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Search for Potential Markers for Prostate Cancer Diagnosis, Prognosis and Treatment in Clinical Tissue Specimens Using Amine-Specific Isobaric Tagging (iTRAQ) with Two-Dimensional Liquid Chromatography and Tandem Mass Spectrometry

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This study aimed to identify candidate new diagnosis and prognosis markers and medicinal targets of prostate cancer (PCa), using state of the art proteomics. A total of 20 prostate tissue specimens from 10 patients with benign prostatic hyperplasia (BPH) and 10 with PCa (Tumour Node Metastasis [TNM] stage T1−T3) were analyzed by isobaric stable isotope labeling (iTRAQ) and two-dimensional liquid chromatography−tandem mass spectrometry (2DLC−MS/MS) approaches using a hybrid quadrupole time-of-flight system (QqTOF). The study resulted in the reproducible identification of 825 nonredundant gene products ($p \leq 0.05$) of which 30 exhibited up-regulation (≥2-fold) and another 35 exhibited down-regulation (≤0.5-fold) between the BPH and PCa specimens constituting a major contribution toward their global proteomic assessment. Selected findings were confirmed by immunohistochemical analysis of prostate tissue specimens. The proteins determined support existing knowledge and uncover novel and promising PCa biomarkers. The PCa proteome found can serve as a useful aid for the identification of improved diagnostic and prognostic markers and ultimately novel chemopreventive and therapeutic targets.

Keywords: prostate cancer • proteomics • biomarkers • iTRAQ • LC−MS

Introduction

In 2006, a total of 234 460 new cases of prostate cancer (PCa) were diagnosed in the U.S.A.[1] Currently, screening for prostate cancer involves a digital rectal exam and the serum determination of the levels of the prostate-specific antigen (PSA). Although screening with PSA has provided improvement in the diagnosis of PCa, it presents suboptimum sensitivity and specificity as an early stage marker.[2,3] As such, there is a need for more discriminatory protein biomarkers to assist therapeutic intervention and to define higher risk candidates for preventative intervention.

Because the process of carcinogenesis involves the synergistic change in multiple pathways inside the cell, an effective means to investigate and understand them is to engage a global approach that identifies and considers multiple changes simultaneously. Proteomics allows the large scale analysis of protein identity and expression. The objectives of this study were the development and application of a quantitative proteomic method involving the use of two-dimensional liquid chromatography hyphenated with high resolution, tandem mass spectrometry (2DLC−MS/MS) techniques in combination with the use of isobaric tags for relative and absolute quantification (iTRAQ).The principal advantages of 2DLC−MS/MS methods using iTRAQ labeling include the ability to conduct multiplex experiments, whereby up to four samples can be analyzed concurrently under the same experimental conditions, resulting in reduced systematic error and increased electrospray ionization efficiency leading to higher sensitivity; in addition, because protein identification and quantification is based on tandem mass spectrometric (MS/MS) evidence, increased selectivity, specificity, and confirmatory power are achieved.[4]

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The latest findings suggest that carcinogenesis and carcinomas are phenomena that occur in the tissue as a whole and not in individual cells alone.[5,6] It is in the tissue matrix whereby an evolving crosstalk between epithelium and stroma takes place. This interactivity between the stroma and epithelium constitutes the tumor system, and its study provides a more accurate forum in understanding carcinogenesis. As such, the hyperplasic and cancerous prostatic tissue matrix provides a suitable environment for the determination of novel prostate cancer diagnosis and prognosis markers along with promising new targets for chemopreventive and therapeutic intervention. In the study presented herein, we describe the application of an LC−MS based proteomic analysis of prostate tissue specimens from patients with BPH and PCa.

Materials and Methods

Reagents and Chemicals. The chemical reagents acetonitrile, ethanol, isopropanol, methanol, acetone, and formic acid (HPLC grade) were obtained from Sigma Corporation (St. Louis, MO). The ultrapure HPLC grade water, utilized for the initial peptide fractionation with strong cation exchange high-performance liquid chromatography (SCX HPLC) and subsequent LC−MS analysis procedures, was generated from the Barnstead water filtration system (Dubuque, IA). All iTRAQ reagents and buffers were obtained from Applied Biosystems (Foster City, CA).

Serum PSA Analysis. Serum PSA measurements were performed using the PSA-RIACT reagents' kit (Cisbio International) and Automatic Gamma Counter WIZARD 1470 instrumentation. PSA-RIACT is a solid-phase two-site immunoradiometric assay. Two monoclonal antibodies were prepared against sterically remote sites on the PSA molecule. The first one was coated on the solid phase (coated tube); the second one, radiolabeled with iodine 125, was used as a tracer. The PSA molecules present in the standards or the samples to be tested were "sandwiched" between the two antibodies. Following the formation of the coated antibody/antigen/iodinated antibody sandwich, the unbound tracer was removed by a washing step. The radioactivity bound to the tube is proportional to the concentration of PSA present in the serum sample.

Patient Characteristics and Tissue Procurement. A total of 20 patients were included in the study. The patient consent forms along with tissue procurement procedures were approved by the biomedical ethics committees of the Laiko Hospital of Athens and the Athens University Medical School (ref. No. 456/19−06−06).

The prostate was macroscopically evaluated by the urologist and the pathologist immediately after the operation. In the cases of prostate cancer, a 7 mm core borer was entered posterolaterally on the right and left side of the gland, from the base to the apex. As a result, two large cylinders of tissue were removed. The procedure was performed blindly because prostate cancer is difficult to identify grossly, so there is no advantage of widely opening the gland while attempting to visualize the tumor. On the basis of this protocol, there was a 70% success on the isolation of cancerous material with various concisions. Tissues extracted from the core borer were then cut lengthwise. One half was stored in liquid nitrogen within a 5 min interval and then subsequently transferred to the proteomics laboratory for storing at −80 °C prior to sample preparation. The mirror face half was evaluated after standard preparation of 5 μm thick, formalin fixed paraffin embedded sections, stained with hematoxylin and eosin, to confirm the primary isolation of cancerous tissue. In cases of benign prostatic hyperplasia (BPH) tissue specimens, two slices, each at 7 mm width, were cut directly from the removed prostatic adenoma and followed the predescribed storage and histological evaluation.

Patients were categorized in Groups A and B: Group A comprised 10 patients, who underwent open radical retropubic prostatectomy for the management of PCa, with a mean age of SD ± 66 ± 4 years (range 59−71), mean serum PSA ± SD 9.3 ± 4.6 ng/mL (range 3.2−19). Group A patients met the following criteria: Tumour Node Metastasis [TNM] stage T1−T3 prostate cancer, occupying ≥70% of the tissue section's total plan, Gleason score ≥6, without excessive morbidity, concurrent malignancy, and without being under any androgen blockade therapy. Pathological stage distribution was pT3a in 7 patients and pT3b in 3 patients. Group B included 10 patients who received a simple suprapubic prostatectomy for the management of BPH, with a mean age ± SD of 70 ± 9 years (range 49−80) and mean PSA ± S.D: 4.1 ± 1.9 ng/ml. Patient baseline characteristics are shown in Table 1.

Tissue Specimen Processing for Proteomic Analysis. The sample workflow and iTRAQ labeling scheme used for this study are illustrated in Figure 1. Detailed descriptions on tissue sample processing, protein extraction, and the protein Bradford assay are provided in the Supporting Information. Subsequent sample processing, such as solution phase digestion, iTRAQ labeling, peptide fractionation, and desalting, was conducted in accordance to the manufacturer's specifications and guidelines.[7] A brief description thereof is provided in the Supporting Information.

LC−MS/MS Analysis. A detailed description of the LC−MS/MS protocol is available in the Supporting Information. Briefly, all LC−MS/MS experiments were performed on a QSTAR XL system (Applied Biosystems - MDS Sciex) retrofitted to an 1100 nano-HPLC system equipped with a micro well plate autosampler (Agilent Technologies, Karlsruhe, Germany). Individual desalted, filtered, and lyophilized SCX fractions each containing about 5−10 μg peptide material were freshly reconstituted in 10 μL mobile phase A (2% ACN, 0.5% Formic acid). A 3 μL volume of the resulting sample solution was injected and then eluted at 200 nL/min onto a 0.075 × 200 mm reverse phase capillary column (Zorbax C18, 300 Å pore, 3.5 μm particle, Agilent Technologies, Karlsruhe, Germany) retrofitted onto the nanoelectrospray source (Applied Biosystems - MDS Sciex) and connected to a 1P-4P coated, 8 μm tip × 360 μm OD × 75 μm ID PicoTip nanoelectrospray emitter (New Objective, Dingoes, NJ). An in-line MicroFilter (Upchurch Scientific, Oak Harbor, WA) and a 0.30 × 5 mm reverse phase guard column (Zorbax C18, 300 Å pore, 5.0 μm particle, Agilent Technologies, Karlsruhe, Germany) were connected between the pump outlet tubing and the capillary column.

Proteomic Data Analysis. Protein identification and quantification for the iTRAQ experiments was performed with the ProteinPilot 2.0 software program (Applied Biosystems, Foster City, CA) using the Paragon (Applied Biosystems, Foster City, CA) protein database search algorithm.[8] The data analysis parameters were as follows: Sample type: iTRAQ (peptide labeled); Cys Alkylation: MMTS; Digestion: Trypsin; Instrument: QSTAR ESI; Special factors: None; Species: None selected (searching human CDS database); Quantitate tab: checked; ID Focus: Biological modifications - searches for over 170 potential modifications (i.e., phosphorylations, amidations, pyro-glu, semitryptic fragments, etc.); Database: The Swiss-Prot and

Table 1. Patient baseline characteristics

no. patient	age (years)	PSA (ng/mL)	stage	Gleason score	% prostate cancer procurement
		Group A prostate cancer			
1	70	5.0	pT3aNoMx	3 + 3	70% left lobe
2	69	9.7	pT3bNxMx	4 + 5	100% right lobe
3	63	14.5	pT3aNoMx	3 + 5	80% left lobe
4	69	3.2	pT3bNoMx	3 + 4	100% left lobe
5	59	8.7	pT3aNxMx	3 + 4	80% right lobe
6	63	6.8	pT3aNxMx	3 + 4	70% left lobe
7	68	11.0	pT3aNoMx	3 + 5	80% right lobe
8	71	7.0	pT3aNxMx	3 + 4	100% right lobe
9	60	19.0	pT3bNoMx	4 + 5	80% left lobe
10	68	7.6	pT3aNoMx	3 + 4	70% right lobe
		Group B benign prostate hyperplasia			
1	75	3.2			
2	49	4.2			
3	68	2.1			
4	69	5.5			
5	75	1.7			
6	68	2.8			
7	76	5.7			
8	76	3.7			
9	78	8.0			
10	66	3.8			

Trembl databases are updated on a weekly basis; Search Effort: Thorough ID; Minimum Detected Protein Threshold [Unused ProtScore (Conf)]: 1.5 (95.0%). The relative quantification was based on the ratio of the reporter ions corresponding to the PCa tryptic peptides (115.1 and 117.1) over the ratio of the reporter ions corresponding to the BPH tryptic peptides (114.1 and 116.1). Proteins giving tryptic peptides with an average reporter ion ratio ≥ 2 were classified as up-regulated and those with an average reporter ion ratio ≤ 0.5 were classified as down-regulated ($p \leq 0.05$).

Functional distribution of the identified proteins was performed using an in house built software program called TAGOO (Tool for Automatic Grouping of Gene Ontology annotations), which is based on the Gene Ontology (GO) Annotation project.[9]

Immunohistochemistry. Immunohistochemical staining was performed based on the EnVision+ System-HRP. Formalin-fixed paraffin-embedded blocks from six randomly selected cases (4 PCa and 2 BPH) were cut into 4 μm thick sections. Tissue sections were deparaffinized, rehydrated, and incubated in 0.01 M citrate buffer pH 6 for 30 min in a microwave oven at 800 W and treated with 3% hydrogen peroxide for 15 min and rinsed. After cooling for 20 min, they were incubated with the primary monoclonal rabbit antihuman antibody for alpha-methylacyl-CoA racemase (AMACR) (1:100 dilution, clone 13H4, Dako), the mouse monoclonal antibody for melanoma cell adhesion molecule (MCAM) (1:50 dilution, NCL-CD146, Novocastra), and the polyclonal antibody for prostate-specific membrane antigen (PSMA) (1:60 dilution, Zymed) for 1 hour in room temperature and then incubated for 30 min with the antimouse HRP labeled polymer (EnVision+ System-HRP, DakoCytomation). Finally, sections were treated with a diaminobenzidine (DAB) chromogenic substrate (BioGenex) for 10

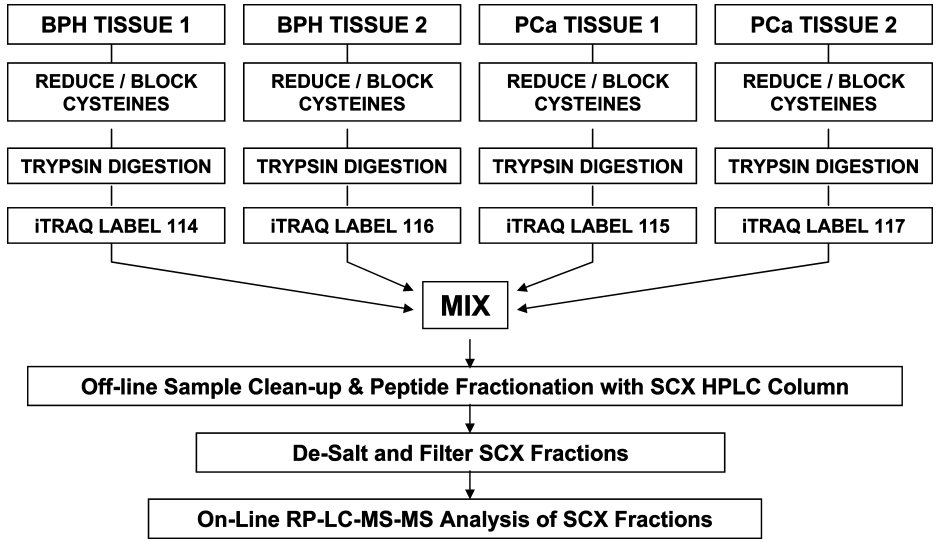

Figure 1. Depiction of the experimental design workflow used for the multiplexed comparative analysis of the BPH and PCa tissue specimens. This workflow was conducted 5 times for a total of 10 different BPH and 10 different PCa specimens. Clinical parameters for these specimens are indicated in Table 1.

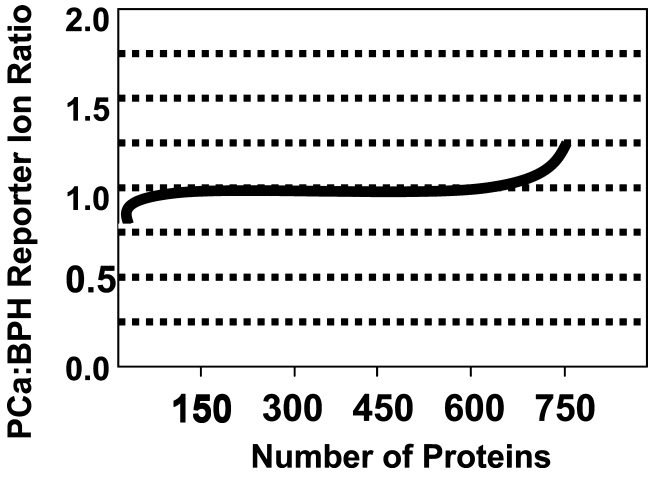

Figure 2. Distribution of the mean PCa/BPH reporter ion ratios (115:114; 115:116; 117:114; 117:116) corresponding to the tryptic peptides of 760 of the 825 proteins reproducibly identified in all five experiments performed.

min, counterstained with hematoxylin, dehydraded, and coverslipped.

Results

The study resulted in the identification of a combined total of 1420 proteins of which 825 proteins were reproducibly identified (≥95% confidence, $p \leq 0.05$, refer to Identified Proteins in the Supporting Information) in all five of the 4-plex experiments (encompassing all 10 BPH and 10 PCa clinical specimens listed in Table 1). The distribution of the mean PCa/BPH reporter ion ratios corresponding to the tryptic peptides of 760 of the 825 proteins reproducibly identified ranged between 0.7 to 1.3 as illustrated in Figure 2. The tryptic peptides of the remaining 65 proteins gave a mean PCa/BPH reporter ion ratio of either ≥2 or ≤0.5 and were classified as differentially expressed as listed in Table 2 (interassay confidence $p \leq 0.01$, refer to iTRAQ Reporter Ion Ratios in the Supporting Information). Analogous reporter ion ratio cut-offs for differentially expressed proteins were used in other iTRAQ MDLC−MS/MS approaches for the identification of potential markers in the endometrial cancer.[10,11]

A distribution of the proteins in accordance to their theoretical molecular weight (MW) and theoretical p*I* values, along with the differentially expressed counterparts is depicted in Figure 3. As seen from this figure, a significant number of proteins having a theoretical MW size <15 and >200 kDa were identified along with proteins having very acidic (pI < 4) and very basic character (pI > 10). These characteristics demonstrate the ability of the LC−MS based proteomic method, as used in this present study, in analyzing such categories of proteins. TAGOO tool facilitated the categorization of the 825 proteins in 3 categories: biological process (BP), cellular component (CC), and molecular function (MF). Pie-charts for each category are depicted in Figure 4A−C, respectively.

As a means to illustrate a typical protein identification and relative quantification result, Figure 5 depicts the data output for Kallikrein-3 (prostate specific antigen, PSA) to which a minimum of 8 uniquely occurring tryptic peptides were reproducibly determined in all five of the 4-plex experiments. This represents a protein sequence coverage of >45% and testifies to the method's specificity, selectivity and sensitivity in detecting PSA in prostate tissue specimens with high confidence (>99%, $p < 0.01$). One of these uniquely occurring peptides, namely AVCGGVLVHPQWVLTAAHCIR, was fragmented to its constituent iTRAQ reporter and backbone fragment ions that allowed its relative quantification based on the signal intensity values of the reporter ions and amino-acid sequencing of the peptide based on the b- and y- product ion signal pattern. This process was conducted on all 8 uniquely occurring peptides thus contributing to the quantification statistics and identification confidence. The relative concentration trends for PSA found between the PCa and BPH tissue specimens correlated well with those found in the corresponding sera PSA concentration trends (determined with ELISA, table 1). For example, Figure 5C depicts a signal intensity profile of approximately 160:100:150:80 assigned for the reporter ions m/z 114.1:115.1:116.1:117.1 produced by the fragmentation of the AVCGGVLVHPQWVLTAAHCIR tryptic peptide of PSA extracted from tissue specimens with respective serum PSA concentrations of 5.7, 3.2, 5.5, and 2.1 ng/mL. Figure 5D depicts a signal intensity profile of approximately 50:175:80:150 assigned for the reporter ions m/z 114.1:115.1:116.1:117.1 produced by the same tryptic peptide of PSA but extracted from tissue specimens with respective serum PSA concentrations of 1.7, 19.0, 3.7, and 11.0 ng/mL. Similar agreement between tissue and serum PSA concentration trends were observed for all clinical specimens analyzed in this study.

A total of 29 proteins were identified with 1 tryptic peptide. These gave a sequence confidence of ≥95% (refer to Supporting Information). In all of these cases, the AA sequence was verified to be accurate based on *de novo* sequencing interpretation.

Discussion

A basic objective to our proteomic study was to identify promising biological markers based on the differential protein expression between cancerous and adenomatous tissue from benign prostatic hyperplasia. However, we should consider BPH and specifically fast-growing BPH as having a cancerous potential, through the metabolic syndrome's pathogenesis, as recent studies have suggested.[12] It would be ideal to use normal, healthy prostatic tissue for the comparison, but such a protocol could create practical and bioethical limitations. Be that as it may, the merit of the proteomic profiling of the BPH tissue in itself may provide insight to the identification of asymptomatic or early stage markers of carcinogenesis. The application of an LC−MS based proteomic method to prostate cancer research was based on the central hypothesis that carcinogenesis involves multiple biological pathways inside the tissue microenvironment and hence requires a more global approach for the discovery of diagnosis/prognosis markers and therapeutic/chemopreventive targets that reflect these pathways.

To our knowledge, this is the first study that exploits the attributes of multiplex isobaric labeling with iTRAQ reagent chemistry, multidimensional liquid chromatography, nanoelectrospray ionization and high resolution tandem mass spectrometry (iTRAQ MDLC−MS/MS) for the quantitative proteomic profiling of prostate tissue (BPH and PCa). Such an approach was partly motivated to provide greater confidence in the discovery of promising molecular markers that feature verifiable, MS based, qualitative and quantitative aspects for their characterization.

The reproducible analysis of 825 proteins featuring a broad spectrum of ontological properties (molecular function, biological function, and cellular component) in all 20 clinical

Table 2. Differentially Expressed Proteins

primary accession no.	protein name	% coverage	number of unique peptides	mean ratio (±SD)[a]
sptlP62851	40S ribosomal protein S25	35.2	3	2.13 ± 0.44
sptlP62081	40S ribosomal protein S7	34	5	5.24 ± 0.66
sptlP01009	α−1-antitrypsin precursor (α −1protease inhibitor)	49.8	13	3.19 ± 0.97
sptlP62633	Cellular nucleic acid-binding protein (CNBP) (Zinc finger protein 9)	33.3	2	2.33 ± 0.79
sptlQ59FG9	Chondroitin sulfate proteoglycan 2 (Versican) variant (Fragment)	12.6	19	2.28 ± 0.58
sptlP83916	Chromobox protein homologue 1 (Heterochromatin protein 1 homologue β)	11.4	2	3.36 ± 0.50
	Cofilin-1 (Cofilin, nonmuscle isoform) (18 kDa phosphoprotein) (p18)			
sptlP23528		67.3	5	3.29 ± 1.20
sptlQ07021	Complement component 1 Q subcomponent-binding protein (p33)	17	4	4.55 ± 1.70
sptlQ05639	Elongation factor 1-α 2 (Elongation factor 1 A-2) (Statin S1)	42.5	11	2.68 ± 0.73
sptlP42892	Endothelin-converting enzyme 1 (ECE-1)	14.4	5	2.35 ± 1.03
sptlP15311	Ezrin (p81) (Cytovillin) (Villin-2)	39	9	2.26 ± 0.49
sptlQ4LE83	FASN variant protein (Fragment)	41.9	45	2.78 ± 0.91
sptlQ02790	FK506-binding protein 4 (Peptidyl-prolyl cis−trans isomerase) (Rotamase)	22.7	6	2.46 ± 0.29
sptlQ04609	Glutamate carboxypeptidase 2 (Folate hydrolase 1) (Prostate-specific membrane antigen)	24.1	8	2.82 ± 0.95
sptlP22352	Glutathione peroxidase 3 precursor	29.6	3	2.19 ± 0.57
sptlP78417	Glutathione S-transferase ω-1	28.2	6	2.36 ± 0.58
sptlP08238	Heat shock protein HSP90-β	41.2	15	3.20 ± 0.61
sptlQ4VB24	Histone cluster 1, H1e	69.9	9	3.50 ± 0.82
sptlP16403	Histone H1.2 (Histone H1d)	73.1	8	2.98 ± 1.07
sptlQ04760	Lactoylglutathione lyase (Aldoketomutase) (Glyoxalase I)	44.3	4	2.06 ± 0.56
sptlQ9UHK6	α-methylacyl-CoA racemase	15.4	3	2.51 ± 0.62
sptlP06748	Nucleophosmin (NPM) (Nucleolar phosphoprotein B23) (Numatrin)	31	4	2.96 ± 1.01
sptlQ5VSY7	Periostin, osteoblast specific factor	41.4	19	3.83 ± 1.59
sptlQ15185	Prostaglandin E synthase 3 (Telomerase-binding protein p23) (Hsp90 co-chaperone) (Progesterone receptor complex p23)	36.9	4	3.14 ± 0.56
sptlP28066	Proteasome subunit alpha type 5 (Multicatalytic endopeptidase complex ζ-chain)	15.8	2	2.07 ± 0.70
sptlQ96A72	Protein mago nashi homologue 2	10.1	1	2.18 ± 0.43
sptlP49221	Protein-glutamine γ-glutamyltransferase 4 (Prostate transglutaminase) (Prostate-specific transglutaminase)	23.5	11	3.45 ± 1.00
sptlQ9BS26	Thioredoxin domain-containing protein 4 precursor	10.3	3	2.54 ± 0.45
sptlP07919	Ubiquinol-cytochrome c reductase complex 11 kDa protein	29.7	3	2.90 ± 0.59
sptlP31946	14−3−3 protein β/α	38.4	5	0.52 ± 0.10
sptlO43294	Androgen receptor-associated protein of 55 kDa (ARA 55)	30.7	8	0.38 ± 0.11
sptlQ9NVD7	α-parvin (Calponin-like integrin-linked kinase-binding protein) (CH-ILKBP)	22.6	5	0.53 ± 0.08
sptlP12821	Angiotensin-converting enzyme, somatic isoform (precursor) (CD143 antigen)	16.5	19	0.32 ± 0.09
sptlQ63ZY3	Ankyrin repeat domain-containing protein 25 (SRC-1-interacting protein)	26.1	15	0.46 ± 0.11
sptlQ53GY1	BCL2-associated athanogene 3 variant (Fragment)	19.5	5	0.32 ± 0.11
sptlP16070	CD44 antigen precursor (Phagocytic glycoprotein I) (Hyaluronate receptor) (Epican)	17.7	8	0.23 ± 0.07
sptlP43121	Cell surface glycoprotein MUC18 precursor (Melanoma cell adhesion molecule) (CD146 antigen)	27.9	7	0.49 ± 0.08
sptlQ8IVF4	Ciliary dynein heavy chain 10 (Axonemal beta dynein heavy chain 10) (Fragment)	18.5	24	0.35 ± 0.05
sptlP20908	Collagen α−1(V) chain precursor	43	32	0.41 ± 0.19
sptlP12109	Collagen α−1(VI) chain precursor	42	17	0.43 ± 0.15
sptlO75955	Flotillin-1	28.1	5	0.33 ± 0.10
sptlO60829	G antigen family C member 1 (Prostate-associated gene 4 protein)	12.7	1	0.25 ± 0.09
sptlO76070	γ-synuclein (Persyn) (Breast cancer-specific gene 1 protein) (Synoretin)	32.3	2	0.18 ± 0.07
sptlP09488	Glutathione S-transferase μ-1	22.1	2	0.42 ± 0.09
sptlP09211	Glutathione S-transferase π-1	34.4	4	0.29 ± 0.10
sptlP50502	Hsc70-interacting protein (Hip) (Putative tumor suppressor ST13) (Progesterone receptor-associated p48 protein)	27.1	10	0.33 ± 0.11

Table 2. Continued

primary accession no.	protein name	% coverage	number of unique peptides	mean ratio (±SD)[a]
sptlP14735	Insulin-degrading enzyme (Insulysin) (Insulinase) (Insulin protease)	14.4	8	0.38 ± 0.12
sptlQ16270	Insulin-like growth factor-binding protein 7 precursor (IGFBP-7) (Prostacyclin-stimulating factor) (PGI2-stimulating factor)	21.3	21.3	0.42 ± 0.10
sptlQ9UBX7	Kallikrein-11 precursor (Hippostasin)	11.7	1	0.41 ± 0.09
sptlQ04695	Keratin, type I cytoskeletal 17 (Cytokeratin-17)	47.4	8	0.38 ± 0.14
sptlP29536	Leiomodin-1 (64 kDa autoantigen 1D)	43	13	0.21 ± 0.07
sptlP46821	Microtubule-associated protein 1B (MAP 1B)	21.1	20	0.46 ± 0.11
sptlQ6UWY5	Olfactomedin-like protein 1 precursor	20.4	4	0.33 ± 0.09
sptlQ15124	Phosphoglucomutase-like protein 5 (Aciculin)	45.8	15	0.47 ± 0.10
sptlP43034	Platelet-activating factor acetylhydrolase IB subunit α	13	2	0.42 ± 0.09
sptlP15309	Prostatic acid phosphatase [Precursor]	44	8	0.42 ± 0.14
sptlP10301	Ras-related protein R-Ras (p23)	23.9	3	0.31 ± 0.10
sptlP09455	Retinol binding protein I, cellular	9	1	0.28 ± 0.12
sptlQ96GX7	Selenium binding protein 1 (SELENBP1 protein)	17.8	4	0.36 ± 0.11
sptlP19447	TFIIH basal transcription factor complex helicase XPB subunit (DNA-repair protein complementing XP-B)	26.6	10	0.32 ± 0.13
sptlP09936	Ubiquitin carboxyl-terminal hydrolase isozyme L1 (Ubiquitin thioesterase L1)	22.4	3	0.38 ± 0.12
sptlP18206	Vinculin (Metavinculin)	56.5	27	0.46 ± 0.12
sptlP25311	Zinc-α−2-glycoprotein precursor	56.9	12	0.43 ± 0.13
sptlQ15942	Zyxin	51.6	12	0.45 ± 0.13

[a] Mean ratio corresponds to the protein reporter ion intensity originating from PCa (115.1; 117.1) relative to BPH (114.1; 116.1) with inter assay significance was $p < 0.01$. The ± SD was determined from $N = 20$ measurements (5 data pairs corresponding to the PCa/BPH reporter ion ratios (115:114; 115:116; 117:114; 117:116) for all 10 PCa and 10 BPH clinical specimens studied. The number and identity of the unique peptides observed for each protein was reproducibly observed in all 5 of the 4-plex experiments in both BPH and PCa specimen categories. Tryptic peptides observed for a subset of the 4-plex experiments were excluded.

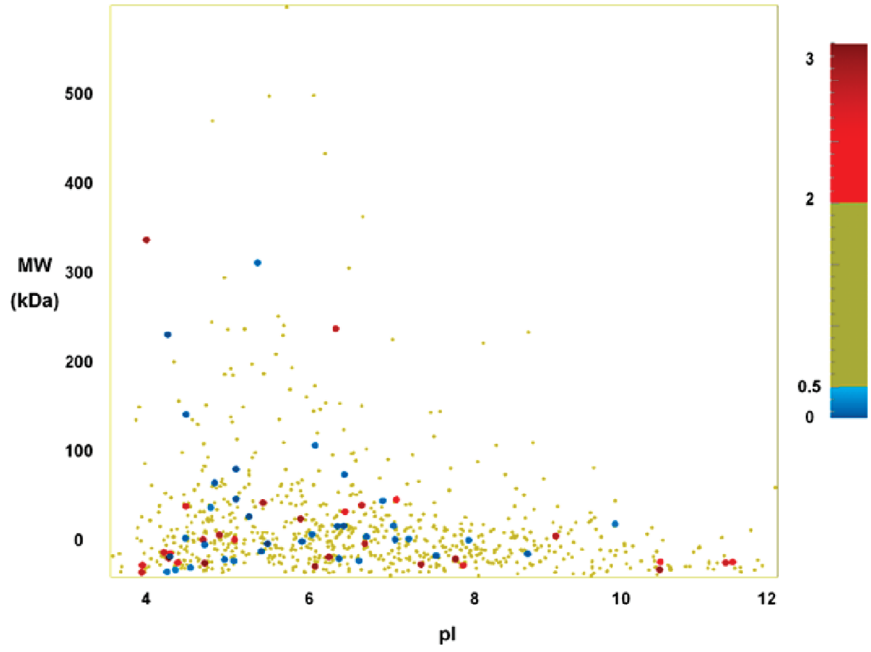

Figure 3. Protein map distribution in accordance to their molecular weight (MW, kDa) vs isoelectric point (p*I*) of the 824 proteins found by the LC−MS based proteomic study. Color coding was used to depict differentially expressed proteins.

specimens examined, reflects an exceptionally well conducted proteomic study compared to other proteomic studies that have examined a smaller number of tissue specimens.[13–17] It must be noted that tissue specimens exhibit extensive biological variability especially when they originate form diverse human populations. As such, the clinical specimen consistency and minimization of the biological, preanalytical and analytical variability leading to the analytical outcome presented, were achieved by the tissue procurement, sample processing, and instrumental performance optimization (chromatographic and nano-ESI ionization efficiency, MS and MS/MS sensitivity, resolution, accuracy, and precision) protocols as applied in this study. A limitation to the iTRAQ 2DLC−MS/MS approach is its reliance on the effective liquid chromatographic separation of the large number of tryptic peptides generated. Extensively coeluting peptides result in saturating the chromatographic capacity of either the off-line first dimensional strong cation exchange column or the online second dimensional reverse

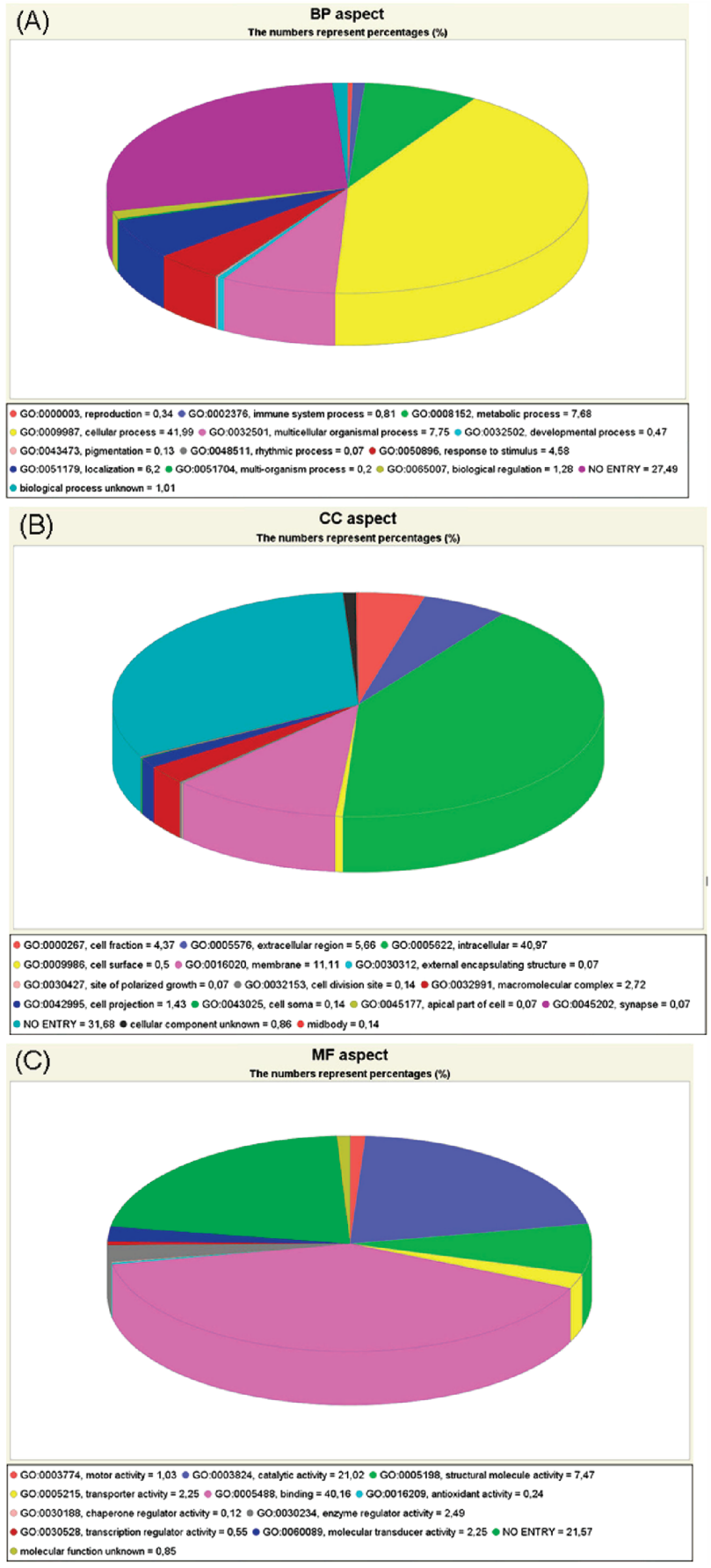

Figure 4. Functional distribution of the proteomically identified proteins in accordance to (A) biological function (BP), (B) cellular component (CC) and (C) molecular function (MF). The no-entry percentage denotes lack of information from the GO.

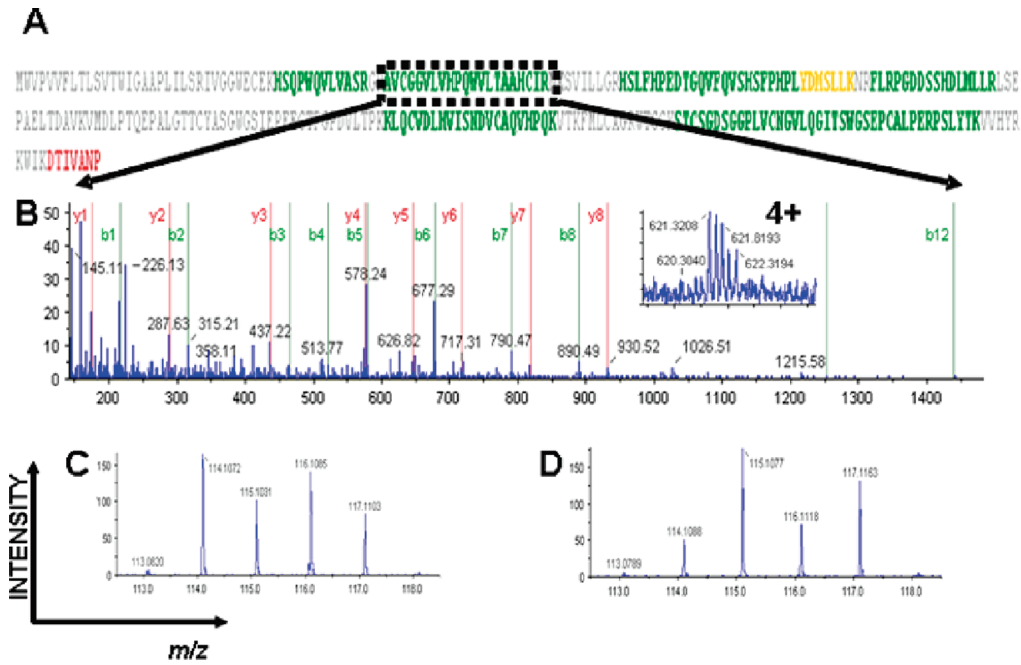

Figure 5. Prostate specific antigen diagnostic peptide sequencing and quantification using iTRAQ. (A) PSA protein with indicated (in green) the peptides detected. (B) MS/MS product ion spectrum of the 4+ charged peptide, AVCGGVLVHPQWVLTAAHCIR, at *m/z* 621.3208 with indicated b-ion and y-ion series. (C) and (D) Expanded view of the low-*m/z* reporter ion region showing the relative abundances of the signature iTRAQ ions at *m/z* 114.1, 115.1, 116.1, and 117.1 for two different experiments. Refer to text in Results section for details.

phase column, which in turn leads to decreased analysis selectivity and sensitivity. In addition, extensively coeluting peptides result in erroneous product ion MS/MS spectra affecting relative quantification efficiency and protein identification accuracy. Therefore, the use of good chromatographic technique was paramount for the optimum application of the iTRAQ 2DLC−MS/MS technique in this present study.

Currently, in addition to the iTRAQ approach, other proteomic approaches such as cysteine-specific isotope-coded affinity tags (cICAT), stable isotope labeling with amino acids in cell culture (SILAC), difference gel electrophoresis (DIGE) and trypsin-mediated ^{18}O isotope labeling have been used for the study of differentially expressed proteins in combined specimen samples.[18–24] However, intrinsic limitations exist for these other approaches. Methodological limitations for the cICAT approach lie in the fact that only proteins containing cysteine residues on tractable peptides upon proteolyzis can be quantified. As such, tractable tryptic peptides identified in this present study would not have been quantified had the cICAT approach been used (for example, refer to product ion MS/MS spectra in Figure 6). Another, cICAT limitation is its dependence on avidin columns which in turn may lead to sample loss due to low chromatographic capacity and irreversible binding or sample contamination due to nonspecific binding. An additional limitation of the cICAT approach lies in the fact that the peptide quantification is based on the relative signal response of the precursor peptides (heavy and light peptides). Therefore, the improved signal-to-noise and signal-to-background ratios achieved by tandem MS techniques (i.e., product ion MS/MS) are not exploited by the cICAT approach as is typically the case for the iTRAQ approach. The cICAT proteomic approach has been used in cell culture based prostate cancer research.[18–20] One such study involves the analysis of secreted proteins from the LNCaP neoplastic prostate epithelium resulting in the quantitative profiling of 524 proteins of which 9% of these were found to be differentially expressed.[18] Another study involving the analysis of perturbed protein networks in LNCaP prostate cancer cells in response to androgen exposure resulted in the identification of 1064 proteins of which approximately 21% of these proteins were differentially expressed.[19] A similar study using the iCAT approach resulted in the identification of 139 proteins of which approximately 77 of these proteins were claimed to be differentially expressed.[20] The iTRAQ multiplex approach has also been applied toward the study of the quantitative proteomic profiling of the LNCaP cell and a highly metastatic variant thereof (LNCaP-LN3). This study resulted in the quantitative profiling of 176 proteins of which 14 were deemed significantly differentially expressed.[21]

In the SILAC approach the tag is incorporated *in vivo* or metabolically via the nutrient media used during cell culture work and, therefore, is not applicable to human clinical tissue specimens (i.e., tissue, serum, or plasma). A SILAC based proteomic approach has been applied toward the quantification of relative protein abundance in PC3 type prostate cancer cells resulting in the identification of 440 proteins of which 82 were found to be differentially expressed.[22]

The trypsin-mediated ^{18}O stable isotope labeling (^{18}O labeling) approach involves the use of $H_2{}^{18}O$ water during the solution phase trypsinization of the proteins extracted from one specimen category (i.e., control, treated, or diseased states). This process leads to the exchange of two equivalents of ^{16}O at the carboxyl terminus of the resulting tryptic peptides with two equivalents of the ^{18}O stable isotope classified as the heavy peptides. The ^{18}O labeling approach was applied to proteins extracted from BPH and PCa cells isolated from a single formalin-fixed prostate cancer tissue specimen.[23] This study resulted in the quantitative profiling of 68 proteins. The SILAC and ^{18}O labeling approaches also rely on the relative signal response of the precursor peptides for their relative quantifica-

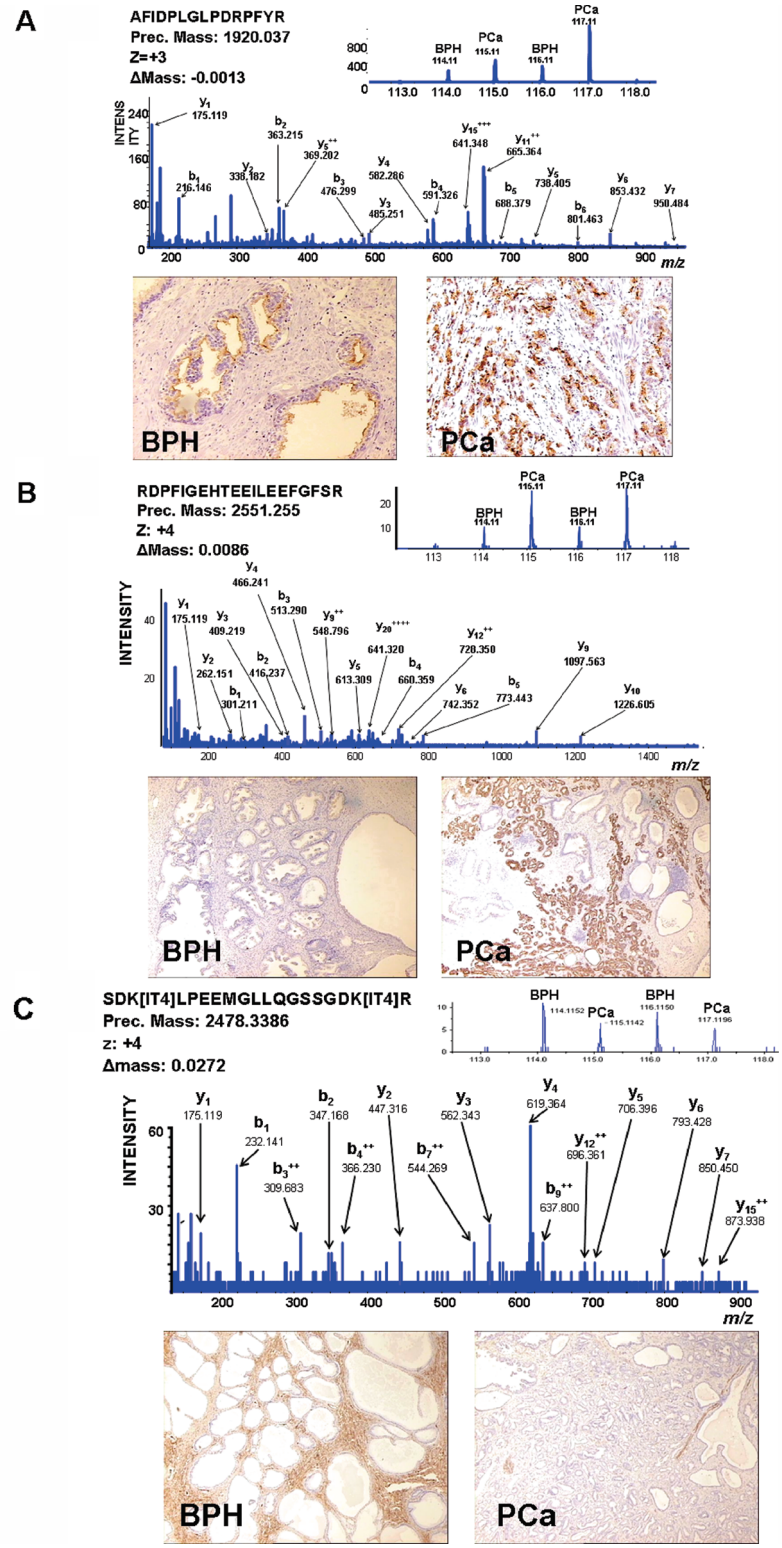

Figure 6. Representative peptide sequencing and quantification using iTRAQ with indicated amino acid sequence, precursor molecular weight, charge state, Δmass, annotated b-ion and y-ion series, and an expanded view of the reporter ion region showing representative relative abundances of the signature iTRAQ ions at *m*/*z* 114.1, 115.1, 116.1, and 117.1. Immunohistochemical conformation is shown below each product ion mass spectrum. (A) Prostate-specific membrane antigen (PSMA). IHC findings: There weak apical PSMA staining in the BPH specimen. Strong positive cytoplasmic and apical PSMA expression was observed in Gleason score 7 (4 + 3) PCa tissue specimen. A 100× magnification was used for these tissue images. (B) α-methylacyl-CoA racemase (AMACR). Immunohistochemical findings: there is no expression for AMACR in the BPH specimen. Strong positive AMACR cytoplasmic expression was observed in Gleason score 6 (3 + 3) PCa tissue specimen. (C) CD146 Antigen. Immunohistochemical findings: strong positive expression of CD146 was observed in stromal cells and vascular endothelial cells in the BPH specimen. Tumor stroma was negative for CD146 expression. For the PCa specimen, there was positive immunoreactivity in scattered small vessels. A 40× magnification was used for all tissue images.

tion and thus shares the same limitation with the cICAT approach in this regard. Another common limitation of the cICAT, SILAC and ^{18}O labeling approaches is that only two samples can be analyzed per experiment thus leading to reduced cost-effectiveness and increased experimental error relative to the iTRAQ approach used in the present study.

The DIGE approach represents a variant of the classical 2-D gel electrophoresis (2DGE) technique whereby CyDye fluors that are fluorescence spectroscopically resolvable and correlated for mass and charge are used as tags to covalently modify proteins. This results in the same protein originating from multiple biological specimens to be labeled with any of the fluors and to migrate to almost the same location of a 2-D gel. Using this approach, up to three different fluor labeled samples can be combined and 2DGE separated in a single experiment thus allowing better spot matching and reduction in gel-to-gel variations. One drawback to the DIGE approach lies on the fact that relative protein quantification is conducted on the basis of differences in the fluorescence response of the fluor tags which are by their nature MS-incompatible due to their ionization suppression effects. The protein identification process frequently reverts to the classical, nonfluor labeled 2DGE approach on a separate specimen basis and using larger sample amounts (i.e., 1 mg) for the preparatory gels. As such, the protein identification is subject to the intrinsic gel-to-gel variation uncertainty.[24] The DIGE proteomic approach was applied to the study of perturbed protein networks in LNCaP prostate cancer cells in response to both androgen and antiandrogen exposure resulting in the quantitative profiling of 107 proteins.[25]

A comparison of the outcome of the above studies to the present one leads to the following conclusions. Our study included a substantially larger sample set of clinically traceable prostate tissue specimens (10 BPH and 10 PCa) resulting in the reproducible quantitative profiling 825 proteins. The other studies using the other multiplexed quantitative proteomic approaches (cICAT, iTRAQ, SILAC, ^{18}O labeling, and DIGE) targeted cell culture based specimens with the exception of the ^{18}O labeling based study targeting BPH and PCa cells extracted from a single formalin-fixed prostate tissue specimen. The number of specimens studied in all off these cases was insufficient ($\leq$2 for each specimen category) and the number of proteins quantitatively profiled was substantially smaller compared to the present study. Additionally, these studies targeted specific type epithelial cells (i.e., LNCaP cells). In contrast, our multiplexed quantitative proteomic study was applied to whole human prostate tissue specimens in order to account for the well established stromal vs epithelial cell interaction in the manifestation of prostate cancer. When all the iTRAQ advantages are also taken into consideration, our study constitutes a well executed proteomic study of clinical BPH and PCa tissue for the search of potential treatment biomarkers.

The sum total of these technological features provided extensive resolving power to our analytical approach to very selectively identify and quantify individual diagnostic peptides (that uniquely identifies the protein of origin) in the presence of thousands of other peptides and extraneous biomolecules that typify biological sample extracts. As a case in point, our proteomic approach was able to confidently and reproducibly identify 7 different enzyme isoforms of glutathione-S-tranferase ($\mu-1$, μ-2, μ-3, π-1, θ-1, θ-2, and ω-1). Additionally, our methodological approach identified the $\mu-1$ and $\pi-1$ isoforms to be down-regulated (0.42 ± 0.09 and 0.29 ± 0.10, respectively) and the $\omega-1$ isoform to be up-regulated (2.50 ± 0.45) which act as scavengers of reactive oxygen (ROS) and reactive nitrogen oxide (RNOS) species implicated for the inflammation response.[26–30]

Prostaglandin E synthase 3 (3.14 ± 0.56) is involved in the production and metabolism of the prostaglandins, which directly stimulate the growth of malignant cells and may serve as a potential therapeutic drug target.[31] Alpha-1-antitrypsin is also a well-documented marker of inflammation, which binds to PSA, forming the complexed form of PSA, which is increased in PCa.[32]

A simultaneous change in expression in multiple proteins involved in steroid receptor physiology was observed. Specifically, androgen receptor-associated protein ARA 55 (0.38 ± 0.11), various chaperone and cochaperone binding partners (FK506-binding protein (2.46 ± 0.29), Heat shock protein HSP90β (3.20 ± 0.61), the aforementioned prostaglandin E synthase 3 (3.14 ± 0.56) and HSC 70-interacting protein (0.33 ± 0.11)). These proteins, in a cooperative way, regulate steroid receptor function which plays a critical role in the carcinogenesis process.[33–38] The steroid receptor network of proteins play a crucial role in the behavior of the multifunctional fatty acid synthase (FASN) found to be upregulated in our study (2.78 ± 0.91).[39,40]

Among the significantly up-regulated proteins in the prostate cancer specimens analyzed, was α-methylacyl CoA racemase (AMACR) (2.51 ± 0.62). This protein has been found to be selectively expressed in neoplastic glandular epithelium and is a well-established and highly specific marker for prostate cancer cells, even in the earliest stages of malignant progression.[41–43]

The nuclear protein nucleophosmin (2.96 ± 1.01) is an RNA-associated nucleolar phosphoprotein believed to be a target of CDK2/cyclin E in the initiation of centrosome duplication.[44] Consistently with our findings, in prostate cancer studies, it has been reported to be more abundant in malignant and growing cells than in normal nondividing cells. Furthermore, it has been considered as a potential tumor marker for human prostate cancer.[45]

Periostin was also significantly up-regulated (3.83 ± 1.59) in the malignant specimens included in our study, similar to other cancers (i.e., breast and colon).[46,47]

We also identified significant changes in the Actin and microtubule cytoskeletal proteins: cofilin 1 (3.29 ± 1.20) and ciliary dynein heavy chain 10 (0.35 ± 0.05), respectively, which participate in the stabilization of cell shape or promotion of cell that may become altered during acquisition of the metastatic phenotype by cancer cells.[48]

Decreased levels of Zn-α−2 glycoprotein (AZGP1) were found in malignant prostate epithelium (0.43 ± 0.13) and were suggested to have a predictive value for the clinical recurrence of cancer after radical prostectomy.[49] When relative measurements are considered on an individual basis, the AZGP1 results may be useful for the prognosis and management of clinical recurrence cases.

Zyxin suppression has recently been implicated in the migration of prostate cancer cells.[50] Henceforth, the down-regulation of zyxin as found in this study (0.45 ± 0.13) may contribute to the metastatic potential of the prostate cancer cells.

Notably, several prostate specific cancer markers were also found to be differentially expressed, namely, prostate-specific transglutaminase (3.34 ± 1.00), prostate associated gene 4 protein (0.25 ± 0.09), prostatic acid phosphatase (0.42 ± 0.14),

and prostate specific membrane antigen (PSMA) (2.82 ± 0.95). The presence of the prostate-specific transglutaminase in PCa has been recently reported.[51] Along with the prostate specific androgen receptor-associated protein ARA 55, these prostate-tissue markers make for ideal candidates for their targeted LC−MS based analysis in plasma or serum. Provided that these proteins are secreted into the systemic circulation, these prostate tissue proteins will aid in the design and development of more targeted and hence more sensitive LC−MS based assays that could be used in the clinical setting.[52,53]

Proteins found to be down-regulated in our study that indicate a compromised nutritional status include: retinol binding protein I (0.28 ± 0.07), selenium binding protein 1 (0.32 ± 0.12) and DNA-repair protein complementing XP-B cells (0.30 ± 0.05). These proteins may serve as potential chemoprevention targets whose expression may be restored by chronic dietary supplementation or nutritionally sound dietary changes.[54–58]

To provide an independent confirmation for some of the proteins found to be differentially expressed in our proteomic study, we performed immunohistochemistry to formalin-fixed paraffin-embedded blocks from six randomly selected specimens (4 PCa and 2 BPH) corresponding to the clinical samples already analyzed with our iTRAQ MDLC−MS/MS method using available antibodies. Figure 6 illustrates representative product ion spectra of protein tractable tryptic peptides with corresponding immunohistochemical confirmation for (A) PSMA, (B) AMACR, and (C) cell surface glycoprotein MUC18 precursor (CD146). As expected, both PSMA and AMACR stained positively in PCa. BPH samples presented a weak apical positivity for PSMA whereas AMACR was negative. A strong positive expression of CD146 was observed in the stromal cells and vascular endothelial cells of all BPH specimens[58] whereas the stroma was CD146 negative in all PCa specimens. This particular expression of CD146 exemplifies the importance of the stromal environment in prostate cancer biology and is subject of an ongoing separate clinical study in our group.

The functional interdependence (cross-talk) characterizing key enzyme systems in tissue microenvironments was captured from our methodological approach. In general, the proteins already discussed along with others found to be differentially expressed (Table 2) are associated, directly or indirectly, to stroma-modulating factors (such as growth agents and proteases). These factors regulate the expression of fibroblast, vascular endothelial, epidermal, platelet-derived, and other components. In summary, the differentially expressed proteome observed in this study reflects a perturbed tissue homeostasis condition characterized by a positive feedback loop involving the tumor activated reactive stroma, growth factor release, angiogenesis, inflammation, further tumor growth, and eventual metastasis.[5,6,59–64]

On the basis of literature research, the existing knowledge of differentially expressed proteins found in prostate-tissue specimens has been generated following the application of time-consuming biochemical assays (i.e., microarray analysis, immunohistochemical analysis, western/Northern blots, etc.) that required prior knowledge of the enzymes involved and lacked the selectivity and reproducibility necessary for a system-wide assessment of the cancer molecular markers constituting the tissue microenvironment. Although biochemical assays correctly identify certain aspects in the oncogenic process, what is frequently missed are all the systemic effects this process evokes to groups of proteins. In contrast, our LC−MS based quantitative proteomic approach with all its intrinsic analytical attributes allowed a more global identification and characterization of both known and hitherto unknown enzymes involved in prostate oncogenesis. This constitutes a more valid discovery phase or hypothesis generating approach that serves as a promising research avenue toward the identification of disease biomarkers and the better understanding of tumor biology.

Conclusions

An advanced LC−MS based proteomic method encompassing high sensitivity, selectivity, and reproducibility was applied to clinical BPH and PCa specimens resulting to a major contribution toward the global proteome study of PCa tissue. The large number and extensive biological distribution of the identified proteins supports existing knowledge and uncover novel and promising PCa biomarkers. The majority of the PCa related proteins currently known have been identified in cell culture using biochemical assay and microarray techniques. Our proteomic study confirms these findings in prostate tissue. Our findings also support the interplay or cross-talk that exists between stromal and epithelial proteins.

The PCa proteome described in this study can serve as a useful aid for the identification of potentially improved diagnostic and prognostic markers and ultimately novel chemopreventive and therapeutic targets. The above observations are consistent to the notion that cancer is manifested by the aberrant behavior of multiple signaling pathways. Our method allows for the analyses of multiple key proteins involved in these pathways that may, in turn, permit the monitoring of therapeutic and chemopreventive intervention. Additionally, many unforeseen proteins have been identified in the benign prostate hyperplasia specimens that may play a role as predisposing factors for prostate cancer. This proteome can be potentially used by clinicians as an aid in the exploitation of novel prognostic and diagnostic biomarkers and treatment targets.

The proteomic findings of this study can be targeted either individually or on a panel basis in clinical sera specimens in the development of mass spectrometry (MS) based assays in a clinical setting. The use of MS based bioassays typically exhibit over 99% confidence and constitutes a major advancement in clinical practice that may complement biochemical assay based methods (i.e., ELISA screening). As such, the findings of this study will serve to inform and engage clinicians about the premise in the use of MS-based proteomic approaches in clinical practice.

Our results are extremely promising and warrant further study with the analysis of viable proteins biomarkers and surrogate partners in clinical sera or plasma specimens. Novel biomarkers, emerging from the LC−MS based methodologies may provide better specificity and sensitivity than PSA.

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Supporting Information Available: Sample preparation and LC−MS/MS. Detailed description on all aspects of the sample tissue processing and LC−MS/MS analysis tasks. Identified proteins with ≥95% confidence ($p \leq 0.05$). Tryptic peptides used for the identification of the proteins listed in Table 1. Single peptide-based identifications including product ion MS/MS spectra, peptide sequence, modifications, precursor mass, charge and mass error, and identification score. Example product ion MS/MS spectra showing both the quantitative reporter ion and qualitative peptide backbone ion profiles of representative sequenced tryptic peptides having ≥80% confidence consistently observed in all 20 tissue specimens. Individual iTRAQ measurements ($p < 0.01$) for each experiment correspond to the PCa/BPH reporter ion ratios (115:114; 115:116; 117:114; 117:116). This material is available free of charge via the Internet at http://pubs.acs.org.

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