytohormone abscisic acid (ABA) has a central role in the regulation of seed dormancy, and protein phosphorylation is quite important in ABA signaling (Umezawa et al, 2010). Here we took a phosphoproteomic approach to elucidate ABA signaling pathways in D and AR seeds.

Methods

D and AR seeds of barley cv. Golden Promise were collected, and half-cut seeds were subjected to 50 µM ABA for 0, 1, 3 and 10 h. Then barley embryos were isolated, and crude extracts were prepared using 8M Urea buffer. After tryptic digestion and HAMMOC enrichment, phosphopeptides were analyzed with a LC-MS/MS system (AB-SCIEX TripleTOF 5600). Peptides and proteins were identified by automated database searching using Mascot (Matrix Science). Each phosphopeptide was quantified using Skyline v3.5 software (University of Washington). Then phosphopeptides were classified by motif analysis using Motif-x algorithm, or GO annotation by BLAST2GO.

Results and Discussion

We identified 1,210 phosphopeptides generated from 673 phosphoproteins in barley seeds. Quantitative analysis detected 319 and 219 ABA-responsive phosphopeptides in D and AR seeds, respectively. Some of ABA signaling proteins was highly phosphorylated in D, suggesting that ABA response in D could be active much more than that in AR. Motif analysis detected the RxxS motif which is phosphorylated by SnRK2, and the SF motif as the most abundant phosphorylated motif in barley seeds. The RxxS motif and SF motif were phosphorylated to the maximum level at 10 h and 3 h in D and AR seeds, respectively. However GO terms of detected proteins are similar between D and AR seeds. Taken together, the results suggested that ABA responsiveness in D and AR seeds should be different at the phosphosignaling level.

Conclusion

Phosphosignaling pathways in barley embryo were surveyed by phosphoproteomic analysis, in which significant differences were found between D and AR seeds. Further analysis will be required to analyze functions of phosphoproteins and discover biologically important proteins involved in barley seed dormancy .

Keywords: Barley (Hordeum vulgare), seed dormancy, Abscisic acid, Phosphoproteome

TO10-05

Proteomics in Food safety: monitoring competition between Listeria monocytogenes and Lactococcus lactis by Imaging Mass Spectrometry

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Introduction and Objectives

Microbial competition is a mechanism aimed at eliminating one of the microbial populations from their common habitat, especially when competition is focused on a single resource and when the populations do not otherwise interact. Different factors can contribute to the outcome of microbial competition, such as efficiency of nutrient acquisition, strategies for surface attachment and the production of molecules to kill the niche competitors, limit their growth or modulate their metabolism. Specifically, production of compounds that kill or limit the growth of competing strains can promote niche monopolization. The released compounds include secondary metabolite antibiotics, peptides or low-molecular-mass organic compounds. In microbiology, changes in specialized metabolite production are measured using phenotypic assays. Moreover, advances in mass spectrometry-based techniques including imaging mass spectrometry (IMS) enable to directly visualize the production of specialized metabolites from microbial colony without using tags or labels. In this work, a combination of IMS and LC-MS/MS was used to study the competition between Listeria monocytogenes (LM) and Lactococcus lactis (LAC) to investigate the metabolic profile of each bacteria in the interacting microbial colonies.

Methods

Strains Lactococcus lactis ATCC 11454 and Listeria monocytogenes ATCC 19115 have been used in the present study. Bacterial colonies were allowed to grow for 48 h at 30°C before transferring to a Bruker MALDI Plate. All colonies were subjected to MALDITOF IMS by a Bruker Daltonics Autoflex Speed . IMS data analysis was performed using Bruker Daltronics FlexImaging. General chemical extraction of the samples was performed. The agar from entire single plates was sliced into small pieces and extracted with methanol plus 0.1% formic acid. LC MS/MS analysis was performed using a Bruker Daltronics Maxis qTOF.

Results and Discussion

IMS analysis revealed several interesting compounds during interaction of microbial colonies. At least six compounds are uniquely expressed during the interaction between LM and LAC. Among these, we focused our attention on three compounds: Cyclo-(Leu-leu), Cyclo-(Phe-Tyr), Cyclo-(L-Phe-L-4-Hyp). These compounds are cyclic peptides, isolated by Lactobacilli, with a biological activity.In particular, they play an important role in bacterial cell to cell communication. Probably, these peptides have a role in inducing of the transcription of gene coding for Nisine.

Conclusion

Microbial competition between Lm and Lac was monitored by combination of IMS and LC MS/MS in order to investigate the metabolic profile of each bacteria in the interacting microbial colonies. Several interesting compounds, uniquely expressed during interaction of microbial colonies, were obtained. These results could be useful to setup new molecular strategies in the control of bacterial species for a better food safety.

Keywords: Microbial competition, imaging mass spectrometry, specialized metabolites, food safety

WO09-02

Single-species proteomics, multi-species metaproteomics, trends and opportunities to read-across in environmental assessment

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Introduction and Objectives

Marine environmental assessment has been performing for more a decade ago applying proteomics to single species bioindicators. Proteomic-based methods can assemble multivariate information providing a global view of effects of exposure. On the other hand, the analysis of biodiversity, ecosystem function and its consequence for ecosystem services has recently addressed from metaproteomics on earth microbiomes. The question now is not only if the metaproteomics analysis from environmental samples can read the drivers of ecosystem function and but also if the dynamics of the microbiome could be correlated with the single species response.

Methods

We present a shotgun proteomic analysis of marine mussel (Mytilus edulis) after long-term exposure to low salinity and propranolol in a Baltic Sea microcosm and compare with a metaproteomics analysis of Baltic Sea sediment co-exposed with mussels in the microcosms. We tested if: i) applying shotgun proteomic analysis to M. edulis gills samples following a proteogenomic strategy increases the identification of marine model organism and unravel the response; ii) metaproteomic-based assessment can estimate variation in which are the drivers of the adaptation to the exposure to pollution and to abiotic changes. We have performed a laboratory experiment building a microcosm and exposed for a 6 week.

Results and Discussion

The proteomics method with a proteogenomic strategy supports a large systematic identification of M. edulis proteome covering over 2000 proteins. The quantitative proteomic analysis unrevealed whichmolecular mechanisms could drive the adaptation to low salinity stress. For the metaproteomic analysis, using a combination of two different search engines: Unipept or MEGAN against different database our results indicated that the salinity has an important impact into the response. The complexity of soil microbial community could be correlated to both changes in the biodiversity and the molecular responses of the communities and therefore interprete and predict the ecosystem response to identical stressors.

Conclusion

By integrating data from individual species response with ecosystem response to identical stressors, we can explore the prediction capabilities of these methodologies to develop metaproteomic based environmental assessment. The two experiment strategies converge reinforcing the idea of the impact of low salinity in combination with anthropogenic pollutants and anticipate critical physiological condition. The change in the salinity in the microcosm has a stronger impact in the microbial responses than the changes in the concentration of stressor.

WO09-02

Keywords: metaproteomics, microbiome, assessment, proteogenomics

WO09-03

Gastric Bypass surgery clearly perturbs the community structure and the functional composition of the intestinal microbiota

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Introduction and Objectives

Roux-en-Y gastric bypass surgery (RYGB) is performed to help reduce body weight to less harmful levels in severely obese patients. Following RYGB patients are only able eat small amounts of food and the nutrients are not efficiently digested in the small intestine, leading to a restriction in overall caloric intake. Globally this operation is performed over 150,000 times each year. How this surgical rearrangement of the gut impacts on the intestinal microbiota has only been investigated to a small degree, especially research on the functional level is lacking.

Methods

In this study we investigated in rats the effect of RYGB on the microbiota from the ilium as well as the colon and compared these to animals with sham surgery. From the two gut localities, we investigated the microbiota inhabiting the mucus layer and the intestinal lumen separately. To resolve the community structure in regard to taxonomy and enzymatic functionalities 16S rRNA gene sequencing and metaproteomics was performed.

Results and Discussion

The results reveal profound changes in the taxa distribution and the enzymatic functional capacity of the microbiome in the ilium as well as the colon after RYGB. For example, for taxa distribution we observed in the ilium and colon greater prevalence of Actinobacteria especially Bifidobacteriales after RYGB with Firmicutes at lower abundances. Enterobacteriales was also more prevalent in the colon of RYGB then in the control. An example for changes on a functional level in the ilium was that the relative numbers of Actinobacteria proteins involved in amino acid metabolism or carbohydrate metabolism were higher in RYGB. In the colon proteins from Clostridia belonging to the function of carbohydrate metabolism or the function energy production were seen at lower levels in RYGB whereas proteins from Actinobacteria which are involved in carbohydrate metabolism or cell motility were observed at higher relative numbers.

Conclusion

With these results and further research it may be possible to design specific diets or medical interventions to limit the perturbation in the microbiota following RYGB or to remove specific pathogenic taxa inhabiting the intestinal tract after surgery.

Keywords: Microbiome; surgery; animal model; microbial proteomics; intestinal tract

WP13-01

The virulence mechanism mediated by a metalloprotein in Streptococcus pneumoniae

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Introduction and Objectives

Streptococcus pneumoniae is an important human pathogen causing a variety of severe diseases, which predominantly relies on strong adhesion factors and toxic proteins to infect humans. Iron (Fe^{3+}) and Manganese (Mn^{2+}) are essential for bacterial virulence. A potential metal transporter protein named Lpm was significantly overexpressed in the S. pneumoniae in Mn^{2+} depleted medium which was found by the proteomics study of our group. Our aim is to explore the function of Lpm protein and to search for a possible drug target.

Methods

We constructed the lpm mutant strain to investigate the biological function of Lpm. The adherence factors such as ply, cbpA, pspA, pcpA were detected by qRT-PCR. The 2-DE combined with MS based proteomic study was performed to find the location of Lpm and to explore the interaction network of this protein. The ATP content in the cells were determined and compared.

Results and Discussion

In contrast to wild type (WT) strain, the knock out of lpm weakened the cell adherence or invasion ability to host cells. The proteomic result showed that 31 proteins were over-expressed and 44 proteins were down-regulated in lpm mutant strain. A large number of down-regulated expressed proteins are involved in cell energy metabolism and other depressed proteins are participated in amino acid synthesis metabolism, glycolysis metabolism and other macromolecular biosynthesis meaning that the depletion of lpm gene interfered with the energy metabolism pathway. The lpm gene knockout caused the decrease of ATP level in bacteria.

Conclusion

These data derived from the biochemical and proteomics analyses clarified the virulence related biological function of a novel protein Lpm in S.pneumoniae and provided insightful clues for further investigations on virulence mechanism in bacteria.

Keywords: Metal transporter protein/ Virulence mechanism / 2-DE / Energy metabolism pathway

WP13-02

Comparative proteomic analysis of virulence variations in Xanthomonas campestris pv. campestris strain 17, 11A and P20H

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Introduction and Objectives

Xanthomonas campestris pv. campestris (Xcc) is a Gramnegative plant pathogenic bacterium causing black rot in crucifers, resulting in tremendous loss in agriculture. The ability of Xcc to infect plants successfully depends on certain factors including extracellular enzymes, exopolysaccharides and biofilm production. A newly isolated pathogenic XC17 from an infected cabbage leaf for some phenotypic characteristics have previously been implicated as factors contributing to pathogenicity. A wild-type strain of X. campestris, XC11, was found to have lost its pathogenicity spontaneously after frequent subculturing for years. This non-pathogenic derivative, designated XC11A. X. campestris P20H was a non-mucoid mutant previously isolated from XC11A by mutagenesis with nitrous acid. Recently the proteomics have been developed more widespread, the techniques can offer insights into the quantity and quality of the final gene products. In this study, we aim to elucidate the physiological and molecular mechanism response to virulence variations in XCC by using proteomics method.

Methods

We first used two-dimensional gel electrophoresis (2-DE) to resolve the expressed proteome in total proteins of XCC, and then used nanospray liquid chromatography/linear ion trap mass spectrometer (nano-LC/LTQ-MS) to identify the differentially expressed proteins in total proteins of XC17, XC11A and XCP20H. Meanwhile, proteins of extracellular, membrane and intracellular were extracted separately for proteomics identification via LTQ-MS and triple TOF 6600 mass spectrometer analysis.

Results and Discussion

The result shows many proteins and signaling molecules involved in TCSTSs of XC17 had higher expression than XC11A and XCP20H. Therefore, TCSTSs could promote pathogenic genes expression. There were also higher expression of transcription and translation factor proteins, DNA polymerases, ribosome, and tRNA ligase of XC17. And extracellular enzymes and enzymes involved in the synthesis of exopolysaccharide of XC17 had upregulated expression as well. Simultaneously, NADH dehydrogenase and ATPase of membrane proteins had higher level expression, resulted in supplying more engery for secretion of extracellular enzymes by type II secretion system. Since XC17 had higher expression of outer membrane beta-barrel proteins (OMPs) which preventing access of toxic molecules from extracellular environment into bacterial cell, such that XC17 had better survival ability in the face of stress.

Conclusion

Through the above results, pathogenicity of XCC originated in complex regulation of physiological processes. Although both XC11A and XC17 could secrete EPS, XC11A

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secreted far less. Moreover, XCP20H had the least protein expression whether synthesis of EPS or other pathogenesis related proteins, such that XCP20H also lacked of pathogenicity. These identified proteins may be helpful in elucidating the molecular basis of virulence variations in Xcc.

Keywords: Xanthomonas campestris pv. campestris / black rot / SDS-PAGE / 2D-PAGE / LC-MS/MS

WP13-03

Biodegradation of Crude Oil and Diesel by Novel Microorganism, Arthrobacter sp. MWB-30

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Introduction and Objectives

Among these microorganisms, several Arthrobacter species are able to act as an effective degrader of PAHs contaminants. Although Arthrobacter species has previously been shown the successfully degrade various single pollutants and roughly studied their metabolic pathway, it is more complicated to assess into the crude-oil and diesel due to their material complexities. . In addition, even metabolic pathway of Arthrobacter identified completely on single components, ultimately expression patterns of enzymes and genes responsible for decomposition should considerably modified by reciprocal action of complicated composition of crude-oil and diesel. Therefore, understanding the degradation pathway of crude-oil and diesel is critical to apply for the bioremediation process and develop the clean-up technology including bacterial modification and setting up the bacterial consortia based on proteomic and genomic approach.

Methods

Crude-oil used in this study was a spilled oil by accident in Tae-an, Korea.. For the evaluation of degradation of hydrocarbon compound in respective oil caused by AMWB30 strain, we conducted GC-MS analysis. For the prediction of the metabolic pathway of AMWB30 strain, annotated coding gene set previously reported were queried in BlastKOALA. Analysis of tryptic-digested peptides was performed using a nano-ACQUITY Ultra Performance chromatography system and LTQ-TOF MS.

Results and Discussion

Arthrobacter sp. MWB-30, isolated from crude-oil contaminated seashore, degraded up to 38 and 29% of hydrocarbons in crude-oil and diesel for 15 days, respectively. Genomic analysis shows that this strain has major enzymes for decomposition of alkanes and PAHs (polyaromatic hydrocarbons) that mainly constitute crude oil and diesel. Basis on the gene expression and proteomic analysis, n-Alkane degradation thought to be initiated by two alkane monooxygenase, novel LadA-like enzyme and cytochrome P450, and tightly regulated by AraC transcriptional regulator. In decomposition of PAHs, novel ring-hydroxylating dioxygenase (AMWB30_04640) plays a critical role in initiation of ring cleavage for various PAHs. Furthermore, degradation of catechol as integrated central intermediate of PAHs should be dominant pathway mediated by extradiol cleavage process in crude oil and diesel decomposition.

Conclusion

Arthrobacter sp. MWB-30 strain can able to initiate broad-rage of n-alkanes degradation via action of various monooxygenases, particularly novel LadA-like monooxygenase participate in long-chain alkane decomposition and degrade diverse polyaromatic hydrocarbons by tightly regulated metabolic enzymes via oil constituent-dependent

manner. This study provides an insight in the complex metabolism and regulatory mechanisms occurring in the presence of crude-oil or diesel for the first time in Arthrobacter species.

Keywords: Arthrobacter, n-Alkane, polyaromatic hydrocarbon, degradation

Isobaric Tagging-Based MS Quantification of HIV-1/gp120/tat in Astrocytoma: Implications for HIV-associated neurodegeneration

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Introduction and Objectives

One of the consequences of HIV-1 infection among patients is HIV-associated neurocognitive disorder (HAND). It is suggested that the degree of Neuro-AIDS vary according to HIV-1 clades. HIV-1 clades B and C account for the majority of infections, showing different neuropathogenicity. So far, no mechanism has been established; therefore, it remains the subject of active research. We hypothesized that HIV-1 clade B and C Gp120 proteins exert differential protein peptide fingerprinting and oxidative stress response in human astrocytoma cells.

Methods

2D-Gel Electrophoresis and Tandem Mass tag (TMT) isobaric labeling quantitative mass spectrometry approach was used on human astrocytoma cells treated with Gp120 proteins from HIV-1 clade B and C.

Results and Discussion

Several protein signatures were significantly up regulated by Gp120 such as Endoplasmic Reticulum (ER), oxidative stress markers, structural, cytoskeleton, and a numerous metabolic factors. HIV-1 clade B and C proteins induced expression of CXCR4 and CCR5 co-receptors and a release of key inflammatory cytokines such as: IL-6, IL-8,and IFN-y. Moreover, Gp120 C induced nitrite release, the production of reactive oxidative species while Gp120 B induced the production of key ER stress markers such as Calreticulin (ER-60), Protein Disulfide Isomerase (PDI), Glucose regulating protein 78 (GRP78), among others.

Conclusion

Our findings demonstrate that HIV-1 clade B Gp120 protein appear to induce an endoplasmic reticulum UPR while HIV-1 clade C seems to be associated with a oxidative stress mechanism in astrocytoma cells.

Keywords: HIV/AIDS, HAND, neurocognitive disorder

Novel characteristics of highly virulent ribotype 027 - Quantitative and qualitative proteomic analysis of C. difficile clinically relevant ribotypes

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Introduction and Objectives

Clostridium difficile is a causative agent of C. difficile infection (CDI) with significant mortality and economic burden. The virulence factors have been studied extensively by the means of the genomic based techniques but only limited number of studies was performed on the proteomic level. The availability of high-resolution mass spectrometry together with the relevant software tools enabled the label-free analyses (LFQ) of culture supernatant of selected representative PCR-ribotypes of C. difficile. The aim of this study was to determine the phenotypic characteristics clearly differentiating virulent ribotypes, e. g. 027 from the less virulent with the absence of the Clostridium difficile binary toxin (CDT) genes or the genes for the Clostridium difficile toxins A (TcdA) and B (TcdB).

Methods

The panel of C. difficile strains in the experiment comprised of ribotypes occurring in Europe: ribotype 027, referred as hypervirulent, 176, genetically related to 027 lineage and ribotypes 001,014,005, 012, 078. Non-toxigenic PCR-ribotype 010 was involved as negative control. Biological replicates were cultured and amount of the bacteria /analyzed by Optical Density/ of each cultivate was set to the same value at the beginning of each cultivation. The supernatants were precipitated and subsequently processed based on the Filter Aided Sample Prep (FASP) protocol followed by trypsin digestion and subsequently separated and analysed using liquid chromatography on-line coupled with mass spectrometer Q-Exactive (Thermo Scientific). The MS/MS data processed and label free quantification was then performed employing MaxQuant and Perseus platforms.

Results and Discussion

Regarding the known virulence factors, TcdA and B were proved as a significantly produced only in the PCR-ribotypes 027 and 176, genetically related cluster linked with severe outcome of CDI and outbreaks. In addition, the presence of both Clostridium difficile binary toxin CDT /A and B type/ was proved in the secreted fraction with a high level. Interestingly, neither TcdA/TcdB nor CDT were detected in the PCR-ribotype 078, so called as hypervirulent, apparently representing different phenotype. Finally, TcdA production was proved in the PCR-ribotypes 005 and 012 while in the ribotypes PCR-001, 078 and 001 it remained undetected. Moreover, the significant abundance of some flagellar proteins was observed in the highly virulent ribotypes 027, 176 and further in the ribotyopes 014 and 005 suggesting the critical role of structures involved in motility and adhesion in CDI. Moreover, further proteins clearly connected with the essential pathophysiological pathways were able to distinguish highly virulent group comprised of

ribotypes 027/176 and 078.

Conclusion

The employment LFQ showed its relevancy in clinical microbiology and enabled not only the confirmation of previously known virulence factors but also proposed the some features and relations.

Keywords: Clostridium difficile, Label-free quantification, virulence factors

WP13-06

iTRAQ-Based Proteomics Revealed the Formation Mechanism of Drug-Resistant Bacteria

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Introduction and Objectives

Staphylococcus aureus is a prevalent human pathogen. The emergence of highly resistant strains increases the treatment difficulty of S. aureus infections.

Methods

In this study, we domesticated S. aureus ATCC29213 with a stepwise increase of ciprofloxacin dosage, which is similar to clinical medication. iTRAQ quantitative proteomics was used to identify the differentially expressed proteins (DEPs) between sensitive strain with drug-resistance strain.

Results and Discussion

Conclusion

In sum, those changes at the metabolism pathway and SpA was relvealed having the relationship with the formation mechanism of drug-resistant bacteria .These findings provide novel insights into the molecular mechanisms of antibiotic resistance in bacteria.

Keywords: Staphylococcus aureus / iTRAQ quantitative proteomics / SpA

WP13-07

A comprehensive proteomics analysis for Streptococcus equi subspecies equi Se071780 to antigen discovery and vaccine development

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Introduction and Objectives

Streptococcus equi subspecies equi (S. equi) is a pathogen known as a major causative agent for equine strangles. Equine strangles is a contagious upper respiratory tract infection of horses and other equines, causing abscessation of lymph nodes in the heads and neck of host. Some of infected host can be carrier of S. equi and spread out this pathogen widely. This disease has been considered seriously due to causing economic losses to the equin industry. However, until now, very few effective vaccines and diagnostic kits are available.

Methods

In this study, we completed and characterized the genome of S. equi strain Se071780 by comparative analysis using genome of S. equi strain Se4047. Using this genome database as reference, comprehensive proteomic analysis of S. equi strain Se071780 was performed. According to subcellular fractionation, soluble protein fraction (Sol), membrane protein fraction (Mem), secretory protein fraction (Sec), and extracellular vesicle fraction (EV) were prepared.

Results and Discussion

By liquid chromatography-tandem mass spectrometry (LC-MS/MS analysis), total 1176 proteins were identified: Sol 721, Mem 698, Sec 463, and EV 408. The prediction of protein localization of each subcellular proteomes and molecular pathway were progressed by bioinformatics tools.

Conclusion

Our results elucidate the overall proteome profiles of S. equi strain Se071780 and provide candidates for potential vaccine targets.

Keywords: Streptococcus equi subspecies equi , S.equi , extracellular vesicles, EVs, Equine strangles, equines, horse, vaccine target, vaccine antigen, comprehensive proteomic analysis, proteomics, genomics, proteogenomics, subcellular fractionation, LC-MS/MS

WP13-08

Using multiple data analytical approaches to determine the metaproteome of the human gut

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Introduction and Objectives

The human gut microbiome is a dense, complex and diverse microbial community. We evaluated the microbial diversity of the gut microbiome of an adult male by ultra-high-performance liquid chromatography coupled to a quadrupole mass filter Orbitrap analyzer (Q Exactive).

Methods

Proteomic material was prepared from four feacal samples that were collected over the period of a week, and another four over the same period three months later. Initial analyses using a proteome database generated from existing feacal 16S RNA data we identified 14301 non-redundant peptides that represented only 16% of the submitted spectra. Since annotation of tandem MS raw data is limited to known proteomes for spectra-peptide matching, we approached the data analyses using a variety of available tools such MetaProteome Analyzer (MPA), de novo sequencing followed by MS-BLAST, and Unipept with re-indexing against Uniprot databases. The data derived from the latter analyses is leveraged against matched peptides compiled from proteomes of existing feacal 16S cDNA data and pre-existing published gut metaproteomes.

Results and Discussion

These different metaproteomic approaches evaluating the microbial diversity of the human gut show considerable correlation and overlap in the final analysis.

Conclusion

This unbiased metaproteomic analysis approach may thus provide a suitable complimentary workflow to 16S cDNA sequence analysis, and a combination of the different strategies may improve the depth of coverage of the gut microbiome.

Keywords: Metaproteomemicrobiomehuman gut

WP13-09

Alkaline protease activity and alk protein level in clinical isolates of A. flavus

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Introduction and Objectives

Mycotic keratitis is a leading cause of blindness due to fungal infections in topical countries. Understanding the virulence mechanism will help enormously in clinical management of the disease. In order to determine the pathogenic proteins of the fungus we examined the exoproteome of A.flavus since exoproteins are the earliest proteins elaborated by infecting pathogen during infection. In this report we describe the detailed analysis of alkaline protease, which exists as multiple isoforms and virulence related.

Methods

ALK was purified from exoproteins of A.flavus CI662 by cation exchange column and the identity was confirmed by western blot. Multiple proteoforms were resolved on a 2D PAGE followed by Mass Spectrometry (MS) analysis and confirmation by 2D western blot. Expression level between different clinical isolates and saprophyte was quantified by 1D western at two different temperatures 30? and 37?C.

Results and Discussion

ALK is a 403aa protein of which 126aa correspond to signal and propeptide. Removal of signal and propeptide results in a 28 kDa (277aa) protein. 1D PAGE of purified alkaline protease showed two proteins bands majority at 37 and 28kDa and both were confirmed to be alkaline protease by immunoblot. In 1D MS showed 37 kDa has propetides and mature peptides were identified. But in 28 kDa only mature peptide region was identified. Molecular weight shift is due to presence of propeptide region. Both 37 and 28 kDa proteoforms displayed protease activity. Purified ALK resolved in to 23 spots in a 2D PAGE, of which 20 spots identified as alkaline protease. Among 20 spots, 18 spots have both wild type and protein with intron2 region and 6 spots have wild type and protein with intron2 and 3. ALK shows both proteolytic and autoproteolysis property.Relative quantification of ALK level in clinical isolates were higher than saprophyte at 37?C. Transcript data also confirm the higher expression level of ALK in clinical isolates than the saprophyte. RT-PCR data clearly showss that in CI1123 at 37?C produce ALK transcripts with introns retained and the functional validity of these alternatively spliced mRNA is confirmed by mass spectrmetry of expressed proteins.

Conclusion

ALK exists in two major protein bands in 37 and 28 kDa in 1D PAGE and the purified protein could be resolved into multiple proteoforms. One mechanism we discovered is

intron retention leading to multiple transcripts and corresponding proteoforms. Additional modifications are not ruled out at this time. The implication of the existence of multiple proteoforms of this protein in infection is not clear

Keywords: ALK-Alkaline protease, Multiple proteoforms, A.flavus, Mycotic keratitis

WP13-10

Metalloproteome study on the iron transportation mechanism in bacteria.

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Introduction and Objectives

Iron is an essential element for almost all bacteria. Streptococcus pneumoniae causes a series of disaeses worldwide, and even death. Efficiently acquiring iron from the environment is critical for S. pneumoniae to sustain growth and cause infection. There are only three known iron-uptake systems in Streptococcal species responsible for iron acquisition from the host, including ABC transporters PiaABC, PiuABC and PitABC. Besides, no other iron-transporting system has been suggested. In this study, we aimed to find out new iron transporters and deep understand the iron transport mechanims in gram-positive bacteria.

Methods

In this work, we employed our newly established translating mRNA analysis integrated with proteomics to evaluate the possible existence of novel iron transporters in the constucted piaA/piuA/pitA triple mutant (Tri-Mut) S. pneumoniae D39.

Results and Discussion

Three important iron transporters have been clearly characterized. With ribosome associated mRNA sequencing-based translatomics focusing on translating mRNA and iTRAQ quantitative proteomics based on the covalent labeling of peptides with tags of varying mass, we indeed observed a large number of genes and proteins representing various coordinated biological pathways with significantly altered expression levels in the Tri-Mut mutant. Highlighted in this observation is the identification of several new potential iron-uptake ABC transporters participating in iron metabolism of Streptococcus. In particular, putative protein SPD_1609 in operon 804 was verified to be a novel iron-binding protein with similar function to PitA in S. pneumoniae.

Conclusion

These data derived from the integrative translatomics and proteomics analyses provided rich information and insightful clues for further investigations on iron-transporting mechanism in bacteria and the interplay between Streptococcal iron availability and the biological metabolic pathways.

Keywords: S.pneumoniae; Iron transport; Translatome; Proteome

WP13-11

iTRAQ-Based Proteomics Revealed the Bactericidal Mechanism of Sodium New Houttuyfonate (SNH) against Streptococcus pneumoniae

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Introduction and Objectives

Streptococcus pneumoniae (S. pneumoniae) is a major human pathogen causing pneumonia, meningitis, bacteremia, and otitismedia worldwide, posing a major threat to human health worldwide. Due to the increasing incidence of antibiotic resistant clones and the limitations of exiting vaccines, it is urgent to screen for novel drugs to combat pneumococcal infection. Sodium New Houttuyfonate (SNH), an addition product of active ingredient Houttuynin from the plant Houttuynia cordata Thunb, inhibits a variety of bacteria, yet the mechanism by which it induces cell death has not been fully understood.

Methods

In the present study, we utilized iTRAQ-based quantitative proteomics to analyze the protein alterations in S. pneumoniae in response to SNH treatment.

Results and Discussion

Conclusion

These results indicate that SNH indeed induced H2O2 formation to contribute to the cell lethality, providing new insights into the bactericidal mechanism of SNH and expanding our understanding of the common mechanism of killing induced by bactericidal agents.

Keywords: Streptococcus pneumoniae , Sodium new houttuyfonate, Proteomics, iTRAQ, H2O2, Bactericidal mechanism

WP13-12

In-depth metaproteomic studies of human and mouse gut microbiota

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Introduction and Objectives

The gut microbiota has been shown to be closely associated with human health and disease. While next-generation sequencing can be readily used to profile the microbiota taxonomy and metabolic potential, metaproteomics is better suited for deciphering microbial biological activities. However, the application of gut metaproteomics has largely been limited due to the low efficiency of protein identification. In this study, we aim to provide an in-depth analysis of the human and mouse gut metaproteomes.

Methods

A total of 32 stool samples were collected from either high-fat diet (HFD)- or low-fat diet (LFD)-fed mice; 8 mucosal-luminal interface (MLI) samples were collected during endoscopy, from the ascending colon of 8 different children. The bacteria were then isolated, lysed, trypsin digested and subjected to a 4hr gradient MS run for each sample on the Q Exactive mass spectrometer. Gut metaproteome identification was performed using a multi-pass database search against an in-house gut microbial protein database and quantification using widely used MaxQuant software.

Results and Discussion

An average of 38% and 33% of the acquired tandem MS spectra were confidently identified for the studied mouse stool and human MLI samples, respectively. In total, we accurately quantified 30,749 protein groups for the mouse metaproteome and 19,011 protein groups for the human metaproteome. Functional analysis showed that the most abundant COG categories were G (Carbohydrate transport and metabolism), C (Energy production and conversion) and J (Translation, ribosomal structure and biogenesis) in both human and mouse microbiota. The response of gut microbiota to HFD in mice was then assessed, which showed distinct metaproteome patterns for HFD mice and identified 849 proteins as significant responders to HFD feeding in comparison to LFD feeding. COG category O (Posttranslational modification, protein turnover, chaperones) was significantly increased in HFD group, which suggest that the microbiota in the gut of the HFD-fed mice may experience substantial stress from either the host or the diet. The latter may be an important part of the disrupted homeostasis of diet-microbiota-host interactions during high-fat feeding.

Conclusion

We present a metaproteomic approach for highly efficient intestinal microbial protein identification and quantification in mouse and human intestinal samples, which generated the largest number of gut microbial peptide and protein identifications from a single experiment, and will thus facilitate the application of metaproteomics for better understanding the functions of gut microbiota in health and disease

Keywords: gut microbiota, metaproteomics, protein identification, quantification

WO05-01

The Benefits of Hybrid Fragmentation Technologies (EThcD) for the in-depth analysis of the Immunopeptidome

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Introduction and Objectives

Human Leukocyte Antigen (HLA) class I molecules present short peptides derived from the degradation of cellular proteins on the cell surface. Recognition of pathogen- or disease-related HLA class I-presented peptides by CD8 T lymphocytes cells leads to the activation of a cytotoxic response and clearance of the affected cells. The repertoire of peptides presented by HLA class I molecules is dominated by self-peptides, all with defined sequence motifs that determine binding to expressed class I alleles. The overwhelming majority of the naturally processed and presented HLA class I peptides are unmodified, however a small fraction of peptides do harbor post-translational modifications (PTMs). The importance of HLA class I presentation of peptides with PTMs and their specific recognition by T cells has been demonstrated in infectious diseases, autoimmune diseases, and cancer. The main aim of the here reported work has been to develop and optimize mass spectrometry based methods to improve the analysis of Human Leukocyte Antigen (HLA) class I and class II molecules, enabling also the analysis of their post-translational modification, such as phosphorylation, methylation and OGlcAcylation

Methods

HLA class I-peptide complexes were immunoprecipitated using the mouse monoclonal IgG2a antibody W6/32. HLA class I-eluted peptides were fractionated by strong cation exchange (SCX) chromatography. The peptides were analyzed on an Orbitrap Fusion by data-dependent EThcD, CID, ETD, or HCD. All RAW files were analyzed using Proteome Discoverer, searching against the human Swissprot database with no enzyme specificity using the SEQUEST HT search engine.

WO05-01

Using the combination of HCD, ETD and EThcD on an Orbitrap Fusion we analyzed the immunopeptideomes of a variety of cells. These analysis allowed us to identify more than 10,000 HLA peptides in a single experiment. The depth of the ligandome help us to redefine the binding motifs of the probed alleles and uncover the presence of numerous HLA peptide harboring post-translational modifications. We focus especially on phosphorylation, arginine methylation and OGlcAcylation

Conclusions

By using optimized workflows and especially EThcD the identified repertoire of HLA peptides can be significantly boosted, enhancing the identification of HLA class I and class II molecules, enabling additionally the analysis of post-translational modifications they harbor, such as phosphorylation, methylation and OGlcAcylation.

Keywords

EThcD, HLA peptides, immunopeptidome, phosphorylation, methylation and OGlcAcylation.

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WO05-02

Digestomics of Raw Peanut and Characterization of Gastric-Phase Released Peptides of Peanut Allergens

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Introduction and Objectives
Grounded whole grain raw peanut was subjected to simulated in vitro oral and gastric digestion. After separation of solid undigested material, proteins were extracted from the digestion mixture and analysed by SDS-PAGE, 2D-PAGE, followed by mass spectrometry and proteomics. IgE-binding properties of digest was quantified by inhibition ELISA using pooled sera from peanut-allergic patients. Moreover, peptides were purified and tested for specific IgE inhibition of individual patients sera.

Methods
Grounded whole grain raw peanut was subjected to simulated in vitro oral and gastric digestion. After separation of solid undigested material, proteins were extracted from the digestion mixture and analysed by SDS-PAGE, 2D-PAGE, followed by mass spectrometry and proteomics. IgE-binding properties of digest was quantified by inhibition ELISA using pooled sera from peanut-allergic patients. Moreover, peptides were purified and tested for specific IgE inhibition of individual patients sera.

Results and Discussion
Ara h 1 and Ara h 3 were partly extracted during digestion process and were digested by pepsin resulting in digestion resistant peptides. In contrast, due to their low solubility in acidic conditions, Ara h 2 and Ara h 6 remained in solid undigested material and resistant to digestion. Digestion-resistant peptides of Ara h 1 and Ara h 3 were capable of binding IgE antibodies. Gastric phase digestion of whole grain peanut results in partial extraction and digestion of Ara h 1 and Ara h 3 giving large digestion-resistant peptides with preserved IgE-binding epitopes, while Ara h 2 and Ara h 6 remained trapped and stayed intact in the solid material until they are liberated in intestinal phase.

Conclusion
The results of our study suggest that the processes of protein extraction from the food matrix and their enzymatic digestion occur simultaneously, and that both intact and large digestion-resistant immunopeptides of peanut allergens are able to reach lower parts of gastrointestinal system.

Keywords:
Peanut allergens, digestion, proteomics, food matrix, digestion-resistant peptid

WO05-03

Ultra-Fast Analysis of Allergens using capillary electrophoresis coupled to mass spectrometry and Ultraviolet Photodissociation

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Introduction and Objectives

The identification of proteins in food matrices that cause sensitization in individuals or allergic reactions in those individuals already sensitized, represent a major concern for the food industry. We explore the application of CE/MS/MS as an accurate and sensitive way to rapidly test for protein allergens in food. The workflow is based in a simple protein extraction step and the use of capillary electrophoresis (CE) hyphenated with a 3D printed nano electrospray ion source to an Orbitrap Fusion Tribrid mass spectrometer with an UVPD source to do the analysis of the extracted proteins. The high mass accuracy and resolution, coupled to the efficient isoform separation and the different fragmentation modes allows for the rapid identification of the allergen and its origin.

Methods

Reference samples from commercial fish species were included in the work. Protein extraction was carried out by mechanically homogenizing 1 g of muscle. Water soluble proteins were centrifuged, the supernatant heated and centrifuged again. Soluble proteins were cleaned using stage tips, and then transferred onto the Agilent 7100 CE capillary electrophoresis system. An Orbitrap Fusion Tribrid mass spectrometer was coupled to CE system using a custom made ion source that incorporates an electro-osmotic flow driven methanol/formic acid sheath liquid to improve the signal in the mass spectrometry. Proteins were loaded at 5 psi for 10 s and separated using an uncoated fused silica capillary (50 cm X 50um I.D) at -30 Kv. MS/MS acquisition was performed using ETD, EThcD, HCD or UVPD fragmentation at 120K@m/z 200. Data analysis was performed using Thermo Scientific Deconvolution 4.0 and Prosight PD node in Proteome Discoverer 2.0 software.

Results and Discussion

The main goal of this work was to develop a simple and fast strategy that allows the identification of allergens and the authentication of fish species at the same time. The method consists in a two-step workflow. First we purified the thermo-stable proteins from the sarcoplasmic protein fraction. The second step consist in a top-down proteomic approach of the purified proteins. Samples loaded onto CE system, a CE separation was performed and the proteins were then analyzed doing a top-down using a trybrid mass spectrometer. HCD, CID, EThcD and UVPD fragmentation modes were evaluated to achieve the highest sequence coverage possible. Unique mass to migration time maps were built representing a unique signature for each of the fish allergens. The most abundant protein was a 11 kDa protein, identified as parvalbumin (PRVB).This thermo-stable protein is considered as the major fish allergen.

Conclusion

WO05-03

The total time for the end to end analysis can be done in less than 10 min. Overall, this strategy offers a very reliable and widely used top down proteomics method, and sets the basis for the development of CE chips that could be used for the food industry to detect allergen and to authenticate food.

Keywords: Fish allergens, Top-Down Proteomics, UVPD, Capillary Electrophoresis

WO05-04

Characterisation of specificity of different commercial proteases for food hydrolysates

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Introduction and Objectives

Commercial proteases are often not specific and there is lack of information from the producers about their main cutting sites with only limited description about their origin. Choosing the right protease is often fundamental for obtaining the desired food functional and nutritional values. The absence of characterisation can create confusion and difficulty to predict the outcome of a hydrolysis product, therefore the main goal of this work was to characterize the activity of some common commercial proteases.

Methods

Porcine haemoglobin was hydrolysed with 4 common commercial proteases, Promod 439L, Protex 6L, Foodpro alkaline protease and papain P1 at different time points (1, 2, 4 and 6 hours) at their optimum pH and temperature. The activity was stopped by use of protease inhibitors. The hydrolysates were centrifuged and filtered and the protein part was then run into an LC/MS-MS (Ultimate 3000+ HPLC coupled with a Qexactive mass spectrometer) and the spectra analyzed using database search (Proteome Discoverer). The obtained sequences were then categorized based on their relative abundance.

Results and Discussion

After hydrolysis the samples were characterized. As foreseen, the results showed that all the commercial proteases do not have a specific cleavage site; nevertheless it is possible to see a pattern. Out of four proteases tested, three (Promod 439L, Protex 6L and Foodpro alkaline protease) showed similar results in term of peptide sequences and activity, with only Papain behaving differently. Interestingly, the major differences in peptide sequences were seen between the different hydrolysis times, more than the different proteases, allowing us to speculate that these three products contain similar proteases, or with very similar activity.

Conclusion

This work shows that among the commercial proteases tested, three have similar activities, most likely due to the use of similar bacterial strains. Papain, being a single protease is the only one with different activity.

Keywords: Haemoglobin, commercial proteases, cleavage sites

WO05-05

Characterization and Modulation of Fish Allergens

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Introduction and Objectives

European aquaculture encounters great challenges as consumers demand competitive prices, high quality and environmental friendly products. Fish production has 20-50% of operational costs in feeding, and by this way it is important to have specific feeding strategies to optimize growing with a minimum of waste, allowing optimal fish health (Rodrigues et al, 2012). Fish meal replacement by plant-protein ingredients in diets causes deficiencies in some amino acids, which has an impact on growth performance and the ability to resist to stress (Conceicao et al., 2012). To compensate for these deficiencies, crystalline amino acids may be added to the diet, but this supplementation is expensive for the industry and therefore evaluation of the functional effect is important. In our experiment we will use three amino acids; histidine – involved in biochemical changes which occur during inflammation (Conceicao et al., 2012) – threonine – involved in the immune system response (Conceicao et al., 2012) and tryptophan which metabolism increases during inflammation (Le Floc'h et al., 2011). An evaluation of these diets on the overall immune status of European seabass will be performed by differential proteomics of skin mucus.

Methods

An evaluation of the effect of a deficiency in amino acids not commonly added to fish feeds (histidine, threonine and tryptophan), in the context of an alternative formulation (0% fishmeal), on European seabass immune status was performedEuropean seabass were exposed to three different diets - an aggressive formulation (CTRL) with 90% of the putative requirements of these three amino acids, CTRL with supplementation (HTW1) with 110% of the requirements and CTRL with supplementation (HTW2) with 150% of the requirements. Fish were fed by hand, ab libitum, twice a day and held at natural temperature, salinity and photoperiod. After 12 weeks fish were anaesthetized and mucus was collected by scraping, avoiding contamination with blood, urine and feces. Samples were kept at -80ºC until further analysis. Proteins were extracted using a lysis buffer and quantified by the Bradford method. Using 2D-DIGE, proteins were first focused on 24 cm pH 4-7 drystrips (GE Healthcare) followed by separation according to their molecular weight on 12% bis-acrylamide gels. Differentially expressed proteins were identified using mass spectrometry.

WO05-05

Results and Discussion

Several proteins with differential expression in European seabass mucus were identified by LC-MS/MS and found to be mainly involved in oxidative stress and immune system. We foresee, after validation of these results by RT-PCR, to create an evaluation service of nutritional and immune status of fish in aquaculture, based on molecular biomarkers,and depending on the aquafeeds employed.

Conclusion

Based on our results we can conclude that proteomics is a useful tool to access nutritional and immune status of fish in aquaculture.

Keywords:

Fish nutrition, Amino acids, European seabass, Aquaculture, Fish proteomics

WP14-01

Identification of 2-oxohistidine interacting proteins using E. coli proteome chips

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Introduction and Objectives

Cellular proteins are constantly damaged by reactive oxygen species generated by cellular respiration. Due to its metal-chelating property, the histidine residues is easily oxidized in the presence of Cu/Fe ions and H_2O_2 via metal-catalyzed oxidation, usually converted to 2-oxohistidine. We hypothesized that cells may have evolved antioxidant defenses against the generation of 2-oxohistidine residues on proteins, and therefore there could be cellular proteins which specifically interact with this oxidized side chain.

Methods

Using two chemically synthesized peptide probes containing 2-oxohistidine, high-throughput interactome screening was conducted using the E. coli K12 proteome microarray containing >4200 proteins. Ten interacting proteins were successfully validated using a third peptide probe, fluorescence polarization assays, as well as binding constant measurements.

Results and Discussion

We discovered that 9 out of 10 identified proteins seemed to be involved in redox-related cellular functions. We also built the functional interaction network to reveal their interacting proteins. The network showed that our interacting proteins were enriched in oxido-reduction processes, ion binding, and carbon metabolism. A consensus motif was identified among these 10 bacterial interacting proteins based on bioinformatic analysis, which also appeared to be present on human S100A1 protein. The preferential binding of S100A1 with 2-oxohistidine over histidine was successfully validated using all three peptide probes, suggesting that the capacity to recognize 2-oxohistidine modification may be evolutionarily conserved from bacteria to humans. Besides, we found that the consensus binding motif among our identified proteins, including bacteria and human, were located within α-helices and faced the outside of proteins, which meant that the motif had a chance to interact with the other proteins.

Conclusion

WP14-01

The combination of chemically engineered peptide probes with proteome microarrays proves to be an efficient discovery platform for protein interactomes of unusual post-translational modifications, sensitive enough to detect even the insertion of a single oxygen atom in this case.

Keywords: 2-oxohistidine, histidine, protein oxidation, redox proteomics, protein microarray

WP14-02

Quantitative phosphoproteomic characterization of the effect of RN/HAL genotypes on post-mortem energy metabolism in pigs

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Introduction and Objectives

During the conversion of muscle to meat, the post-mortem energy metabolism in muscle undergoes substantial changes which have significant influence on the final quality of the meat. Two mutant genotypes AMPKγ3R200Q (RN, Rendement Napole) and RYR1 (Hal, Halothane positive) have been intensively investigated in pigs and it's well known that these two genotypes have significant impact on the post-mortem metabolism. Protein phosphorylation is a key regulator of the energy metabolism and the aim of this study is to investigate how the two genotypes affect changes in protein phosphorylation during the post-mortem metabolism.

Methods

Muscle samples from the four different genotypes (wildtype, RN, HAL and RN/HAL) where collected after slaughter (45 min) and frozen in liquid nitrogen. Muscle samples (200 mg) were homogenized in 1 ml extraction buffer (6M Urea, 2M thiourea, 2% SDS, 1% DTT, one tablet of phosstop / 10ml and one tablet of complete / 10ml). Samples were digested with trypsin and 100µg of peptides from each genotype were labeled with iTRAQ (4 plex). Three biological replicates were analyzed for each genotype. Phosphor peptides were enriched using the TiO2 strategy. The enriched phosphopeptides and non-modified peptides from flowthrough were pre-fractionated with HILIC and analyzed with LCMSMS (Easy nLC / Q-Exactive Plus, Thermo). Data were analyzed using Proteome Discover.

Results and Discussion

In this study, 11025 unique phosphorpeptides and 2381 proteins were identified and quantified. Results revealed that there was clear effect of the HAL genotype on phosphorylation level, compared with the wildtype or the RN genotype. However, at the protein level, clear differences appeared evident with the RN genotype. These data suggest changes occurring in post-mortem metabolism with the HAL genotype are mainly due to regulatory events mediated through protein phosphorylation events while differences in post-mortem metabolism in RN pig muscle are more related to changes in protein levels.

Conclusion

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These data show that two different genotypes (HAL and RN) influence the post-mortem energy metabolism in pigs through different pathways. The HAL genotype affects the activity of the glycolytic enzymes through phosphorylation events, whereas the RN genotype affects glycolytic flux through the amount of glycolytic enzymes expressed in the muscle.

Keywords: Pig muscle, AMPK, RN (Rendement Napole), RYR1, HAL (Halothane positive), Post-mortem energy metabolism, Phosphor proteomics.

WP14-03

Proteomic analysis of antique glue on a Stradivari violin

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Introduction and Objectives

Antique violins made in Cremona, Italy by Antonio Stradivari (1644-1737) have unsurpassed qualities strongly preferred by musicians and collectors. The tonal quality and visual beauty of Stradivari violins have survived for over three centuries and still cannot be reproduced. There are strong historical and practical interests in understanding what materials were used to construct these highly valuable violins. Protein-based glues are used for two purposes on the violin. The first is to join different pieces of wood together, and the second is to seal the wood surface before varnishing. Some have proposed that Stradivari used casein (cheese) glue to join wood together, instead of common glues based on collagen.

Methods

During our research into wood treatment of a 1725 Stradivari violin, we accidentally encountered a piece of glue used for neck modification around 1800. Although not the original glue applied by Stradivari, it nevertheless represented the first and the only old glue sample analyzed from an antique violin to date. It was analyzed by direct trypsinization of solid glue and shotgun mass spectrometry.

Results and Discussion

We identified the old glue as highly purified collagen, with dozens of peptides which matched modern cow, goat/sheep, horse, and pig collagen sequences. No casein or other proteins were found.

Conclusion

Our results ruled out the possibility of fish glues such as “isinglass” or the weaker rabbit glue, but suggested a formulation of “mix animal glue” possibly originating from slaughter houses of large domesticated animals.

Keywords: collagen, glue, Stradivari, violin

WP14-04

Rapid and Simultaneous Determination of γ-oryzanol, Vitamin E Isomers in Brown Rice Sample by Ultra-Performance Liquid Chromatography Mass

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Introduction and Objectives

Brown rice contains high amounts of fat soluble phytochemicals with potential health benefits for human. In this work, a rapid ultra-performance liquid chromatography mass spectrometry method for the separation and characterization of vitamin E isomers and γ-oryzanol in brown rice was optimized and established. A complete separation of all compounds was achieved within 10 min. Brown rice, being the major component of rice grain and rich in bioactive phytochemicals, would be an excellent source of health maintaining food for daily consumption, in the different grain parts of rice varieties have provided useful information for the food industry because of their potential to serve as nutraceutical and functional ingredients.

Methods

All rice samples were supplied from Taiwan agricultural research institute council of agriculture. (Taiwan, Taichung), and before analysis were stored in the fridge at 4 °C. The extract was filtered through a 0.22 μm membrane filter, followed by collection of the filtrate and storage at −20°C until LC-MS analysis.Anaylates for LC-MS/MS analyses were introduced into the mass spectrometer via Xevo TQ tandem quadrupole mass spectrometer (Waters Micromass MS Technologies, Manchester, UK) coupled with a Waters ACQUITY UPLC system (Waters, Milford, MA, USA). The chromatographic separation was performed on a Kinetex PFP column (150 x 2.1 mm, 2.6mm) (Phenomenex, CA, USA) operated at 40Ċ. All mass spectrometric analyses were performed on a Waters xevo TQ mass spectrometer, with an ESI source in the positive ion and negative mode were used under the following operating conditions.

Results and Discussion

Using a Kinetex New F5 column (100 x 2.1 mm, 1.7mm) column by reversed-phase. Almost all of the analytes achieved good linearity with R2 > 0.98, with method detection limits of 10–160 ng/mL for the eight vitamin E congeners and limits of 25-1600 ng/mL for the fourγ-Oryzanol congeners . Accuracy ranged from 94.3% to 98.9%. The validation of the method was examined performing intraday (n= 3) and interdays (n= 3) assays with inter-day and intra-day precisions of less than 15%. It was found to be satisfactory. The findings of this study will improve an UPLC-MS/MS method for brown rice, the method was applied to identify the eight vitamin E isomers and γ-oryzanol in brown rice.

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Conclusion

A UPLC method for the simultaneous determination of vitamin E and γ-oryzanols components had been for the first time reported.The developed method was successfully validated and applied to the determination of T and T3 homologues and γ-oryzanols in brown rice and supplement samples. This method has the shortest analysis time for the separation of both tocols and γ-oryzanols while enabling high throughput analysis of samples with reduced consumption of organic solvents. Brown rice, being the major component of rice grain and rich in bioactive phytochemicals, would be an excellent source of health maintaining food for daily consumption.

Keywords: Mass Spectrometry, γ-oryzanol, Vitamin E Isomers, Brown Rice

WO10-01

Comparative proteomic analysis of kidney distal convoluted tubule and cortical collecting duct cells following long-term hormonal stimulation

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Introduction and Objectives

The distal convoluted tubule (DCT) and the cortical collecting ducts (CCD) are portions of renal tubule that are partly responsible for maintaining the systemic concentrations of potassium, sodium, calcium and magnesium. Despite being structurally similar, DCT and CCD cells have different transport capabilities due to a variety of different membrane-associated transport proteins. However, DCT and CCD cells appear to be modulated via the same hormones. The objective of this study was assess the differential response of DCT and CCD cells to long-term exposure to the hormones vasopressin or angiotensin II, both of which modulate DCT and CCD cells differently.

Methods

Mouse kidney distal convoluted tubule cells (mpkDCT) were cultured in heavy SILAC medium (Lys+6, Arg+10) while cortical collecting duct cells (mpkCCD) were cultured in light SILAC medium (Lys+0, Arg+0). Four passages of labelled cells were used to generate four biological replicates for statistical analysis. In addition to control conditions, cells were also stimulated for 4 days with the vasopressin type II receptor agonist 1-desamino-8-D-arginine vasopressin (dDAVP, 1nM) or angiotensin II (ANGII, 1nM). Cells were harvested, equally pooled and subjected to offline high-pH fractionation based two dimensional LC-MS/MS analysis (Q-Exactive). Identification and quantification of proteins was performed by MaxQuant.

Results and Discussion

Proteins that had at least three ratios out of four biological replicates were defined as quantifiable proteins. Proteins that had at least three iBAQ values out of four biological replicates in light or heavy channel and zero iBAQ values in the other channel were defined as unique proteins in a particular cell type. Out of the 4304 quantifiable proteins under control condition, 4190 under ANGII stimulation, 4110 under dDAVP stimulation, 1101, 1566 and 1294 proteins passed the quantitative Benjamini-Hochberg (BH) FDR 1% threshold, respectively. 594 proteins passed the threshold in all three conditions, and all of them go the same direction, with 287 up-regulated in mpkDCT and 307 up-regulated in mpkCCD. The average RSD for the ratios of these 594 proteins under three conditions was 11%, suggesting very good quantification precision and accuracy. The mpkDCT or mpkCCD specific proteins under the three conditions were subjected to further bioinformatics analyses including Panther and DAVID gene ontology analyses, E3 ligase and deubiquitinating enzyme identification, and kinase and transcription factor predictions, with the aim to identify cell specific proteins that define tubule-specific biological processes.

Conclusion

WO10-01

The differential responses of DCT and CCD cells to long-term exposure to the hormones vasopressin or angiotensin II were assessed using SILAC labeling and extensive bioinformatics.

Keywords: kidney, distal convoluted tubule, cortical collecting duct, vasopressin, angiotensin II

WO10-02

Targeted MS-based assay of circulating proteoforms related to aging in human plasma

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Introduction and Objectives

To gain insight into the basic mechanism of aging, studies have attempted to identify signaling proteins that both in animal models and in humans change systematically with aging, are associated with phenotypes typical of the aging process, and appear to differentially respond to stress or trauma by compensation, repair, or adaptation. In many instances, this approach has produced controversial findings, likely because of multiple reasons. The protein product of a single gene takes multiple proteoforms because of alternative splicing or post-translational modifications that affect proteolytic processing cleavage or terminal degradation. Measuring these alternative circulating proteoforms with conventional immunoassays or aptamers in the blood or other biological material has proven to be difficult.

Methods

In order to facilitate the study of these circulating proteoforms with aging phenotypes, we developed a novel multiplexed selected reaction monitoring (SRM) assay and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to measure fifteen plasma proteoforms representing eight important proteins that appear to be important for aging, including GDF11 and GDF8 propeptides and mature proteins, follistatin, follistatin-related protein 3, WFIKKN1, WFIKKN2, oxytocin, and eotaxin.

Results and Discussion

A total of 66 peptides consisting of 198 interference-free SRM ion pairs were monitored and validated by using the optimized MS parameters for the fifteen plasma proteoforms. The lower limits of quantitation was identified for all proteoforms. Mean protein concentrations for selected proteins in plasma were respectively for the following: (i) GDF8 propeptides (0.4 ng/mL) and mature protein (2.0 ng/mL); (ii) GDF11 propeptides (29.7 ng/mL) and mature protein (1.1 ng/mL); (iii) follistatin isoforms 1 & 2 (0.3 ng/mL) and follistatin cleavage form FST303 (8.2 ng/mL); (iv) FSTR3 mature protein (0.4 ng/mL); (v) eotaxin mature protein (0.3 ng/mL); (vi) oxytocin mature protein (1.5 ng/mL); (vii) WFIKKN1 mature protein (0.8 ng/L), and WFIKKN2 mature protein (1.8 ng/mL), in triplicate assays with a total CV<10%.

Conclusion

Targeted MS-based assay has proven valuable for the quantitation of proteoforms. This multiplexed SRM assay should facilitate the study of these proteoforms that are considered to be important in aging.

WO10-02

Keywords: aging, GDF8, GDF11, follistatin, FSTR3, eotaxin, oxytocin, WFIKKN1, WFIKKN2

WO10-03

Can proteomics contribute to solving the riddle of Diabetic Nephropathy?

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Introduction and Objectives

Diabetic nephropathy (DN) is one of the major complications associated with diabetes and is a leading cause of kidney failure. Progression is regularly measured by monitoring the urinary albumin excretion rate (UAER). A moderate increase in UAER is referred to as microalbuminuria and is a serious risk factor for development of DN, although only 15-40% of type 1 diabetes patients with microalbuminuria progress to DN. As such the UAER is an incomplete measure of risk of developing DN and is a poor predictor of progression. The aim of this project was to delve deeper into the proteome changes in DN.

Methods

The N-glycosylated serum proteomes of 60 type 1 diabetic patients (Normoalbuminuria n=20, Microalbuminuria n=20 and Macroalbuminuria n=20) and 20 non-diabetic controls were compared using shotgun mass spectrometry (MS). Principal component analysis and Orthogonal partial least square analysis were applied including individual patient factors like SBP, eGFR, gender, diabetes duration, HbA1c, smoking and total cholesterol. One-way ANOVA was used for statistics and selected reaction monitoring MS of certain peptide intensities was performed for verification.

Results and Discussion

Although the albuminuric groups were matched for mean glomerular filtration rate (GFR), multivariate proteome analyses revealed a distinct GFR dependent separation of the normoalbuminuric patient group into two sub groups. Therefore, all proteins with significant abundances based on the UAER were compared using a classification system, which includes GFR. For several proteins, there was a large variance within the albuminuric group, which was decreased, as the groups were further sub divided. Finding proper biomarkers for prediction of development and progression in DN is a big challenge and the incomplete classification system using UAER could be part of the explanation. It is though complicated to correctly measure the GFR and protein markers reflecting GFR could help in a more accurate determination of kidney status.

Conclusion

In summary, our UAER-based proteomics analysis reveals protein changes in serum that could reflect the GFR. Those proteins could be disease risk markers and provide new insights into disease progression in DN.

Keywords: Diabetic nephropathy, N-glycosylation, multivariate analysis, serum

WO10-04

Quantification of Angiotensin II-Regulated Proteins in Urine of Patients with Polycystic and Chronic Kidney Diseases by Selected Reaction Monitoring

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Introduction and Objectives

Angiotensin-II (AngII) mediates progression of autosomal-dominant polycystic kidney disease (ADPKD) and other chronic kidney diseases (CKD). However, markers of kidney AngII activity are lacking. We previously defined 83 AngII-regulated proteins in vitro, which reflected kidney AngII activity in vivo. In this study, we developed selected reaction monitoring (SRM) assays for quantification of AngII-regulated proteins in urine of ADPKD and CKD patients.

Methods

We demonstrated that 47 of 83 AngII-regulated transcripts were differentially expressed in cystic compared to normal kidney tissue. We then developed SRM assays for 18 AngII-regulated proteins overexpressed in cystic tissue and/or secreted in urine. Methods that yielded CV< 6% for control proteins, and recovery ~100% were selected. Heavy-labeled peptides corresponding to 13 identified AngII-regulated peptides were spiked into urine samples of 17 ADPKD patients, 9 patients with CKD predicted to result in high kidney AngII activity and 11 healthy subjects. Samples were then digested and analyzed on triple-quadrupole mass spectrometer in duplicates.

Results and Discussion

Calibration curves demonstrated linearity (R2>0.99) and within-run CVs<9% in the concentration range of 7/13 peptides. Peptide concentrations were normalized by urine creatinine. Deamidated peptide forms were monitored, and accounted for <15% of the final concentrations. Urine excretion rates of proteins BST1, LAMB2, LYPA1, RHOB and TSP1 were significantly different ($p<0.05$, one-way ANOVA) between patients with ADPKD, those with CKD, and healthy controls. Urine excretion rate of all proteins was the lowest in patients with ADPKD and the highest in patients with CKD. Univariate analysis demonstrated significant association between urine protein excretion rates of most proteins and sex ($p<0.05$, unpaired t-test) as well as disease group ($p<0.05$, ANOVA). Multivariate analysis using multiple correspondence analysis across protein concentration, age and sex demonstrated good separation between ADPKD and CKD.

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Conclusion

We have optimized methods for quantification of AngII-regulated proteins, and we demonstrated that they reflected differences in underlying kidney disease in this pilot study. Future studies will determine whether urine excretion rate of AngII-regulated proteins correlates with cyst burden and kidney AngII activity in larger cohorts of ADPKD patients and those with other kidney diseases.

Keywords: Angiotensin II, renin angiotensin system, polycystic kidney disease, autosomal dominant polycystic kidney disease, selected reaction monitoring, proteomics, urine, chronic kidney disease

WO10-05

Analysis of the HDL Proteome - Myths and Legends and Reality

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Introduction and Objectives

The high-density lipoprotein (HDL) plays an essential role in lipid transport in both health and disease. The HDL is composed out of different lipids, phospholipids, cholesterol and signal lipids, as well as proteins to maintain the structure and for recognition by receptors. Numerous publications describing the protein composition of HDL, up to 100 different proteins have been associated with HDL. Some of these are the well-known traditional high abundant plasma proteins, as albumin, others exceed alone the molecular weight of the HDL particle (175k- 360k), as APO B-100 (500k).

Methods

HDL is purified from individual human plasma by consecutive ultracentrifugation steps. Proteins are identified after tryptic digestion by nano-flow LC-MS/MS. For in-depth analysis a segmented LC-MS/MS approach is applied, while MS is recorded over the full mass range from 200 - 1400 Th, MS/MS is recorded in 50 Th windows in 14 consecutive injections of the same sample of combined HDLs. The identified proteotypic peptides are used to develop a targeted (MRM) LC-MS/MS approach. Identified modifications of the proteins are also taken into account for the MRM development. This analysis results in 25 proteins which are quantified in the individual HDL samples by MRM. Intact HDL proteins are separated by reversed-phase HPLC into 24 fractions and the molecular weight of the proteins is determined by LC-MS.

Results and Discussion

In-depth analysis of purified HDL particles combined from over 70 individuals reveals a limited list of proteins for the development of the targeted Proteomics approach. The analysis confirms APO-A1 and APO-C3 as the major components of HDL, APO-E, M and some others are minor components, possibly not present in every HDL particle. Other proteins as APO-B, albumin, Clusterin are identified as contamination in the HDL preparation. These findings are in agreement with the postulated structure of the HDL particle. The in-depth analysis using the segmented LC-MS/MS approach also identifies a number of modifications of the HDL proteins, some are not described before. The determination of the molecular weight of the intact HDL proteins gives further insights into modifications and truncations of individual proteins.

Conclusion

The combination of in-depth and targeted Proteomics analysis of HDL isolated of individual donors shows a limited number of proteins and a conserved composition within a limited range of HDL. Unlike other publications the HDL is composed only of a few proteins, the basis is APO-A 1 and APO-C3, other proteins are only present in subsets or

WO10-05

are idnetified as contaminants. The targeted approach also allows to identify modifications in individual HDLs and to correlate them to health and disease status.

Keywords: Plasma protein, high-density lipoprotein, HDL, targeted Proteomics, MRM, LC-MS/MS, in-depth analysis, post-translational modifications

WP15-01

Proteomic insight reveals elevated levels of albumin in circulating immune complexes in diabetic plasma.

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Introduction and Objectives

Hyperglycemic condition in diabetes promotes formation of advanced glycation end products, which are known to elicit immune response and form complex with immunoglobulins called circulating immune complexes (CICs). Objective of the study was to understand the role of advanced glycation endproduct (AGE) modified proteins in the elicitation of autoimmune response and formation of CICs.

Methods

1. IgG immune complexome from clinical Control, prediabetic, newly diabetic, diabetes mellitus and diabetic microalbuminurea plasma was isolated using protein G sepharose immunoprecipitation and subjected to in-solution trypsin digestion.2. Further label free based mass spectrometric analysis was used to identify, quantify and characterization of the proteins associated.3. Followed by clinical studies the levels of serum albumin in CICs was quantified in streptozotocin induced diabetic mice treated with or without aminoguanidine (AMG) a prototypic AGE inhibitor.3. Western blotting using anti serum albumin and anti-AGE antibodies was used to confirm mass spectrometric observations.4. Cytokines, the key mediators of immune response, inflammation and disease pathogenesis were quantified by Bio-plex multiplex analysis in mice plasma.

Results and Discussion

1. Clinical study revealed the elevated levels of serum albumin in the CICs from prediabetic, newly diabetic and diabetic microalbuminurea plasma compared to that of control.2. The elevated level of serum albumin in the immune complex was also observed in STZ induced diabetic mice to that of control mice. Treatment of diabetic mice with AMG decreased the level of albumin in CICs.3. Mass spectrometric and western blot analysis revealed serum albumin in CICs was also AGE modified.4. Increased levels of proinflammatory cytokines such as IL-1b, IL-2, and TNF-alpha were observed in diabetes, which were reduced with AMG treatment suggesting the involvement of glycation in immune response.

Conclusion

In conclusion to the best of our knowledge this is the first study reporting elevated levels of serum albumin in the CICs from subjects with different levels of glucose intolerance and diabetic complications and that albumin in CICs is also AGE-modified. The observation in clinical subjects was corroborated in STZ induced diabetic mice model. Further AMG treatment regulates albumin levels in CICs of diabetic mice suggesting the

WP15-01

involvement of glycation in elicitation of autoimmune response and formation of CICs.

Keywords: Advanced glycation endproducts, immune complexome, proteomics, mass spectrometry, label free quantification, cytokines

WP15-02

Profiling of plasma for proteomic signatures of mammographic density

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Introduction and Objectives

Mammographic density (MD) is one of the strongest risk factors for breast cancer. Reflected by the white part of a mammogram, MD is a measure of the amount of epithelial and stromal tissue in relation to fatty tissue in the breast. The molecular basis for how MD relates to risk is however poorly understood, so are the precise mechanisms for how MD is regulated. In order to better stratify women at high risk, there is thus a great need for a better understanding of the biology behind MD.

Methods

Plasma samples from two sets of 600 and 730 breast cancer-free women (age>40) were obtained from the Swedish KARMA project (Karolinska Mammography Project for Risk Prediction of Breast Cancer). Besides samples and density measures, many other life-style factors are available and considered in the design. The samples were screened using multiplexed suspension bead arrays with 780 antibodies raised against 440 unique proteins. These proteins included those from the literature that had previously been studied in the context of breast cancer and proteins annotated for the extracellular matrix.

Results and Discussion

A set of 23 proteins showed a linear association to mammographic breast density within each of the two sample sets ($p<0.05$) after adjusting for age and body mass index (BMI). The corresponding antibodies were further validated in plasma by immuno-capture MS. Among the 23 target proteins, on-target binding could be verified for a majority (60%) of the antibodies in plasma. We also identified BMI to be an important confounding factor that needs to considered appropriately. Current work is to develop sandwich immunoassays for the selected set of targets and to quantify these in additional samples sets.

Conclusion

Biological factors related to mammographic density, such as alterations in the stromal content or extracellular matrix, may be reflected by changes in the composition of blood plasma and important for prediction of breast cancer risk. Here, screening with multiplexed protein arrays and validation by immune-capture MS suggested 14 proteins to be associated to breast density in non-diseased women.

Keywords: mammographic density, affinity proteomics, breast cancer, plasma, suspension bead arrays

WP15-03

Novel proteomics-based pipeline for identifying predictive Biomarkers of chemotherapy-induced toxicity

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Introduction and Objectives

Major advances in early detection and therapy have significantly increased the survival of breast cancer patients. Unfortunately, most cancer therapies are known to carry a substantial risk of adverse long-term treatment-related effects. Little is known about patient susceptibility to severe side effects after chemotherapy. Chemotherapy-induced peripheral neuropathy (CIPN) is a common side effect of taxanes. In this study, we applied advanced proteomics technology to explore the feasibility of identifying predictive biomarkers for chemotherapy-induced toxicity in breast cancer patients.

Methods

In this study, we evaluated the association between protein content in serum exosomes and severity of CIPN. Women with early stage breast cancer receiving adjuvant taxane chemotherapy were assessed with the FACT-Ntx score and serum was collected before and after the taxane treatment. Based on the change in FACT-Ntx score from baseline to 12 month follow-up, we separated patients into two groups: those who had no change (Group 1, N=9) and those who had a >20% worsening (Group 1, N=8). MS-based proteomics technology was used to identify proteins present in serum exosomes to determine potential biomarkers. Mann–Whitney–Wilcoxon analysis was applied and maximum FDR was controlled at 20%.

Results and Discussion

In this study, we showed that a complex protein profile of serum exosome was obtained from serum exosome of breast cancer patients using the highly sensitive Orbitrap Fusion Tribrid mass spectrometer. Over 700 proteins were identified from serum exosome derived from breast cancer patients and the profile showed that we captured a wide-range of proteins known to be in different subcellular locations and serve different functions. To correct for large-scale multiple testing errors, we applied LIMMA analysis to identify differentially expressed proteins. From the results, we made several interesting observations. Based on the Principle Component Analysis (PCA), we observed a distinct separation of group 1 (severe neuropathy) and group 2 (no neuropathy) in serum exosome isolated from baseline blood draw (12 proteins, $q<0.1$). On the contrary, the same statistical parameters yielded only 3 proteins that can distinguish group 1 from group 2 patients at the endpoint blood draw suggesting it is highly feasible to identify predictive biomarkers. By examining exosomal protein profiles before and after the taxane treatment from the same individual, we were able to see progressive changes of these candidate markers longitudinally at the protein level as well as at the peptide level.

Conclusion

Our findings suggest a new hypothesis underlining the susceptibility of BC patients to

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CIPN and offer potential explanation why current CIPN prevention trials have failed to intervene the development of CIPN. These toxicity-associated biomarkers can be further validated in larger retrospective cohorts for their utility in identifying patients at high risk for CIPN.

Keywords: mass spectrometry, breast cancer, drug toxicity biomarkers

WP15-04

Human native Peptidome database for Peptidomics studies

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Introduction and Objectives

Peptidomics is a new field stemming from proteomics and targeting endogenous peptides produced by proteases from precursor proteins. Endogenous peptides are mostly functional peptides like hormones and neuropeptides. They can be beneficial or sometimes hurtful. Tracking endogenous peptides in human body is very crucial for understanding human physiology and pathology and also for the early diagnosis of different human diseases and biomarker discovery. In native peptidome analysis, peptides are cleaved in vivo by different types of unknown endogenous proteolytic enzymes, which require searching against proteome database (Uniprot) considering all cleavage possibilities. This results in expanding the search space in unlimited fashion for peptide search especially when post-translational modifications (PTM) will be considered and subsequently increasing the false positive hits. Moreover, the comprehensive annotation and investigating novelty for the identified native peptide in a given peptidomics study is not possible due to the absence of human native peptidome database.In this regard, the need for comprehensive human native peptidome database is critical for characterization and annotation of native peptides in peptidomics studies. Therefore, the goal of this project is to construct a comprehensive human native peptidome database (HNPD) to search and annotate mass spectra from different peptidomics studies where there is no human native peptidome database exist so far.

Methods

Our database core will be generated from considering molecular processing annotations in protein databases such as Uniprot database. Utilizing theses annotation on precursor proteins will generate different types of native protein and peptides including signal peptide, transit peptide, propeptides, peptides and mature proteins. As well as collecting semi or full native peptides which have already dispersed in different peptide databases such as peptide atlas, Global machine proteome (GMP) database and mapping them to their precursor proteins. The future expansion of the constructed peptidomics database will be achieved through applying a peptidomics workflow which has the ability to identify novel native peptides to feed them in the peptidomics database.

Results and Discussion

The molecular processing of uniprot database generated 5905 distinct signal peptides, 463 distinct transit peptides, 379 distinct native peptides, 759 distinct propeptide, and 32096 distinct mature proteins. in addition to 368,332 one end tryptic peptides collected from peptide atlas database and they considered as semi native peptides cleaved in-vivo at N or C-terminus.

Conclusion

Utilizing the peptidome database in peptidomics studies will contribute to the identification

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of well annotated peptides in less time and generating less false hits.

Keywords: Proteome, peptidome, database, bioinformatics

WP15-05

Urimem, a Membrane that Can Store Urinary Proteins Simply and Economically, Makes the Large-Scale Storage of Clinical Samples Possible

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Introduction and Objectives

By nature, biomarker is the measurable change associated with a physiological or pathophysiological process. Unlike blood which has mechanisms to minimize changes and to keep the internal environment homeostatic, urine is more likely to reflect changes of the body and is a better biomarker source. Because of its potential in biomarker discovery, urinary proteins should be preserved comprehensively as the duration of the patients' corresponding medical records.

Methods

(i) The urinary protein concentration was determined from routine urine tests. (ii) The diluted samples were centrifuged in a thermostatic centrifuge for 10 min at 12000×g and 4°C and the supernatant was saved. (iii) Four to six sheets of wetted circular filter paper were placed onto the vacuum suction filter bottle (10-cm2 filter area). (iv) One activated PVDF membrane (activated in methanol and rinsed with pure water) was placed immediately onto the filter paper, avoiding the generation of bubbles. (v) The vacuum suction filter bottle was installed and loaded with 20 mL supernatant or the flow-through from the 0.45-μm filter membrane. (vi)The vacuum suction filter bottle was connected to the vacuum pump, and the solution allowed passing through the PVDF membrane drop-wise. (vii) After the proteins were adsorbed onto the PVDF membrane, the protein-bound membrane was placed under four 275 W bulbs for 3–4 min to complete the drying process, or left to dry at room temperature. (viii) The dry membrane with tag paper was placed between aseptic sealing membranes to keep the tag paper and dry membrane separate. The membrane was then sealed using a kitchen vacuum sealer and stored.

Results and Discussion

Urinary proteins on the Urimem that were stored under four different temperature conditions for 18 d: room temperature, 4°C, -20°C, and -80°C exhibited similar SDS-PAGE patterns. We believe that the current technique provides a practical method for preserving urinary protein samples from consenting people during every stage of the disease development. Even more objective large scale prospective studies will be possible. This procedure has the potential to change the current landscape of medical research and medical practice.

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Conclusion

A UPLC method for the simultaneous determination of vitamin E and γ-oryzanols components had been for the first time reported.The developed method was successfully validated and applied to the determination of T and T3 homologues and γ-oryzanols in brown rice and supplement samples. This method has the shortest analysis time for the separation of both tocols and γ-oryzanols while enabling high throughput analysis of samples with reduced consumption of organic solvents. Brown rice, being the major component of rice grain and rich in bioactive phytochemicals, would be an excellent source of health maintaining food for daily consumption.

Keywords: Urimem; Urinary proteins; Simple; Economical; Biomarker.

WP15-06

Rapid and high-throughput identification of acute hepatic porphyria by SALDI-TOF MS analysis

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Introduction and Objectives

The porphyrias are a group of rare inherited metabolic disorders of heme biosynthesis and acute hepatic porphyria is the most serious type of porphyria. In hospital, the concentrations of aminolevulinic acid (ALA) and porphobilinogen (PBG) in random urine are used for the diagnosis of acute porphyria, owing to the concentration of ALA and PBG will get higher in patient urine. Recently, mass spectrometry has better performance on sensitivity and specificity, it offered an alternative tool for porphyria clinical studies. Surface-assisted laser desorption/ionization time-of-flight mass spectrometry (SALDI-TOF MS) are known for its high throughput, high sensitivity and the ability of the quantification of small molecules.In this study, our purpose is to establish a rapid and high-throughput platform for the screen of acute hepatic porphyria patients by using SALDI-TOF MS.

Methods

In this study we first used PBG standard to find the best experimental method, than mixed PBG and ALA standards based on this method, finally used the real sample for testing

Results and Discussion

In results, fullerene C60 has the best performance compared with other traditional organic matrix (CHCA and DHB) and carbon materials (graphene nanoplatelets grade 5, graphene nanohorns, single-layer graphene oxide, 2-4 few-layer graphene oxide, 4-8 multi-layer graphene oxide). Then, the parameters of C60 are optimized by using PBG, including the solution (ddH2O), concentration (10 ppm), SALDI-TOF MS mode (negative mode) and sample preparation (thin-layer method).

Conclusion

The limits of detection were 2.5 ppm for ALA and 1 ppm for PBG. Overall, we establish a simple, sensitive and high-throughput technique for the detection of ALA and PBG in urine, it can help us to screen the acute porphyria patients.

Keywords: SALDI-TOF MS, acute hepatic porphyria

WP15-07

Developing a Strategy to Identify Low-Abundance Native Peptides in Plasma

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Introduction and Objectives

Serum/plasma contains thousands of different types of proteins and peptides and can provide with valuable information on numerous inherent processes. However, the existence of high-abundance proteins and the large dynamic range of serum proteins/peptides make analysis of low-abundance proteins/peptides challenging. In particular, detailed analysis and identification of native peptides remain extremely difficult due to the tremendous variety of cleavage possibilities and posttranslational modifications. Here, we developed an improved strategy to extract and analyze plasma low-molecular-weight peptides, and initiated a large-scale sequencing of circulating native peptides.

Methods

Native peptides in plasma were extracted essentially by the 'differential solubilization (DS) method' as described previously (J. Proteome Res, 9, 1694-705, 2010). Enriched peptides with or without reductive alkylation were pre-separated into 8 or 13 fractions using 'cyclic sample pooling technique' (Biomed Chromatogr 27, 691-694, 2013). Separated peptides were introduced from the nanoLC (flow rate of the mobile phase: 300 nl/min) to a Q-Exactive (Thermo Fisher Scientific). Raw LC-MS/MS data was searched against the SwissProt_2015_02.fasta database (selected for Homo sapiens; 20,199 entries) using Mascot (Matrix Science Ltd) and PEAKS Studio (Bioinformatics Solutions, Waterloo).

Results and Discussion

Our optimized DS method combined with cyclic sample pooling selectively and efficiently enriched plasma peptides of less than 5 kDa that had been unbound and bound to carrier proteins. Assessment of the sensitivity of our proposed method was performed using tryptic peptides of E. coli β-galactosidase [β-Gal] as monitor peptide spiked into plasma samples at a concentration of 1 nM prior to peptide extraction. The maximum intensity of precursor ion of these peptides was 2.5 x 107, and the lower limit of the intensity of precursor ion for identification was 2.8 x 104. Thus, we estimated that the concentration limit for peptide identification from 200 µL of plasma

WP15-07

was pM order. We successfully identified hundreds of novel native peptide sequences with false discovery rate of 1%. Among those identified were well-known bioactive peptides and cleaved products of many cellular component proteins.

Conclusion

Our new strategy successfully identified plasma low molecular weight native peptides and would facilitate human plasma peptidomic analysis.

Keywords: plasma, native peptide, peptidomics

WP15-08

Preliminary Study into the Quantitative Impact of Long-Duration Space Travel on the Cosmonaut Plasma Proteome

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Introduction and Objectives

The effects of spaceflight (SF) on human physiology were studied for years, yet the molecular mechanisms driving physiological changes remain unknown. With that in mind, this study was performed to obtain a deeper understanding of the proteome change during space travel by quantifying a panel of 142 proteins in the blood plasma samples of 18 Russian cosmonauts (mean ± SD age: 44 ± 6 y.o. all male) taken at 3 defined time points: prior to launch, on the first day of recovery, and 7 days later. They had conducted long-duration missions to the ISS (169 - 199 days). The first blood drawing after landing was performed through 25.2± 0.1hrs.

Methods

The chosen 142 proteins, are putative biomarkers of non-communicable disease, and cover a concentration range of more than 5 orders of magnitude in human plasma. Quantitation was performed by mass spectrometry-based approach involving targeted multiple reaction monitoring (MRM) in conjunction with stable isotope-labeled prototypic peptide standards. Several methods of analysis of variance was used: LSD Test, Scheffe, Newman-Keuls, Tukey HSD, Duncan, Unequal N HSD Tests.

Results and Discussion

It was found that 19 proteins significantly change their concentration in the blood due to space flight. We believe that the proteins whose level was reduced and has not recovered to pre-level in a week, are witnessing a structural adaptation that occurred during flight. The proteins whose concentrations after landing were on the pre-flight level, but it underwent changes in the post-flight period, apparently are the members of rehabilitation processes to terrestrial conditions. Differently expressed proteins observed in cosmonauts' plasma during inflight experiments highlighted several non-tissue specific pathways related to oxidative stress, cytoskeleton, cell proliferation, glucose and lipid metabolism, cell damage and repair response, apoptosis, calcium/collagen metabolism, transport of lipoproteins, cellular functions, protein degradation, signal transduction and cell energy metabolism.

Conclusion

The expression of a few proteins related to cytoskeleton, extracellular matrix metabolism, internal cell transport, cell motility, apoptosis and oxidative stress was altered within time exposure to μg suggesting their sensitivity to gravitational changes.

Keywords: spaceflight, cosmonauts, blood plasma, proteins.

Acknowledgments

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WP15-09

Proteomics Analysis of Rat Kidney under Maleic Acid Treatment by SWATH-MS Technology

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Introduction and Objectives

Maleic acid is an industrial-grade chemicals, often used in adhesives, stabilizers and preservatives. It's unknown that whether long-term consumption of maleic acid-modified starch is harm to the human. However, most studies in animal models against maleic acid leads to renal tubular damage, but they did not understand the relevant changes in protein expression. The proteomics technology is widely used in various research fields, a comparison among each sample, it can help us to find out the difference in protein expression. Label-free quantitative analysis as one of the main methods for proteomics, and Sequential Window Acquisition of all THeoretical fragment ion spectra (SWATH) is the most innovative of the label-free quantitative technology. It can be more accurate than the previous label-free quantitative technology. Therefore, we use ultra performance liquid chromatography (UPLC) coupled with Q-TOF MS, and SWATH technology is used to investigate the effect of maleic acid on rat kidney amount of protein expression changes and to find relevant pathways and biomarkers.

Methods

1 µg of each protein sample was added 100 fmole BSA as an internal standard for RT calibration. And sample were analyzed using the Thermo U3000 LC System using trap and analyze column chip packed with Thermo Acclaim PepMap RSLS, C18, 2 µm, 100 Å, 75 µm i.d. × 15 cm nanoViper reverse phase. Eluent was analyzed using the TripleTOF 6600 system (AB SCIEX) using parallel 150 min gradients of DDA and DIA. SWATH conditions were 25 amu windows/90ms over mass range of 350-1250 amu. All data were processed using ProteinPilotTM and PeakView® for visualization.

Results and Discussion

We further compare control and treat groups. Pick out the proteins that fold change >1.5, and p-value < 0.05. GO analysis and path discussed show that most of the proteins involved in metabolic process, oxidoreductase activity, and biological regulation.

Conclusion

22 proteins were simultaneously expression differences in control versus treat groups.

WP15-09

And many literature suggests these proteins are associated with kidney damage. We will be further verified by MRM in the future.

Keywords: Maleic acid, kidney, proteomics

WP15-10

The discovery of HDL-associated protein biomarkers to predict stroke

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Introduction and Objectives

High-density lipoprotein (HDL) is a lipid and protein complex that consists of apolipoproteins and lower level HDL-associated enzymes. HDL can protect against atherosclerosis via its cholesterol acceptor and effects in antioxidation, anti-inflammation, and antiapoptosis. ApoA1, a major lipoprotein on HDL, has antioxidant and anti-inflammation properties to prevent atherosclerotic vascular disease (ASVD). Oxidation, carbamylation and glycation of ApoA1 have been reported to be associated with impairment of cholesterol efflux and can lead to cholesterol plaque accumulation, increasing risks of ASVD and stroke. Therefore, in this study HDL from a large size of stroke and disease control samples was analyzed with MALDI-TOF to discover HDL-associated protein biomarkers for stroke.

Methods

Plasma samples from healthy (n=50), diabetes (n=50), hyperlipidemia (n=50), uremia (n=50), chronic stroke and acute stroke subjects (n=100) were collected. HDL was purified with salt density ultracentrifugation and analyzed with MALDI-TOF for protein profiling. The modified ApoA1 was further separated with Bis-Tris gradient gel and identified with MALDI-TOF and nanoLC-MS/MS.

Results and Discussion

The expression levels of major lipoproteins on HDL were analyzed with intact protein profiling. The results showed that the expression level of major lipoproteins are similar among all disease groups (diabetes, uremia and hyperlipidemia). Compared to healthy, diabetes, and hyperlipidemia subjects, uremia and chronic/acute stroke subjects displayed more intense modified-ApoA1 bands. These modified Apoa1 bands are highly correlated with oxidation, glycation and carbamylaton, which were also compared in all subjects.

Conclusion

Lipoprotein composition on HDL may not altered significantly and specifically to stroke disease. However, we found that higher expression level of modified ApoA1 bands on gradient gels is specifically appeared in stroke and uremia (easily developed to atherosclerosis) and may be used to evaluate HDL dysfunction and risks of atherosclerosis development to predict stroke.

Keywords: High-density lipoprotein, Stroke, ApoA1, Glycation, Carbamylation

WP15-11

EVALUATION OF CARDIOVASCULAR SYSTEM STATE BY URINE PROTEOME AFTER MANNED SPACE FLIGHT

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Introduction and Objectives

It is well known that microgravity conditions induce various changes in the cardiovascular system (CVS), including cephalic fluid shift, change in stroke volume, modification of cardiac cycle, general deconditioning and orthostatic intolerance. Adaptive adjustments to preserve the body vital constants under long-term microgravity conditions are manifested by qualitative and quantitative changes of proteins in body fluids.

Methods

In order to find markers to assess the functional state of the cardiovascular system before and after spaceflight (first and seventh day after landing), we analyzed the urine proteome composition of 10 Russian cosmonauts aged of 35 to 51 years who have completed 169 to 199-day spaceflight onboard the International Space Station (ISS). Also the analysis of urine samples of 6 cosmonauts' back-up group was conducted. A special sample preparation was performed, followed by liquid chromatography-mass spectrometry. We also performed analysis of over-represented biological processes using BiNGO program for CVS proteins.

Results and Discussion

It was found that changes in cosmonauts' urine proteome comprehensively reflect the adaptive responses of cardiovascular, renal and neuroendocrine systems to long-duration microgravity conditions. It was shown that physiological changes were connected with response to wounding, regulation of inflammatory response, blood coagulation, response to light stimulus, negative regulation of bone mineralization and others. Besides, some of biological processes could be related with specific space flight factors such as solar radiation or low wavelength electromagnetic radiations from the Earth or the space environment. Proteins participating in inflammation, response to wounding, blood coagulation processes could be related with microdamages in organism caused by G-forces during descent.

Conclusion

The use of bioinformatics analysis to the reconstruction of protein-protein interaction networks and the identification of overrepresented Gene Ontology biological processes related to the cardiovascular system allowed us to establish relationships between proteomic data and physiological effects observed in cosmonauts after the flight.

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Keywords: cosmonauts, cardiovascular system, urine proteome.We wish to thank "Bruker Daltonics“ for administrative and financial support. This work was supported by Russian Foundation of Basic Research (Grants 15-04-02463 A). High resolution mass spectrometry study was funded by Russian Science Foundation grant no. 14-24-00114.

WP15-12

Human plasma proteome analysis using meter-long monolithic silica columns with match-between-runs

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Introduction and Objectives

Plasma is the favorite specimen sample source for biomarker from discovery to clinical practice. Despite the great improvement of proteomic technologies, biomarker discovery in human plasma is restricted by the wide dynamic range in protein abundance with over ten orders of magnitude, results in low coverage of proteome. Although numbers of approaches using depletion of highly abundant proteins combined with multidimensional separation have been reported, it is still challenging task to unveil the human plasma proteome. Here, we describe two kinds of strategies for increasing the coverage of human plasma proteome.

Methods

We examined two different strategies for shotgun proteome analysis of depleted plasma (top 12 proteins). One is based on reversed phase StageTip chromatography at high pH for pre-fractionation (Hp-RP). De-glycosylated peptides (0.02mg) were loaded onto C18-beads packed StageTip and separated into five fractions by 11%, 14%, 17%, 20% and 45% acetonitrile in 200 mM ammonium bicarbonate (pH=10), followed by nanoLC-MS/MS. The other is based on match-between-runs (MBR) using cultured cells as reference. The tryptic peptides from Hela and human plasma samples were analyzed by 200 cm monolithic silica C18 column for 600 mins. The MBR function in MaxQuant was used to identify additional peptides.

Results and Discussion

Without depletion of top 12 abundant proteins, only 224 proteins were identified in single shot LC-MS/MS analysis. By using the Hp-RP approach, the number of identified proteins was increased to 421. Although the depletion of top 12 abundant proteins markedly increased the number of identified peptides from 1616 to 8128 (5-fold), the number of proteins was just slightly increased from 421 to 792 (1.9-fold). It might be due to the wide dynamic range in protein abundance even after depletion of 12 proteins. In order to detect low abundant proteins in plasma, we further used the MBR-based approach for quantifying the MS1 signals by the feature of MBR in MaxQuant. In the MBR approach, the tryptic peptides from Hela and depleted plasma samples were analyzed by 200 cm monolithic columns with 600 min gradient. Without MBR, the identified proteins/peptides were only 395/4476, while the number of identified proteins/peptides were increased to 1330/5948 with MBR using the reference peptides from Hela cells. The average number of identified peptides per protein by MBR (1.6) was much less than that by MS/MS (11.3). This result indicates the abundance of proteins identified by MBR is extremely low. We further implement these approaches to parallel reaction monitoring (PRM) for validation of those matched peptide in plasma samples. 88% of matched peptides could be identified

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with MS/MS fragment ions (b or y ions) by skyline software. The results indicated that MBR method could enhance the low abundance proteins identification.

Conclusion

We enhanced the coverage of human plasma by MBR together with single shot LCMS using meter-long monolithic silica columns

Keywords: Plasma, MBR, monolithic

WP15-13

Comparative Proteomic Analysis of Human Calcium Oxalate and Calcium Phosphate Kidney Stone Matrices

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Introduction and Objectives

About 5% of American women and 12% of men develop kidney stones. Most stones are composed of calcium oxalate (CaOx) and calcium phosphate (CaP). CaOx is the main mineral constituent, with CaP comprising <=10%. When CaP becomes the main constituent (>50%) of stones, the stones are referred to as CaP stones. Our goal is to better understand whether differences in constituent matrix proteins, their physicochemical properties, and pathways/functions associated with the unique proteomes are related to mechanisms of stone formation.

Methods

We analyzed the protein composition of representative stones from respective stone formers using label-free quantitative mass spectrometry via LC-MS/MS. 29 replicate human kidney stones from 15 de-identified patients were analyzed: 10 CaP (brushite, female) and 10 CaP (brushite, male), and 9 CaOx (male). Stones were powdered, proteins extracted, and analyzed using the IdentiQuantXL™ platform as recently described1 and developed to individually and accurately align the retention time of each peptide and to apply multiple filters for exclusion of unqualified peptides to enhance label-free protein quantification. Peptide retention time determination using clustering, extraction of peptide intensity using MASIC, peptide coefficient of variation calculation, and peptides correlation were all conducted within the software platform to “filter out” unqualified peptides. Using only qualified peptides, protein intensity was calculated using the formula: Protein Intensity = (intensity of peptide 1) / (peptide 1 sharing times) + …+ (intensity of peptide n)/(peptide n sharing times). For a peptide shared by different proteins, the intensity of this peptide was divided by the number of times the peptide was shared.

Results and Discussion

1,941 unique protein database entries representing 1,812 unique gene products were identified, quantified, and statistically compared. Of these, 1,004 proteins were detected and quantified in all three groups, mean abundances compared by ANOVA, and fold-differences calculated. Hierarchical clustering and PCA revealed a clear pattern of distinction between stone types (CaOx vs. CaP) but not gender (CaP). Correspondingly, male CaOx stone matrix had the largest number (305) of unique protein types. Significant differences in the mean abundance of both commonly detected and previously unreported stone matrix proteins were observed between all groups and suggest differential stone-formation mechanisms.

Conclusion

CaOx and CaP kidney stone organic matrices contain highly complex mixtures of cellular

WP15-13

and extracellular proteins that differ both by gender and stone type in a statistically significant manner, despite a high degree of variability.Reference: [1]Witzmann, et al. (2016). Label-free proteomic methodology for the analysis of human kidney stone matrix composition. Proteome Sci. 14, 1-10.

Keywords: kidney stone; calcium oxaate; calcium phosphate; quantitative mass spectrometry; label-free

WP15-14

Proteomics of urine from healthy volunteers by SWATH-MS

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Introduction and Objectives

SWATH-MS technology is moving increasingly into clinical research field to discover robust biomarkers for human diseases. “All-in-one” urine test project has been undergoing in our institute for discovery of predictive biomarkers for all diseases. To achieve this project, qualitative and quantitative proteomic data of health volunteers through generations and genders are crucial. We have collected more than 400 urine samples from health volunteers. In this study, 40 samples collected before and after sauna bathing were selected to certify whether our current protocols work well for protein preparation from frozen urine samples, protein digestion with trypsin and qualitative proteomics by SWATH-MS.

Methods

Forty frozen urinary samples, which were collected before and after bathing sauna with/without nano-mist for 2 weeks to examine effects on urine proteomes, were thawed at 37°C water bath and mixed well by vortexing and subjected for protein preparation by methanol/chloroform precipitation method. The pellet was dried and then dissolved in 8M urea Tris-HCl buffer (pH8.0) for in solution digestion with trypsin for 16 hours at 37°C. Digested peptides were purified by using C18 MonoSpin column and 500 ng peptides dissolved in 0.1% formic acid were used for qualitative and targeted proteomics by LC-mass spectrometry (SCIEX 5600+ and Esigent HPLC 400). Protein identification was performed by ProteinPilot™ (SCIEX) search engine with paragon algorisms and SWATH data was analyzed by PeakView™ (Ver. 5.0) combined with SWATH tool (ver. 2.0). Consensus spectral library was created from our own urine identification result. To demonstrate difference in biological functions after sauna bathing, urinary proteins changed more than 2-fold in amount were analyzed by Panther™ and DAVID™ bioinformatics tools.

Results and Discussion

In the 40 urine samples, 1341 proteins and 21165 distinct peptides were identified in total with high stringency (Local FDR<1%). Approximately 80% of urinary proteins were not significantly changed after the sauna bathing, and 18.7% of proteins changed within 2 folds, and 1.8% (24 proteins) changed in 2- 3 folds and only 0.2% changed more than 3-folds. Analysis of pathways for biological processing with these changed urine proteins suggested increase in glycolysis process after the sauna bathing (without nanomist). Enhancement of focal adhesion and lysosome related proteins were observed after sauna with Nano-mist in same time.

Conclusion

Urine qualitative and targeted proteome have been performed successfully by our protocols. And we hope the protocols will support “All-in-one” urine test project well in

WP15-14

future. These health volunteer urinary proteome data may help robust non-invasive biomarkers discovery.

Keywords: Urine, Nano-mist, Biomarker, SWATH.

WP15-15

Proteomics of Diabetic Nephropathy Glomerulus

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Introduction and Objectives

Diabetic nephropathy (DN) is a major serious complication of diabetes and also a major chronic kidney disease (CKD) to progress to the end-stage chronic kidney failure, which needs dialysis therapy. Although the number of DN patients is increasing all over the world, no specific treatments for DN have developed yet. As the glomerulus is an initial site injured in the kidneys of the patients, the glomerulus of DN kidneys were analyzed by proteomics to understand the pathophysiology and to proceed to develop new specific treatments for DN.

Methods

Autopsied formalin-fixed paraffin-embedded (FFPE) human kidneys from DN patients and non-kidney disease patients as a control were used in this study. Kidney lesions of DN were divided into three groups by histology: early, intermediate and advanced stages (n=5 each). The kidneys were sectioned at 10 μm thickness and 50 glomerular sections were collected from each kidney by laser-microdissection. The glomerular sections were then autoclaved and digested directly with trypsin (OSDD method) and peptides were purified with C18 spin columns. The peptides were analyzed by Orbitrap Fusion mass spectrometry and proteins were identified by Mascot search engine and quantitated by modified normalized spectrum index (SIN). Proteins, which increased in DN glomerulus more than two-fold comparing to control glomerulus, were analyzed by DAVID pathway analysis.

Results and Discussion

More than 1500 proteins were identified (FDR <0.01) by MS of each glomerulus sample and compared with the control glomerulus proteome profile. The numbers of proteins identified both in control and early stage DN were more than 2,000, those in intermediate stage more than 1,600 and those in advanced stage more than 1,100. Among these proteins, the proteins increased more than two-fold in the value of SIN comparing to control were analyzed by the pathway analysis. The pathway analysis depicted significantly enhanced pathways in each DN stage and the results may contribute to promote development of specific therapies to DN.

Conclusion

This is the first study in the world analyzing proteomes of DN glomerulus and demonstrating significant uniqueness in the pathophysiologic processes. Our study

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will contribute to promotion of molecular pathology-based new treatments for DN in the future.

Keywords: Diabetes, Proteome, Glomerulus, Kidney

WP15-16

Application of High Throughput Urinary Proteomic Strategy in the Diagnosis of Acute Appendicitis with Confusable Acute Abdomens

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Introduction and Objectives

Urine has been considered to be a good source for disease biomarker discovery with the advantages of being non-invasive and easily attainable. However, application of urinary biomarkers in clinical disease diagnosis is extremely rare. Acute appendicitis is the most common surgical emergency, but differential diagnosis of acute appendicitis from other acute abdomens with similar symptoms remains a major challenge. We aim to applicate the high throughput urinary proteomic strategies to the discovery of diagnostic biomarkers of acute appendicitis, to improve the diagnosis and treatment of this disease in clinic.

Methods

In this study, a high throughput proteomic method had been optimized for urine biomarker discovery. In brief, urine proteins were extracted by two-step ultracentrifugation, separated by SDS-PAGE and identified by LC-MS/MS. To get better effect on disease classification, we developed a novel outlier-based method for disease diagnosis using the normal range derived from healthy individual database.

Results and Discussion

With the optimized proteomic method, more than 1000 proteins could be identified in each urine sample with good reproducibility. Urine samples from 87 acute appendicitis (AA) cases and 43 control acute abdomen (CON) cases were processed. For data analysis, traditional methods such as identification of differentially expressed proteins and classification modeling based on machine learning algorithms (including random forest, support vector machine and naive Bayes algorithms) were used, but none of these methods could yield an accuracy of more than 80%. Then we developed a new data analytic strategy: AA-specific and CON-specific outlier pools were established respectively, and a diagnostic algorithm including indexes of outlier frequency difference and total outlier hit numbers was optimized. With this new strategy, the diagnostic accuracy was 95.0% in training set containing 30 AAs and 30 CONs, and 90% in validation set containing 57 AAs and 13 CONs, which is better than traditional methods.

Conclusion

A high throughput and stable system had been established for urine protein extraction and identification. And a new outlier-based algorithm for diagnosis of acute appendicitis and other acute abdomen were developed with better results than the traditional analysis methods. Our study on urine biomarker discovery showed great potential in clinical application.

Keywords: Urinary proteomics / Acute appendicitis / Acute abdomens/ Biomarker / Outliers

WP16-01

Biological Characteristic of Human Enterovirus SpeciesA in Human Nucleolin Transgenic Mice

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Introduction and Objectives

Human enterovirus species A (HEV-A), one of the four species of HEV in the genus *Enterovirus* of the *Picornaviridae* family, is known to manifest hand-foot-mouth diseases in young children and may cause severe neurological disorders such as encephalitis and meningitis. This virus family consists of 16 stereotypes, such as *Coxsackieviruses* (CV) A2–A8, A10, A12, A14 and A16 and *Enterovirus* 71. Receptors are necessary in the early stages of virus infection. The virus recognizes surface receptors and entry into the host cells. In our previous study, we have identified nucleolin (NCL) as an EV71 bnding receptor by glycoproteomic approaches. We found that EV71 interacted directly with nucleolin, and an anti-nucleolin antibody reduced the binding of EV71 to host cells. In addition, the knockdown of nucleolin decreased the attachment of EV71 to RD cells. The binding ability of EV71 strains to nucleolin-expressing mouse cells were also significantly elevated.

Methods

In order to understand whether nucleolin mediates in the early stages of HEV-As infection, we evaluated the binding ability of HEV-As *in vivo*.

Results and Discussion

Human nucleolin gene was subcloned to Fusion Red and Flag-tagged BAC vector to create human nucleolin transgenic(Tg) mice. We infected the Tg mice with a mouse-adapted EV71 MP4(C2), EV71 human strains 87-N6356(C2), 94-N2873(C4), 97-M448(B5), and coxsackievirus CA16 via intraperitoneal (i.p.) injection. The biological characteristic such as body weight, survival and clinical score of limb paralysis were observed and compared. Among these viruses, MP4-infected-Tg mice displayed progressive limb paralysis prior to death. Further virus distribution and histopathological examination will be investigated in the limb muscles, brainstem, and spinal cord.

Conclusion

Our results indicated that the hNCL transgenic mice could be a useful animal model for the study of EV71 infection.

Keywords

nucleolin; enterovirus species A

WP16-02

Zika Virus is Not Alone: Proteomics Associates a Bovine-Like Viral Diarrhea Virus to Microcephaly

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Introduction and Objectives

Reports linking high incidence of human microcephaly to the presence of Zika virus do not disclose any experimental data that provide a direct link between microcephaly and Zika virus during fetal brain development. Here, our objective is to use discovery-driven shotgun proteomics based on high resolution and accuracy mass spectrometry analysis to show the detection of a BVDV-like virus in tissue extracts of Zika positive brains.

Methods

Postmortem brain tissue of fetuses with microcephaly PCR positive for Zika virus were extracted with 5% sodium deoxycholate (SDC) / 0.75 mM DTT and protease and phosphatase inhibitors, precipitated with cold acetone, washed, dried, dissolved in 7M urea / 2M thiourea, reduced, alkylated and trypsin hydrolyzed. Peptide mix were separated by RP-nLC coupled to a QExactive Plus; spectra were first analyzed in Proteome Discoverer 2.1 using the Sequest TM algorithm (FDR <1%).against UniProt all virus entries and then reanalyzed in a human and virus concatenated database downloaded from UniProt (Jun_2016). Another database search used used PatternLab 4.0 software and Comet algorithm against the same concatenated human and virus database. Post-processed results accepted only PSMs with less than 6 ppm from the global identification average. At the protein level, the FDR was set to lower than 1% for all search results.

Results and Discussion

The first search identified polypeptides from Bovine Viral Diarrhea Virus (BVDV) and no Zika proteins. The second search - combined virus and human entries - showed two Master Proteins that identified BVDV polyproteins (Q65815 andQ8B512). PatternLab 4.0 and Comet, respectively, using the same virus and human database, identified the same proteins. In summary, the searches identified at least 25 peptides that map to BVDB peptides and none to Zika. Blastp against UniProt viruses' databank identified as first hits several BVDV polyproteins. When blasted against UniProt Human Protein database the same peptides, except one, showed 100 % matches to five different human proteins. However, a unique 21-residue peptide pinpointed by the Proteome Discovery search, TLTGRTITLEVEPSDTIENVK, was the only one to match 100% of the sequence of residues 1597 to 1617 of the genome polyprotein of bovine viral

diarrhea virus 1-Osloss. Importantly, this peptide showed a lower match of 95.2 % to human ubiquitin-60S ribosomal protein L40 differing from all other 24 peptides that matched 100 % with both human and virus proteins. The difference is due to the exchange of R in the virus protein for a K.

Conclusions

Our data raises the suspicion that another agent possessing similar structural features and properties to BVDV might induce the same anomalies to animal and human fetuses. Finally, it did not escape to our attention one working hypothesis for viruses functioning in humans: concerted actions in a synergistic way such that Zika virus breaks down physiological barriers for entrance of the B

Keywords

Microcephaly, Zika virus, BVDV-like virus

WP16-03

Lymph Node Metastasis of Primary Endometrial Cancers: Associated Proteins Revealed by Maldi Imaging

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Introduction and Objectives

Endometrial cancer is the most common gynaecological malignancy in Australia with 2,256 diagnosed cases in 2010 and 381 associated deaths in 2011. The presence or absence of lymph node metastasis is the most important prognostic factor in early stage I endometrial cancer. Of the patients diagnosed with stage I disease, around 10% will have pelvic lymph node metastases (LNM). Despite the small percentage of patients who suffer from metastasis the majority undergo radical treatment including the removal of lymph nodes; a precautionary measure carried out due to our current inability to accurately stage the disease. Lymph node removal is associated with significant complications including lower extremity lymphoedema, occurring in up to 38% of patients. A classification system based around predictive tissue markers of metastasis is therefore essential to determine the optimal treatment strategy for endometrial cancer patients and to reduce disease morbidity. In this study we show that data acquired from the MALDI imaging of primary endometrial carcinomas can be used to successfully predict the presence or absence of LNM with an overall accuracy of 88.4%. The development of such a classification method shows the diagnostic potential of MALDI imaging in determining the metastatic potential of primary carcinomas.

Methods

In the current retrospective study, we have used primary tumour samples of endometrial cancer patients diagnosed with (n=16) and without (n=27) lymph node metastasis to identify potential discriminators. Upon data acquisition, a Canonical Correlation Analysis (CCA) based method was applied to rank the intensities of the acquired MALDI *m/z* values based upon their power to discriminate the primary carcinomas with metastasis from those without. This ranking was used to reduce the

dimension of the data to the top ranked *m/z* values prior to classification by linear discriminant analysis (LDA), and the performance of this classification was judged by leave one out (LOO) cross validation The top *m/z* values were targeted for identification using the complementary techniques of *in-situ* MALDI MS/MS and matching to peptide sequences obtained from traditional nano-flow liquid chromatography electrospray ionization tandem mass spectrometry (nanoLC-ESI-MS/MS). The localization of the peptides from the proteins found to be differentially expressed were visualized using peptide MALDI MSI in whole tissue sections as well as tissue microarrays containing a total of 43 patients. The proteins identified were further validated by label-free quantitative LC-MS/MS and immunohistochemistry.

Results and Discussion

Using peptide MALDI-MSI, we have identified *m/z* values which can classify 88% of all tumours correctly. The top discriminative *m/z* values were identified using a combination of *in situ* sequencing and LC-MS/MS from digested tumour samples. Two of the proteins identified, plectin and α-Actin-2, were used for validation studies using LC-MS/MS data independent analysis (DIA) and immunohistochemistry. Moreover, logistic regression analysis of our patient cohort implicated a correlation between lympho-vascular space invasion and LNM, which has previously been shown. Furthermore, a comparison of ROC curves from different multivariate regression models indicate that the multivariate logistic regression model of MALDI MSI data alone has the potential to predict LNM from primary tumours and potentially perform better than models containing immunohistochemistry data. To the best of our knowledge this is the first time, the performance of different models using pathological parameters, MALDI MSI data and/or IHC data haven been directly compared.

Conclusions

In summary, MALDI-MSI has the potential to identify discriminators of metastasis using primary tumour samples. The differential expression of plectin and α-Actin-2 between the primary carcinomas with and without LNM was further validated using label-free quantitative LC-MS/MS and immunohistochemistry. Moreover, the predictive model using highly ranked *m/z* values identified by MALDI-MSI showed significantly higher prognostic accuracy than the model using immunohistochemistry data.

Keywords

Endometrial cancer, metastasis, MALDI-MSI, label free LC-MS/MS, immunohistochemistry

WP16-04

Human SRMAtlas: A Resource of Targeted Assays to Quantify the Complete Human Proteome

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The ability to reliably and reproducibly measure any protein of the human proteome in any tissue or cell-type would be transformative for understanding systems-level properties as well as specific pathways in physiology and disease. We have completed the generation and verification of a compendium of highly specific assays that enable the identification and quantification of 99.7% of the 20,277 annotated human proteins by the targeted and sensitive mass spectrometric method selected reaction monitoring, SRM[1]. The human SRMAtlas is developed with well-characterized, chemically synthesized proteotypic peptides on a range of mass spectrometers and provides definitive coordinates that conclusively identify the respective peptide in biological samples. We report data on 166,174 proteotypic peptides providing multiple, independent assays to quantify any human protein and numerous spliced variants, non-synonymous mutations and post-translational modifications. The data is freely accessible as a resource at www.srmatlas.org, and we demonstrate its utility by examining the network response to inhibition of cholesterol synthesis in liver cells and to docetaxel in prostate cancer lines.

[1]Kusebauch, U. *et al.* Human SRMAtlas: A resource of targeted assays to quantify the complete human proteome. Cell 2016, 166 (3), 766-778.

WP16-05

Low Microgram Amount Protein Samples Coupled with Novel Polymers for TAILS Terminomics Yield Efficient Enrichments Utilizing Precipitation with Organic Solvents

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Introduction and Objectives

Proteolytic processing of proteins is a pervasive post-translational modification that affects over 50% of all human gene products. Processing of proteins can alter their activation state, cellular localization, binding partners and have profound implications on cellular life. Determining the cleavage position of protease substrates in complex proteomes poses a unique analytical challenge as there is no single chemical group that enables their enrichment using conventional chromatographic or antibody-based methodologies. However a variety of techniques are available, of which Terminal Amine Isotopic Labeling of Substrates (TAILS) was developed by our lab. N-terminal peptides are enriched via the specific binding of internal tryptic peptides to a water soluble polymer (HPG-ALD) 100 kDa in size and removed by ultrafiltration. Despite the effectiveness of TAILS to enrich for N-terminal fragments of proteins from a myriad of tryptic peptides, removal of the polymer from the sample is cumbersome at retrieving all peptides using ultrafiltration. Here we developed new high molecular weight polymers (800 kDa) in combination with precipitation methods to enrich N-terminal fragments in E. coli including acetylated peptides not visible by shotgun proteomics. We apply this down to 150 ug sample with potential for lower amounts and reliable identification.

Methods

HPG-ALD polymers of 800 kDa in size with varying numbers of aldehyde groups were prepared as previously described. The regular 100 kDa HPG-ALD polymer with ~400 ALD groups was also utilized. E. coli K12 proteins were extracted by sonication in 1% SDS, 100 mM DTT, alkylated with N-ethylmaleimide and precipitated with chloroform/methanol. Proteins were dimethylated using light formaldehyde and digested with trypsin. Depletion with polymer was carried out using 40 mM sodium cyanoborohydride. Polymer was removed from samples using ultrafiltration as indicated by the manufacturer's instructions or precipitated using acetone or a combination of acetone/methanol. Following evaporation of organic solvents, peptides were purified using C18 resin and C8 discs in house-packed columns. Peptides were analyzed by LC-

MS/MS on a Thermo Easy nLC-1000 coupled online to a Bruker Daltonics Impact II ultra-high resolution mass spectrometer.

Results and Discussion

Utilization of precipitation of polymers proved that over 96% of the polymer is recovered as measured by thermogravimetric analysis with no detrimental impact on LC-MS/MS performance. Precipitation improved the recoverable number of peptides compared to ultrafiltration in the 100 kDa polymer and enriched for N-terminally acetylated peptides in E.coli. Using methanol as an additive in the precipitation steps improves the recovery of hydrophilic peptides and proves to be fast, consistent and simple to perform while reducing costs of the protocol.

Conclusions

Removal of HPG-ALD polymers by precipitation proves to be a fast, reproducible and reliable method for terminomics.

Keywords

Terminomics, polymer precipitation.

WP16-06

Identification of Cofilin-1 Induces G0/G1 Arrest and Autophagy in Angiotensin-(1-7)-treated Human Aortic Endothelial Cells from iTRAQ Quantitative Proteomics

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Introduction and Objectives

The angiotensin-converting enzyme 2/angiotensin-(1-7)/Mas axis is a pathway that acts against the detrimental effects of renin-angiotensin system. However, the effects of angiotensin-(1-7) on endothelial protein expression and related phenotypes are unclear.

Methods

We performed a duplicate of iTRAQ quantitative proteomic analysis on human aortic endothelial cells (HAECs) treated with angiotensin-(1-7) for 6 hours and related biochemical experiments to validate the hypothesis.

Results and Discussion

Cofilin-1 was identified as the high abounded candidate with consistent > 30% coverage and > 1.2-fold overexpression in the angiotensin-(1-7)-treated group. Gene ontology analysis was shown 'regulation_of_mitosis' was significantly altered and cell cycle analysis indicated that 6 hour angiotensin-(1-7) treatment significantly induced G0/G1 arrest. Knockdown of the *cofilin-1 (CFL1)* gene suggested the G0/G1 phase arrest was mediated by Cofilin-1 modulation of p27 and p21/Cyclin/CDK complex. Interestingly, quiescent HAECs escaped G0/G1 arrest upon angiotensin-(1-7) treatment for 24 hours and angiotensin-(1-7) induced autophagy by upregulating Beclin-1 and microtubule-associated protein 1 light chain 3b-II expression, which was also attenuated by A779 pre-treatment and *CFL1* knockdown. Pre-treatment with 3-methyladenine (3MA), 24h angiotensin-(1-7) treatments induced a significant G0/G1 phase arrest and apoptosis, suggesting a pro-survival role of autophagy here.

Conclusions

In conclusion, Cofilin-1 plays a dominant role in angiotensin-(1-7)-induced G0/G1 arrest and autophagy to maintain cellular homeostasis in HAECs

Keywords

Cofilin-1, Angiotensin-(1-7), HAECs, iTRAQ, Cell cycle, Autophagy

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Zimmermann, Michael	MO04-004
Zoccaratto, Anna	TP02-011
Zolles, Gerd	WO08-002
Zou, Hanfa	TO02-003
Zou, Jin	TP01-005
Zubarev, Roman	MP06-051
Zwak, Sandra	MP07-017
Zwinderman, Aeilko	MP09-006

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