Quantitative Proteomic Approaches for the Determination of Serum Proteome in Patients with Benign Prostate Hyperplasia and Prostate Cancer

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Overview

Purpose: The development of a novel quantitative MS method for the characterization of the low molecular weight (< 50 kDa) serum proteome of potential clinical significance to prostate cancer.

Methods: An LTQ Orbitrap Velos mass spectrometer equipped with progressively stacked-ring ion guide, dual-cell differentially pumped ion trap, and higher energy collision dissociation (HCD) cell with axial field was used for the analysis of the iTRAQ™ 8-plex labeled sample set.

Results: Using ZIC-HILIC pre-separation, a broad coverage of human serum proteome was achieved with respect to protein MW, pI, hydrophobicity and biological significance. The LTQ Orbitrap Velos instrument delivered both identification and quantitation of peptides in a single method/analysis.

Our findings from serum analysis support the notion of epidemiological correlation of metabolic syndrome to prostate cancer proteins found in prostate tissue. Uniquely occurring prostate cancer (PCa) proteins were identified relative to the benign prostate hyperplasia (BPH).

Introduction

Prostate cancer was the 2nd most common form of cancer diagnosed in men in 2008 in the United States and the European Union. Early screening, involving serum prostate specific antigen (PSA) levels, is currently the most reliable way to diagnose prostate cancer. However, with a specificity of abnormal PSA reaching only 20%, the need for more discriminatory protein biomarkers emerges. As such our project objectives include: a) mass spectrometry (MS) based characterization of proteomic profiles in hyperplasic and cancerous prostate serum samples; b) determination of novel biological markers with greater specificity and sensitivity than the current clinical PSA approach, and c) validation of these biomarkers.

We describe the analysis of multiple fractions collected after ZIC-HILIC separation of non-depleted human serum sample digests following the labelling with iTRAQ 8-plex reagent. We focus on high confidence identification of proteins together with relative quantitation to serve as a basis for selecting proteins of interest directing our future research efforts.

Methods

Sample procurement and preparation - Peripheral venous whole blood specimens were obtained from male patients diagnosed with prostate cancer (PCa) with Gleason score (GS) ≥6 (N=15) and male patients diagnosed with benign prostate hyperplasia (BPH) (N=9).[1] For each specimen collected into anticoagulant-free tubes, the cellular components were separated upon centrifugation at 3000 *rcf* after 30 minutes clotting at room temperature. Serum samples were diluted with ultra pure water (with 0.01% SDS) up to 30 mL and filtered (2000 *rcf*) with 50 kDa MWCO Amicon™ ultrafiltration membranes. The resultant filtered samples were then concentrated to 200 µL final volume using 3 kDa MWCO Amicon ultrafiltration membranes. Proteins (at 2 x 100 µg for each pooled specimen) were reduced, alkylated and digested as reported.[1] In our study, the iTRAQ reagents containing the 113, 114 and 115 reporter ions corresponded to the BPH pooled sera, the 116, 117, and 118 reporter ions corresponded to the PCa sera with GS = 6, and the 119 and 121 pooled sera corresponded to the PCa sera with GS = 8 (Figure 1).

Peptide Fractionation - Zwitterion Ion Chromatography – Hydrophilic Interaction Chromatography (ZIC-HILIC; Merck, 4.6 mm X 150 mm; 5-µm particle; 300 Å pore retrofitted with a 2.1 mm X 20 mm guard column with analogous chemistry, particle size and pore size features) was performed using a Dionex HPLC equipped with a multi-wavelength UV absorption detector (Dionex, P460). Peptides were eluted with a linear gradient of 0 – 100% mobile phase B (90/10% v/v water/ACN, 10 mM ammonium formate, pH 3) over a 40 min time period with fractions collected in a peak-dependent manner.

LC-MS: Peptides reconstituted in 30 µL of 0.1% aqueous formic acid (3 µL injected) were loaded using a pre-column (Nanoseparations C18, 3 µm) and separated on the analytical column (same stationary phase, 10 cm x 75 µm ID) with a linear gradient of acetonitrile containing 0.1% formic acid from 0 to 60%. Flow rate 300 nL/min, total run time 90 min.

Mass spectrometric analysis was performed with a Thermo Scientific LTQ Orbitrap Velos. The following settings were used: full scan resolution 30,000; MS/MS scan in HCD cell at 7,500 resolution with 2 microscans; max inject time 250 ms.

Peptide identification/quantitation: Thermo Scientific Proteome Discoverer was used for identification (Mascot™ search engine) and quantitation. Database contained 20,328 human proteins (SwissProt) and parameters were: missed cleavage sites 1; peptide mass tolerance 10 ppm; fragment mass tolerance 20 mmu; modifications MMTS on Cys, iTRAQ on *N*-terminus and Lys, phosphorylation on Ser, Thr, Tyr, acetylation on *N*-terminus, and oxidation of Met.

FIGURE 1. Workflow summary for sample preparation and iTRAQ labeling used

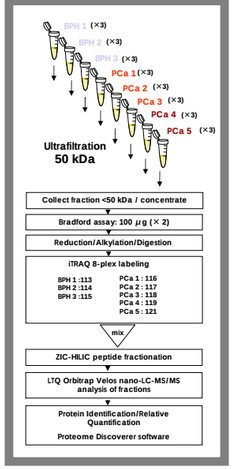
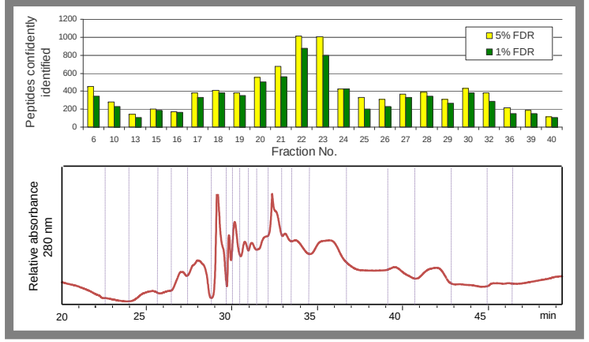


FIGURE 2. Number of peptides confidently identified at specific FDR in individual ZIC-HILIC fractions (top pane). The bottom pane shows the UV trace (280 nm) from ZIC-HILIC separation of iTRAQ-labeled peptide mixture



Results

The analysis of 23 ZIC-HILIC fractions using the LTQ Orbitrap Velos™ system coupled to LC separation on reverse phase C18 nano-column provided identification of 10,727 peptides pertaining to 577 different human proteins. The confidence of these identifications was ascertained by a search in a decoy database, and FDR <5% was used for final results reporting (Figure 2).

The methodology used in this study provide a broad overview of serum proteome as documented by the wide distribution for the MW, pI and hydrophobicity of confidently identified proteins (Figure 3).

The use of 8-plex reagent enabled us to improve the reproducibility by employing several labels for the same sample set (Figure 1). The new collision cell design enabled efficient quantitation of reporter ions. More than 99% of identified peptides showed the iTRAQ reporter ion signatures in their spectra. Data processing and quantitation was done in Proteome Discoverer™ software.

FIGURE 3. Distribution of MW, pI range and hydrophobicity for proteins confidently identified in this study (FDR <5%). Proteins marked in red are those found to be up-regulated in the PCa sera. Proteins marked in green are those found to be up-regulated in both PCa sera and tissue specimens. Positive Gravy Index values denote hydrophobic character whereas negative Gravy Index values denote hydrophilic character.

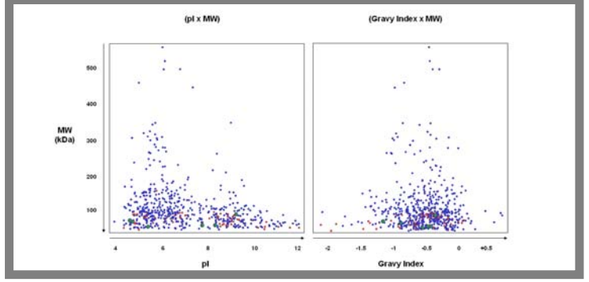
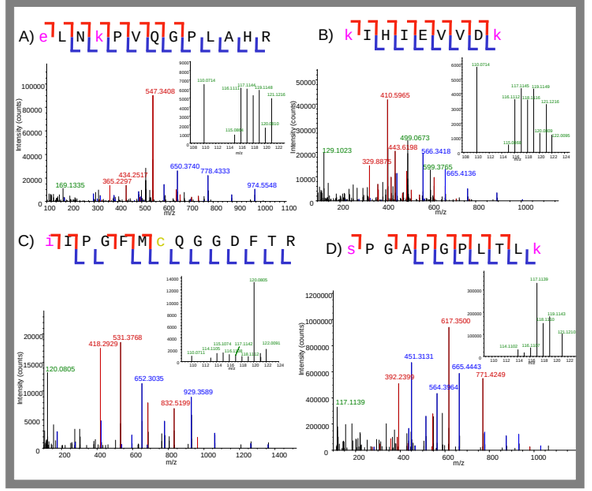


FIGURE 4. Interpreted MS/MS spectra for peptides from von Willenbrand factor A: A) voltage gated sodium channel, B) peptidyl-prolyl cis-trans isomerase A, C) and zyxin, D) the insert provides a detail of reporter ion region. The diagram of peptide sequence shows the extent of sequence coverage for b,y-ion series (iTRAQ-labeled residues in magenta)



The preliminary review of the quantitative results highlighted several proteins that represent novel findings. High levels of von Willenbrand factor protein (vWf) were significantly associated with short cancer-specific survival in Kaplan-Meier analysis of all patients with GS 6 and 7 tumors and constitutes a potential prognosis marker.[2] The vWf was identified for the first time with an MS based proteomic approach. The voltage gated sodium channel (VGSC), a membrane bound porin protein, was found to be a potential new drug target for the senescence of prostate cancer cells.[3] The ability to identify this highly hydrophobic protein was made possible with our novel methodological approach.

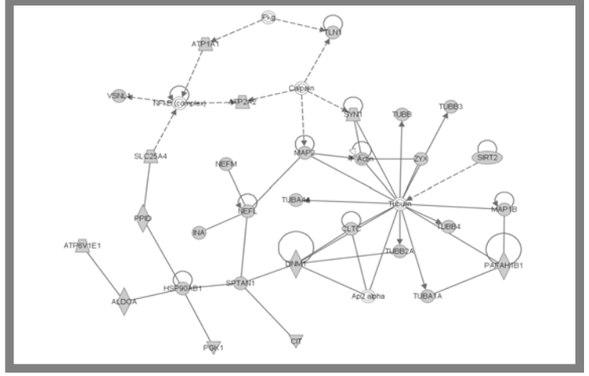
Our current results show that both the vWf and VGSC proteins are essentially uniquely expressed in the PCa specimens (Figures 4A and 4B) signifying their relevance as potential clinical markers for the prognosis and staging of prostate carcinogenesis and its pharmacologic treatment.

The cis/trans isomerization of the peptide bond preceding proline is an intrinsically slow process, although important in both prokaryotes and eukaryotes. *In vivo* this isomerization is catalyzed by peptidyl-prolyl cis/trans isomerase (PPIase).[4] The PPIase was found to be up-regulated in the PCa sera specimens relative to the BPH sera samples (Figure 4C) in this study. This PPIase trend was consistent with that observed in the prostate tissue specimens.[1]

The differential expression of zyxin is implicated in the migration potential of prostate cancer cells. The present study resulted in the significant up-regulation of zyxin in the PCa sera specimens relative to the BPH sera specimens (Figure 4D). This trend was opposite to that observed in the respective prostate specimens.[1] Further biological verification studies will be needed to explain such a discrepancy.

Figure 5 illustrates the functional inter-dependence of a subset of the differentially expressed proteins found in this study in terms of their relevance to established carcinogenesis pathways as determined with the Ingenuity Pathway Analysis software program (Ingenuity Systems, Inc.). As such, expressed proteins found in this present study may constitute mechanism based markers.

FIGURE 5. Clustering of regulated proteins by means of cancer networks. Proteins highlighted in grey were observed to be up-regulated in PCa sera specimens relative to the BPH sera specimens.



Conclusions

Simultaneous confident identification and quantitation of 577 proteins (FDR <5%) was performed using the LTQ Orbitrap Velos. A wide protein physico-chemical and biological space was captured thanks to the high sensitivity, selectivity and specificity attributes of the novel LC-MS method employed in this study.

The ZIC-HILIC separation was used to fractionate the mixture of peptides before their separation on reverse phase nano-LC. With the hindsight, the sample would benefit from more extensive removal of top 10 serum proteins before ZIC-HILIC separation.

A number of differentially expressed proteins found in the sera specimens in this present study were consistent with those observed in respective prostate tissue specimens. These proteins include Cofilin-1, Elongation factor 1-α, PPIase, Heat shock protein HSP90, various Histone isoforms, Nucleophosmin, and 14-3-3 protein β/α.

Our results suggest epidemiological correlation of metabolic syndrome to prostate cancer proteins reported earlier in prostate tissue. This is potential novel therapeutic finding.

The pathway analysis employed in our study underscores the validity and relevance of the approach in providing greater insight to the biology of prostate cancer.

References

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4. Kouri, E.D., Labrou, N.E., Garbis, S.D., *et al.* Molecular and Biochemical characterization of the Parvulin-type PPIases in Lotus japonicus. (2009) *Plant Physiology* PMID:19403733.

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