Biomarker Detection in Urinary Proteome of Prostate Cancer by Nanoflow LC-MS/MS

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ABSTRACT

Introduction. Urinary proteomics provides a wealth of information in the identification of protein markers associated with various diseases such as in carcinoma. With the increasing incidence of prostate cancer and the lack of sensitivity and specificity of prostate specific antigen, the simultaneous identification of an alternative protein biomarker through urinary proteomics is encouraging. Urine, which has similar proteins with serum, makes it an ideal alternative biofluid wherein the collection is easy and non-invasive.

Methods. Urinary proteins were separated by gradient SDS-PAGE followed by in-gel digestion and organic/buffer peptide extraction. The protein biomarkers in prostate cancer patients and control subjects were identified via LC-MS/MS and submitted to Protein Prospector where the peptide fragmentation of sequence was analyzed and compared with the SwissProt database.

Results. A panel of three protein biomarkers for the early detection of prostate cancer were identified: transthyretin, hemoglobin subunit alpha and hemoglobin subunit beta. The presence of these three biomarkers is associated with high Gleason scores and TNM stages but not with PSA level. Uromodulin and mannan binding lectin serine protease 2 are the additional biomarkers that can distinguish prostate cancer from BPH. The study also revealed the divergence of the urinary proteome of the cancer patients from the urinary proteome of the control with BPH suggesting the fundamental differences in benign and malignant growth of the prostate epithelial cells. Another highlight of the study was the identification of oxidation of pro63 of transthyretin in patient 3. The proposed role of the post translational modification in pro63 of transthyretin in the mechanism of prostate carcinogenesis remains to be defined and warrants further study.

Conclusion. Our study was able to establish the homology of urine proteome among the controls and its divergence from the patients afflicted with prostate cancer by simultaneously comparing their urine proteomes leading to the identification of a distinct panel of biomarkers, namely, transthyretin, hemoglobin subunit alpha and hemoglobin subunit beta. Uromodulin and mannan binding lectin serine protease 2 are the additional biomarkers that can distinguish prostate cancer from BPH. Due to limitations in the number of controls and patients, only preliminary findings and their significance were shown. These findings need to be confirmed in future investigations using larger sample size for both the controls and the patients.

Key Words: urinary proteomics, tandem mass spectrometry, nano-high pressure liquid chromatography, prostate cancer, transthyretin

Introduction

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer death in the US in older men more than 40 years of age.[1] It is a highly heterogenous disease in terms of its development,[2] multiple molecular alterations,[3,4] pathology[5] and clinical presentations.[6,7,8] Prostatic carcinoma remains one of the most common cancers afflicting men today[9] characterized by increasing incidence with age.[5,10] Among the Asian countries, Rizal province in the Philippines showed the highest rate although much lower compared with the US and European countries. Recent Asian statistics, however, showed an alarming increase of prostate cancer. A 49.5% change in incidence from 11.1% in 1978-1982 to 16.6% in 1993-1997 was reported in Rizal, Philippines.[4]

The variability of some types of prostate cancer is documented.[5] Some types grow slowly and need minimal or no treatment while other types are more aggressive and spread quickly. Prostate Specific Antigen (PSA) is the standard diagnostic biomarker in prostate cancer diagnosis and prognosis[5] but with limited sensitivity and specificity.[9,11,12,13] Elevated PSA (>4ng/ml) maybe observed in prostatitis and benign prostate hyperplasia (BPH).[14] The PSA test may also give false positive or negative information because it does not allow the differentiation of BPH from prostate cancer.[15] BPH is a non-malignant condition found in

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older men and does not appear to progress directly to carcinoma.[10] PSA is thus inadequate as an ideal screening test for prostate cancer.

Proteomics is an across-the-board study of structure, function, expression, localization and protein-protein interaction.[16] Advances in genomics and proteomics facilitated the identification of protein biomarkers that may reflect the true state of prostate cancer. The proteomics of protein structures contributes to our understanding of gene function, including its biochemistry, processes and their pathways.[17] Proteomics can make a crucial contribution to our understanding of biology and medicine through the global analysis of gene products, i.e., the proteins.[18] This approach makes possible the discovery of new disease-associated biomarkers by comprehensively examining the protein expression profiles in patients such as those with cancer and in individuals without the disease.[16,19-23]

Proteomic strategies can be used to study all expressed genes to discover biomarkers indicative of the physiological and pathological state of cancer cells at specific time points including the early stage diagnosis before cancer progression.[24] Urinary proteomics, through global analysis of protein structures, provides a wealth of information in the identification of protein markers by simultaneously identifying all the contributing proteins in a specific disease.[25]

Urine, a non-invasive obtainable biofluid, is a source for non-invasive biomarker discovery in diseased state.[26] It is a potential source of biomarkers since it contains large amounts of small peptides that can represent a large number of unexplored novel biomarkers for disease monitoring similar with blood.[27] Using urine samples instead of blood contributes to the ease of improving the detection and identification of biomarkers supported by global analysis of proteins using LC-MS/MS and public databases. The present study aims to identify the protein biomarkers in urine of prostate cancer patients via LC-MS/MS with the aid of Protein Prospector (UCSF, SF, CA) and to compare the acquired spectra to theoretical fragmentation of peptides in the SwissProt database (http://www.ebi.ac.uk/uniprot/). XCalibur Software (Thermoscientific) was used to process the chromatograms.

Methods

Patients and Controls

The study was approved by a Local Independent Ethics Committee in accordance with the ethical standards based on the principles enunciated in the Declaration of Helsinki of 1975 revised in 2008. The nature and scope of the study were explained to all the controls and patients prior to signing of the informed consent. The controls (19yo, 86yo/BPH) and patients were included in the study based on the criteria proper to them. The 19yo control was included in the study based on the following criteria: a). non-smoker, b) non-alcoholic drinker, c) no history of any form of cancer in the family and immediate relatives, d) no history of heart attack and diabetes, e) no recurrent urinary tract infection during urine collection, f) willing to be examined by digital rectal examination (DRE), g) willing to provide urine during the early morning voiding, h) willing to go back for follow-up examination, i) no renal disease, j) willing to join with proper informed consent, and lastly, k) not diagnosed with prostate cancer. The control with BPH also met the above criteria and upon being clinically diagnosed with BPH and not suffering from prostate cancer, 86yo/BPH was included in the study. On the other hand, the criteria for patient inclusion were similar for controls except the last item (k), instead, the subject participants were clinically diagnosed with prostate cancer, with Gleason scores $\geq$7, and recommended for radical prostatectomy. The patients were recruited sequentially. Decoy proteins from urine of female participants (19yo and 53yo) were included in the study to represent the negative controls. The criteria for their inclusion were the same as normal/healthy controls. Standard decoy proteins from other matrices (CSF, blood, serum and plasma) were also included. The inclusion of above mentioned decoy proteins represent a level of $p=0.0005$ when at least two peptides were identified and $p=0.001$ when one peptide is determined.

About 5 mL clean catch first urine samples were collected from the six patients (64-74 years old) before radical prostatectomy and the controls. Results of PSA and biopsy (Tumor-Node-Metastases (TNM) staging and Gleason scores) were provided by the patients.

Sample preparation and Total Protein Quantification

The urine samples were centrifuged at 40,000 rpm for 40 min using Beckman Altima Ultracentrifuge. Aliquots of the urine were stored in centrifuge tubes at -70^{0}C and thawed prior to use. The Bradford dye reagent concentrate was diluted to a 1:4 ratio with distilled deionized water as specified by the manufacturer. Three to five dilutions (8-80ug/mL) of bovine serum albumin (BSA) were prepared in triplicate. The standard and urine samples (160uL) were pipetted into separate microtiter plate wells. Diluted dye reagent concentrate (40uL) were added to each well, mixed thoroughly and allowed to incubate for 5 minutes. The absorbance was read at 595nm. The protein concentration was calculated against the standard curve based on BSA.

Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE)

Urine samples (20uL) from patients and controls were boiled for 10min before loading in onto 4-20% Biorad precast gel (Catalog No. 161-1159) in a mini Protean Beckman Apparatus set at 200v, 40mA, 5W being controlled by Biorad Power PAC 100. Silver staining[28] was used to visualize the

proteins. Differentially expressed genes below 42,500 Da were excised manually from gels as source of protein marker to target low-molecular weight proteins/peptide.[29,30,31] The corresponding bands in the gels were excised, divided into 4 sections and collected into four individual microcentrifuges.

In-gel Digestion and Peptide Extraction

Gel pieces were incubated for 10 min with 25 mM NH4HCO3/70% ACN. The dried gel pieces were then washed with 10 mM DTT in 25mM ammonium bicarbonate at 50^0C for 30 min and centrifuged for 5 min at 10,000 rpm at 4^0C. After removing the supernatant, 50mM iodoacetamide in 25 mM ammonium bicarbonate were added and incubated in dark for one hour and centrifuged for 5 min at 10,000rpm at 4^0C. After collecting the supernatant, 25mM $NaHCO_3$/70% ACN was added and incubated for 15 min. Trypsin in 25mM sodium bicarbonate was added. The gels were allowed to swell for 5 min, layered with 25mM sodium bicarbonate and incubated at 37^0C for 4 hours or overnight. The supernatants were collected in new siliconized tubes wherein 50% ACN/5% formic acid were added and incubated for 10 min. After collecting the supernatant, the samples were dried using speed-vac.

Nano-High Pressure Liquid Chromatography and Tandem Mass Spectrometry-Collision Induced Dissociation (HPLC/MS/MS (LTQ Orbitrap-CID)

The peptides were analyzed using nano HPLC (nanoAcquity, Waters) and Thermo Scientific MS/MS with the following HPLC conditions: column: Waters 1.7um, BEH 130, C18, 100um x 100mm. Mobile phase consists of: A: 0.1% formic acid in water; B: 2% water, 98% acetonitrile and 2% trifluoroacetic acid; gradient: 0 min, 2%B; 35 min, 25% B; 37min, 50%B; 38min 2%B.

Peptides were analyzed in positive ion mode and in an information-dependent acquisition mode to automatically switch between MS and MS/MS acquisition. MS spectra were acquired in profile mode using the orbitrap analyzer in the m/z range between 300 and 1800. For each MS spectrum, the six intense multiply charged ions over a threshold of 1000 counts were selected to perform collisionally induced dissociation (CID) experiments. Product ions were analyzed on a linear ion trap in profile mode. The CID collision energy was automatically set to 25%. A dynamic exclusion window of 0.5 Da was applied that prevented the same m/z from being selected for 60s after its acquisition. The data generated were further converted to peaklists.

Data Analysis

The raw data generated by MS/MS were converted into peaklists by an in-house generating software by UCSF (SF, CA) and processed by Protein Prospector (USCF, SF, CA) before submission to Swiss Prot (http://www.ebi-ac.uk/uniprot/) searching and peptide and protein identification. The use of a concatenated database of normal protein sequences and randomized sequences is used to estimate the reliability of results. Search parameters used were: parent tolerance of 30ppm, fragment tolerance of 0.8Da, MS precursor change of 23, with the maximum expectation value for protein and peptide = 0.10 and missed cleavages = 2. The expectation value for Protein Prospector was set to α=0.05. The fixed modification was set to carbamidomethyl C. The variable modification included the following: acetyl (prot N-term), acetyl + oxidation (protein N-term M), Gln-loss (protein-N term M), met-loss (protein N-term M), met-loss + acetyl (protein N-term M) and oxidation (M). Total ion chromatographs (TIC) and precursor ion scans were visualized with XCalibur (Thermo Scientific).

Results

The characteristics of the patients and controls are shown in Table 1. The patients were recommended for radical prostatectomy based on clinical findings, laboratory results and biopsy (Gleason scores and TNM staging). The PSA values and protein concentration showed the lack of association to Gleason scores and TNM staging. Ailments of the control and other ailments of the patients other than prostate cancer are shown in Table 2.

Table 1. Characteristics of the controls and the patients

Control/ Patient	age (yr) /gender	protein concentration (ug/ml)	serum PSA* (ng/ml)	Gleason** Score	TNM*** Stage
Control 1	19/M	9.17	0.01	-	-
Control 2	86/M	13.51	12.00	-	-
Patient 1	67/M	14.26	10.00	7 (4+3)	T2N0M0
Patient 2	66/M	12.61	5.39	7 (3+4)	T2N0M0
Patient 3	65/M	8.18	12.59	9 (5+4)	T3N0M0
Patient 4	74/M	12.57	20.00	7 (3+4)	T2N0M0
Patient 5	64/M	9.92	78.44	8 (4+4)	T1N0M0
Patient 6	65/M	9.78	12.00	9 (4+5)	T3N1M0

**PSA – cut off, 4ng/ml; **Gleason scores – sum of most prevalent and second prevalent histological pattern; higher number, clinically significant; ***TNM – tumor-node- metastases; staging from biopsy analysis; higher number – higher stage of prostate cancer in these locations*

Table 2. Other ailments observed among patients and controls

Medical history	Controls	Patients
prostate adenocarcinoma	-	6
benign prostate hyperplasia	1	0
hypertension	2	3
cervical spondylosis	1	-
hyperuricemia	1	4
allergic rhinitis	1	1
cardiovascular diseases	2	3

The migration pattern of the urinary proteins of the six patients and normal controls (Figure 1) were analyzed using 4-20% gradient SDS-PAGE and visualized by silver staining. The collected proteins were limited to 42,500 Da and below

since the visual differences in band patterns were more remarkable at this weight range. Using this weight cut-off was consistent with other studies using low abundance/molecular weight as disease protein markers.[29,30,31]

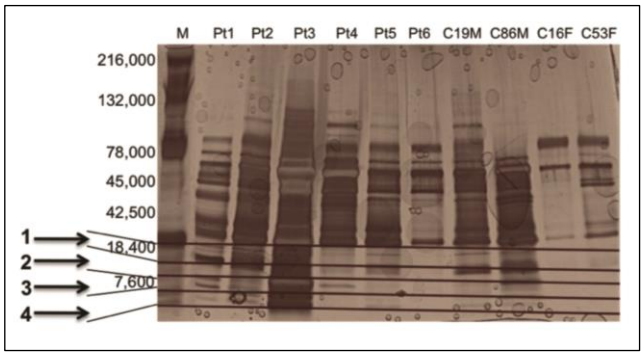

Figure 1. The migration pattern of urine samples from patients before radical prostatectomy after gradient SDS-PAGE (4-20%) is shown in lanes 2-7 (Pt1, Pt2, Pt3, Pt4, Pt5, Pt6, Pt7), and normal controls in lanes 8-11 (C19M, C86M, C16F, C53F) and the protein marker (M) in lane 1. The gel from 18,400 Da and below were divided into 4 locations (1, 2, 3 and 4) and subjected to tryptic digest before LC-M/MS. Lane 1 - protein marker ranging from 216,000 to 7600 Da; Lane 2 – Patient 1; Lane 3 – Patient 2; Lane 4 – Patient 3; Lane 5 – Patient 4; Lane 6 – Patient 5; Lane 7 – Patient 6; Lane 8 – Control, male, 19yo; Lane 9 – Control, male, 86 yo, with BPH; Lane 10 – Control, female, 19yo; Lane 11 – Control, female, 53yo. The bands were visualized by silver stain.

A representative total ion chromatogram (TIC) of the whole spectrum of the tryptic digests from Patient 3 visualized through XCalibur (Thermo Scientific) is shown in Figure 2. It is illustrated in the chromatogram the change of elution time from 31.220 min to 24.889 min upon oxidation of proline (oxidation@8) in peptide sequence AADDTWE**P**FASGK of transthyretin.

The primary structure of transthyretin is shown in Figure 3 highlighting the posttranslational modification in peptide AADDTWE**P**FASGK (oxidation @8) generated by Protein Prospector upon subjecting the peak list to Search Compare against Swiss Prot 2011 01-11 random concat database.

The protein homology (proteins shared) and divergence (unique urinary proteins) in controls and patients are shown in Figures 4a-4d using area proportional Venn diagram.[32] Higher percentage (46.91%) of proteins was shared by male controls when their proteomes were compared to each other (Figure 4a) in contrast to very low percentage of proteins shared by the patients against the controls when compared individually and as a group: 10.87% homology, patients vs 19yo male (Figure 4b); 12.04% homology, patients vs 86yo male with BPH (Figure 4c); and 16.46% homology, patients vs combined proteins of the male controls (Figure 4d). This relationship was illustrated further in Figure 5. The upper portion of the radial chart shows the percent proteins shared by the controls when compared to each other and the lower portion were the percent proteins shared by the patients with the controls. The chart illustrates the contrast - low protein homology or high divergence between the patients and controls, and high protein homology between the male controls.

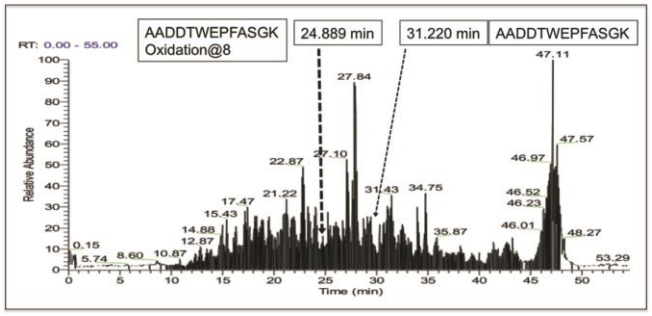

Figure 2. Representative total ion chromatogram (TIC) of urine collected from Patient 3 before radical prostatectomy showing the retention of the wild type peptide AADDTWEPFASGK that eluted at 31.220min and the same peptide which shifted the retention time to 24.889min when an oxidation was observed in proline in the protein transthyretin (P02766, TTR).

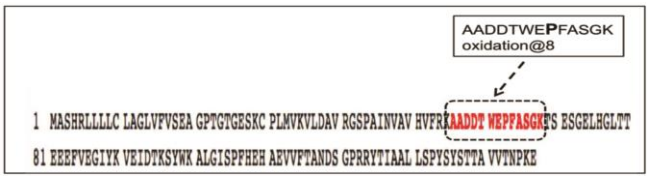

Figure 3. Primary structure of the identified protein (transthyretin, TTR) in urine of Patient 3 that has 147 amino acids, showing the location of the peptides which has a oxidation as modification at proline in the peptide (oxidation@8) shown in the sequence.

Comparison of the unique proteins of the male controls (19yo vs 86M/BPH) revealed 36.42% and 16.67% unique proteins in 19yo male and 86yo male with BPH, respectively (Figure 4a). When each of the controls was compared with the patients, the following unique proteins were revealed: 86.96% in 19yo-male (Figure 4b), 83.33% in 86yo-male with BPH (Figure 4c) and 79.75% in male controls (19yo and 86yo/BPH) (Figure 4d), respectively. These results suggest that a higher percent of proteins were expressed in normal controls than in the patients, wherein the implication warrants further studies using a larger sample size of controls and patients. The summary of percent protein homology and divergence is shown in Figure 6.

Three unique proteins were identified in patients, namely: transthyretin (P02766), hemoglobin subunit alpha (P60095) and hemoglobin subunit beta (P68871) when compared with male controls as a group (Figure 7) that

Table 3. Unique bioactive peptides and the number of peptides present in the urine of prostate cancer patients

Name Protein	Prot ID	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Transthyretin	P02766	1	2	5	2	1	1
Hemoglobin α	P69905	2	4	8	1	5	3
Hemoglobin β	P68871	3	8	12	1	1	2

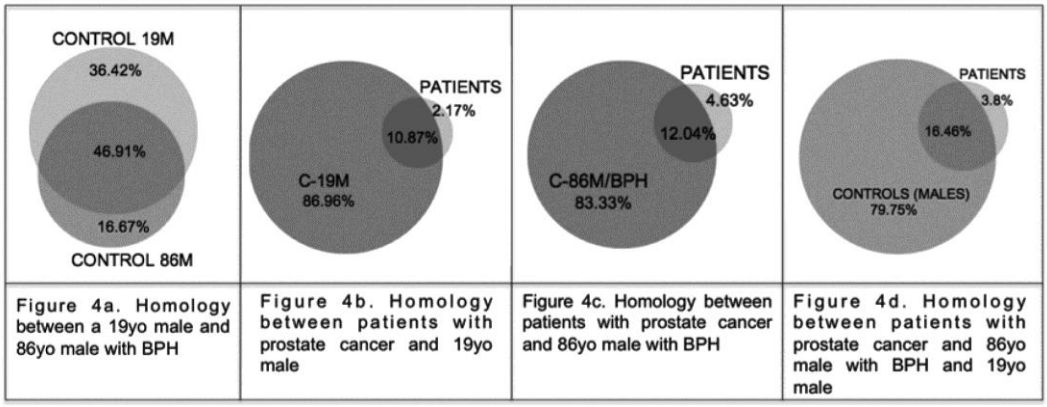

Figure 4a. Homology between a 19yo male and 86yo male with BPH

Figure 4b. Homology between patients with prostate cancer and 19yo male

Figure 4c. Homology between patients with prostate cancer and 86yo male with BPH

Figure 4d. Homology between patients with prostate cancer and 86yo male with BPH and 19yo male

Figure 4. Area-proportional Venn diagram showing the protein homology and divergence in patients and controls.

constitute 3.80% unique proteins (Figures 4d and 8a) and 2.17% when compared with 19yo alone (Figures 4b and 8b). The summary of the number of peptides in these biomarkers is shown in Table 3. Two more proteins, uromodulin (P07911) and mannan binding lectin serine protease 2 (O00187) were detected in the patients' urinary proteome when compared individually with the 86yo male control with BPH but not with the 19yo male control (Figures 8c and 8b). Together these five unique protein biomarkers represent 4.63% unique proteins in patients (Figure 8c).

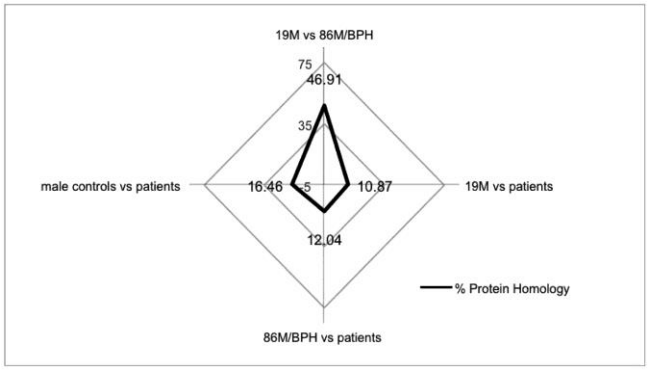

Figure 5. Percent protein homology of the controls when compared with each other and when compared with the patients.

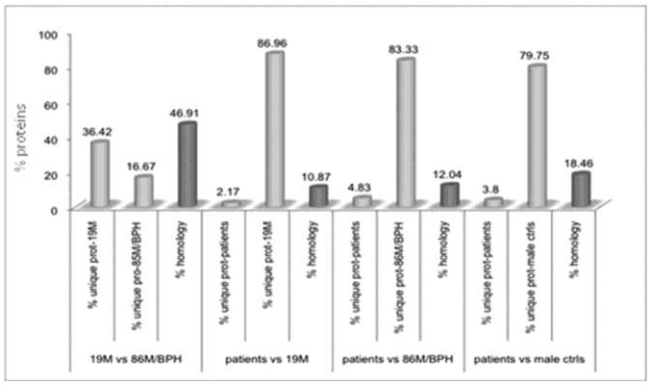

Figure 6. Summary of the unique proteins and homology revealed in male controls when compared to each other and the patients.

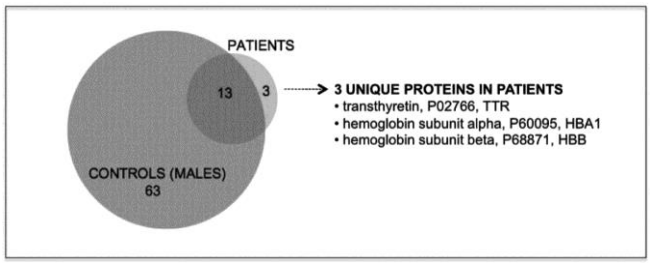

Figure 7. Three protein biomarkers identified in patients: transthyretin, hemoglobin subunit alpha and hemoglobin subunit beta.

Table 1 also shows the lack of association of PSA with high Gleason scores and TNM staging. Furthermore, it shows the association of the three unique biomarkers of the patients with high Gleason scores and TNM staging and their lack of association with PSA values.

Discussion

Mass spectrometry (MS) has essentially replaced the classical technique of Edman degradation in traditional protein chemistry since MS can precisely separate an ion from a collection of other ions, providing a powerful tool to analyze protein mixtures.[33] MS in tandem with liquid chromatography (LC) is a much more versatile technique even for complex protein mixtures offering a higher throughput through simultaneous identification of proteins. The wealth and complexity of data generated by LC-MS /MS via global analysis of protein can no longer be analyzed manually.[18] Thus, the computerized data analysis has become *de rigeur*. In response, the field of bioinformatics has expanded to provide software applications for all aspects of the data analyses needed by LC-MS/MS. Once the data from LC-MS/MS had been acquired, the data have to be processed to convert them into peaklists before database searching. In this way, the LC-MS/MS data search results can be visualized and compared as basis for generating reports for the identification of protein biomarkers in diseased state.[34] This technique is highly utilized in proteomics.

Proteomics is the study of protein structure, function, expression, localization and interactions.[16] Proteomics has been emerging as an important area of cancer research due to the advances in mass spectrometry. Most biomarker discovery studies based on proteomics to date attempted to detect proteins specifically associated with disease by comparative profiling of plasma fractions of healthy control and disease affected donors.[35] From blood, urine is emerging as the new source of biomarkers.

Urine is the biofluid of choice for biomarker discovery and monitoring for kidney diseases and even cancer.[36,37,38] The presence of urinary proteins may result from active secretion by epithelial cells that line the urogenital tract or incomplete reabsorption of plasma proteins by kidney cells. Thus, the urinary proteome contains a mixture of plasma and kidney proteins, whose ratios maybe altered in a diseased state.[30] Since urine contains various metabolites, but also washes out urothelial cells and its protein products throughout the urinary tract, a concept of analyzing urine in order to detect diseases has been developed.[39] Furthermore, urine having large amounts of low molecular weight molecules that represent a rich and a largely unexplored biomarkers is a promising scheme for disease monitoring.[27,31]

In this study, a high protein homology was revealed in male controls, and divergence was shown when controls were compared to patients with prostate cancer suggesting distinct proteins could differentiate the urinary proteome of patients from the controls. These distinct proteins from patients were transthyretin, hemoglobin subunits α and β. An association of these three biomarkers with high Gleason scores and TNM staging was also established in this study.

An interesting finding is the identification of two other proteins seen in the study patients with prostate cancer that were not seen in the 86yo male control with BPH, but surprisingly were present in the 19yo healthy male control. These proteins are uromodulin (P07911) and mannan binding lectin serine protease 2 (O00187) (Table 4).

Transthyretin, haptoglobin and mannan binding lectin serine protease 2 are considered acute-phase reactant proteins (APRP) and had long been associated with cancer.[25] Numerous reports have since linked APRP changes to various types or subtypes of cancer and different stages of the disease.[25,40,41,42] APRP's are proteins whose plasma or serum concentration increases or decreases by at least 25% during inflammatory disorders.[43] Acute inflammatory

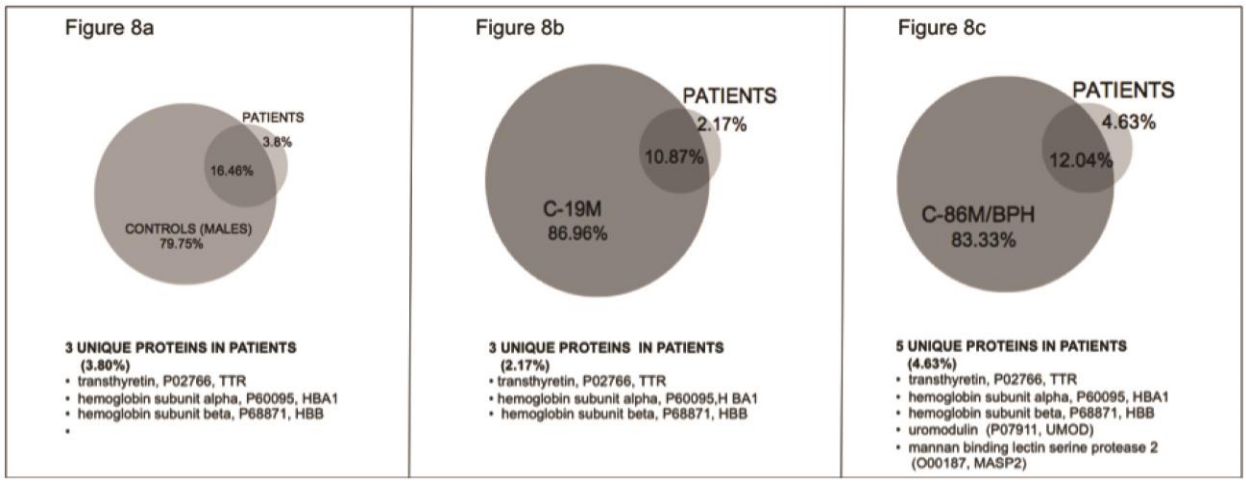

Figure 8a-8c. Comparison of the protein biomarkers identified in patients vs male controls.

Table 4. Comparison of the unique and shared proteins identified in 19yo and 86yo/BPH male controls and patients

proteins	19yo vs 86yo/BPH		19yo vs patients		86yo/BPH vs patients		male controls vs patients	
	19yo	86yo/BPH	19yo	patients	86yo/BPH	patients	male controls	patients
transthyretin	-	-	-	+	-	+	-	+
hemoglobin α	-	-	-	+	-	+	-	+
hemoglobin β	-	-	-	+	-	+	-	+
uromodulin	+	-	+	-	-	+	-/+	-
mannan binding lectin protease 2	+	-	+	-	-	+	-/+	-

diseases are usually self-limiting. However, the inability to switch off the inflammatory response makes chronic inflammation a causative factor in a variety of cancers despite having many of the same mediators generated during acute inflammation.[44] It is now accepted that many cancers arise from the sites of infection during chronic inflammation.[25]

Transthyretin has been associated with a number of pathological conditions, including Alzheimer's disease, Parkinson's disease, schizophrenia and depression[23] as well as its established physiological role of transporting protein for retinol-binding protein and thyroxin.[45]

Therefore, the detailed function of TTR in the progression of prostate cancer warrants further study and confirmation which will require large-scale studies. Further research is also needed to examine the role of TTR in the pathogenesis of prostate cancer.

The association of hemoglobin with prostate cancer as shown in this study is consistent with the association of hemoglobin in hormone refractory prostate cancer.[40] A high level of expression of hemoglobin subunit beta was also reported in chromosome 11p15.5 suggesting that a novel tumor suppressor gene located at this locus may influence the prostate cancer pathogenesis.[46] This association of hemoglobin subunits to prostate cancer illustrates the other role of hemoglobin subunits beyond oxygen binding and transport leading to their role in oxidative and nitrosative stress.[21,47] However, these are preliminary findings and their significance need to be confirmed in future investigations using larger sample sizes for both the controls and the patients.

Both uromodulin and mannan binding lectin protease 2 were cited in previous studies[31,46] as biomarkers used to discriminate BPH and prostate cancer. Uromodulin was shown to be a potential biomarker that will discriminate between patients with benign prostate pathology and those with prostate cancer.[31] Mannan binding lectin serine protease 2, on the other hand, is an enzyme of the innate immune system that was activated once it recognized microorganisms. Subsequently, it cleaves complement factors C4 and C2 that would initiate the activation of the complement system that has a direct link with inflammation.[46] Table 4 shows the comparison of the unique and shared proteins identified in 19yo and 86yo/BPH male controls and patients.

Another highlight of our study was the identification of a novel posttranslational modification in peptide sequence AADDTWEPFASGK of transthyretin showing the oxidation of proline (oxidation@8) revealed in the urine of patient 3 that was pro63 in the full sequence of transthyretin. The modification in this amino acid residue might explain one of the mechanisms of the disease. The result of the oxidation of this imino acid can contribute to energy supply of the cancer cell and thus, enhance the generation of reactive oxygen species (ROS) that is recognized in the apoptotic response. This proposed role of mutant proline during apoptosis and carcinogenesis remains to be defined.[48]

Conclusion

Our study was able to establish the homology of the urinary proteome among the controls and their divergence from the patients afflicted with prostate cancer by simultaneous comparison of their urine proteomes leading to the identification of a distinct panel of biomarkers. This was enhanced by the proper selection of the controls and patients. Furthermore, through the use of the sensitive LC/MS-MS, we were able to simultaneously identify low concentrations of distinct peptides not identified by other studies. At the same time, proper identification of the amino acids that normally undergo variable and fixed modifications before subjecting to database inquiry ensures that naturally occurring posttranslational modifications and those brought about by treatments due to sample preparation are not reported as modifications identified with prostate cancer. All these factors put together led to the proper identification of distinct proteins reflective of the true state of the disease and normal conditions.

This study was also able to report a panel of biomarkers in urinary proteomes of the patients with prostate cancer, namely transthyretin, hemoglobin subunits α and β. Two more proteins were identified in cancer patients but not in BPH, although unexpectedly these were also detected in the healthy male control. Combining the three unique proteins and the additional two other proteins may help differentiate prostate cancer from BPH, and patients with early BPH from normal individuals. Due to the limitations of the number of controls and patients, only preliminary findings and their significance were shown. These findings need to be confirmed in future investigations using larger sample sizes for both the controls and patients.

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