Proteomics-based signature for human benign prostate hyperplasia and prostate adenocarcinoma

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Abstract. Prostate adenocarcinoma often presents at a late stage, due to a lack of early clinical symptoms and lack of accurate objective markers. This study aimed to identify and validate proteomics-based biomarkers useful for prostate cancer diagnosis and to establish a marker-panel for prostate cancer and benign prostate hyperplasia (BPH). Global protein expression patterns in fresh tissue specimens from 8 patients with prostate carcinoma and 16 with BPH were analyzed by two-dimensional gel electrophoresis. Differentially expressed proteins were identified by MALDI-TOF mass spectrometry. We compared our results with those of published studies and defined a set of common biomarkers. We identified 22 differentially expressed proteins between BPH and prostate carcinomas. The up-regulated proteins in cancer compared to BPH included protein disulfide-isomerase, 14-3-3-protein, Enoyl CoA-hydrase, prohibitin and B-tubulin β-2. Keratin-II, desmin, HSP71, ATP-synthase-β-chain and creatine kinase-β-chain were down-regulated. Survey of the literature showed that 15 of our 22 identified proteins have been previously reported to differ in their expression levels between BPH and prostate cancer by other laboratories. The expression patterns of these biomarkers could successfully cluster BPH and adenocarcinomas as well as prostate cancer of low and high Gleason scores. This study validates protein-biomarkers that can be useful for accurate diagnosis and prognostic monitoring of prostate adenocarcinoma. Despite varied prevalence of the disease between different ethnic populations (i.e., high in Sweden, low in Saudi Arabia); the biomarkers indicate that BPH and prostate cancers are biologically 'homogeneous' in their protein expression patterns across wide geographical regions.

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Abbreviations: 2-DE, two-dimensional electrophoresis; PSA, prostate specific antigen; PCa, prostate carcinoma; BPH, benign prostate hyperplasia; TURP, trans urethral resection of prostate; RP, radical prostatectomy; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight

Key words: BPH, prostate cancer, Gleason scores, proteomics, protein expression, biomarkers

Introduction

Prostate cancer accounts for approximately 25% of all newly diagnosed cancers and is the second leading cause of cancer deaths among the male population in America (1,2). While the prevalence of prostate cancer is relatively low in Saudi Arabia, it is the most commonly diagnosed cancer in men in Sweden (3). Even though the molecular variations in prostate carcinomas across wide geographical regions have not been extensively studied, the disease is relatively more common among Afro-Americans and often present with advanced stage disease compared to what is observed in American white men (4,5).

An improvement and widespread availability in the measurement of PSA, such as PSA density, PSA volume and adjusted age-specific PSA ranges has resulted in early disease diagnosis in the Western world (6). However, many malignant cases still elude early detection and patients often present with metastasis at time of diagnosis (7,8) especially in the less developed world. Patients with occult metastases usually do not benefit from radical prostatectomy or radiotherapy and often respond less favorably to hormonal treatment (5,9). Prostate cancer is biologically heterogeneous with unpredictable aggressive behavior and currently the molecular events underlying its development and progression are poorly understood.

Proteomics studies have reported differences in protein expression profiles between BPH and prostate carcinomas. A number of proteins were identified as potential diagnostic or prognostic markers of prostate cancers (10-12). Among the identified proteins from human prostate cancers is Protein disulfide isomerase, Enoyl CoA-hydrase, Prohibitin, Cytokeratin-18, HSP-60, HSP-71 kDa, glutathione-S-transferase-π,

superoxide dismutase, tropomyosin-2 and triose phosphate-isomerase (12,13). The pattern of expression of many of these proteins in prostate cancer is similar to that found in breast and ovarian cancer. This suggests a high degree of similarity in the protein expression profiles of different epithelial tumors (10,11,14). The usefulness of the identified proteins as potential prostate cancer markers are yet to be validated in a large cohort of clinical materials within an ethnic group or across different ethnic populations. With the advancements in molecular biology technology, identification of PCA progression may become more accurate and easier at protein level using protein expression patterns unique to each patient.

The aims of this study was to use the proteomics technique to: i) characterize the pattern of polypeptide expression in BPH and prostate cancer, ii) identify biomarkers useful for diagnosis and iii) establish a marker panel for prostate tumors. Another objective was to compare our results with those of other published studies and to allow us to define and validate a set of common prostate tissue-associated biomarkers across distinct ethnic regions of the world. Furthermore, to determine whether there are ethnic differences in the protein expression patterns of BPH and prostate carcinoma in Sweden and Saudi Arabia.

To our knowledge, this is the first attempt whereby 2-DE fingerprint data were used to evaluate biological variability of BPH and prostate adenocarcinoma samples between different ethnic populations.

Materials and methods

We analyzed fresh surgical biopsies from 16 BPH patients obtained by Trans Urethral Resection of the Prostate gland (TURP) and eight malignant tumors obtained by radical prostatectomy. The patients had no prior hormonal treatment or radiotherapy and the patient characteristics are indicated in Table I.

The gross samples for routine pathology and samples for 2-DE were examined by two observers and independently classified as BPH and carcinomas. Routinely 12 core biopsies are taken for histopathological diagnosis of BPH. Furthermore, all BPH samples were histologically confirmed as epithelial predominant and malignant samples as adenocarcinoma. All patients were recruited at the Department of Urology, KFSH&RC following written and signed informed consent. The Office of Research Affairs of the KFSHRC approved the study.

Sample preparation protocols. We earlier reported that the quality and reproducibility of 2-DE gels largely depends on adequate sample preparation procedures (15,16). The qualities of samples obtained by TURP were comparable with radically resected tissue samples and were judged as representative as previously described (15). Briefly, sample representativity was assessed by cytology and histological evaluation and only samples with >80% tumor cells were included in the study. Following 2-DE separation, low quality sample images judged by markedly low number of total resolved gel spots as well as total optical density were excluded from the analysis as previously described (17). Tumor tissues were homogenized in ice-cold RPMI-1640 medium and the preparation of cells for 2-D gel analysis was performed as previously described (10).

Protein separation: two-dimensional gel electrophoresis (2DE) protein array. High-resolution two-dimensional gel electrophoresis (2-DE) was used for protein separation. 2-D electrophoresis was performed, using precast immobilized pH gradient (IPG) strips (pH 4.0-7.0, linear; Bio-Rad) in the first dimension (IEF). Samples were applied via in gel rehydration of 17 cm IPG strips overnight in sample solution diluted to a total volume of 350 ml with 8 M urea, 2% CHAPS, 2% IPG buffer (pH 4.0-7.0, linear), 0.3% DTT and a trace of bromophenol blue. For analytical runs, 150 μg proteins were loaded on each IPG strip and focusing was carried out for 59500 Vh using six steps of combined ramping of gradient with step and holds constant voltage. After IEF separation, the strips were immediately equilibrated twice over 15 min with 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% glycerol and 2% SDS. In the first equilibration solution, DTT (2%) was included, and 2.5% w/v Iodoacetamide was added in the second equilibration step to alkylate thiols. SDS/PAGE was performed using 12% SDS/polyacrylamide homogeneous large gel format (25 x 20 cm x 1.5 mm). The strips were held in place with 0.5% agarose dissolved in SDS/Tris running buffer and electrophoresis was carried out at constant 100 voltage and temperature (10˚C) overnight until the tracking-dye reached the bottom of the gel. After electrophoresis, gels were stained with silver nitrate solution.

Biological and analytical sample replicates. Histological and cytological evaluations of all samples were done to ensure sample representativity. Two randomly chosen biological samples; run on two duplicate gels resulting in four analytical replicates were used to assess method reproducibility. The gel electrophoresis of the two samples was independently run on two different occasions. The reproducibility was then measured by the average correlation coefficient of the total optical densities between the pairs of gels. Because of limited quantity in amount of protein harvests from clinical samples and combined with a satisfactory average correlation coefficient value of the biological replicate analysis; all biological samples were run on single analytical gel.

Image and data analysis. The silver-stained 2-D gels were scanned at 100 μm resolution (12 bits/pixel) using a GS 800 calibrated laser densitometer and data were analyzed using the PDQUEST™ software v 8.1 (Bio-Rad). For the group sample comparison a 'standard' or 'master gel' was created from one of the samples. The gel with the best resolution and with the highest number of resolved spots was chosen to be the reference gel to link the rest sample gels for differential expression analysis. Polypeptide quantity was expressed as parts per million (ppm) of the total integrated optical density.

Data preprocessing/data analysis. We used a difference of ≥2-fold change as a threshold for marked quantitative difference between sample pairs. Additionally, significantly differentially expressed protein spots were selected using two different statistical methods (Student's t-test and Partial Least Square analysis, features available in PDQuest 2-DE analysis software program). The generated datasets from PDQUEST were normalized prior to multivariate analysis and the data were subjected to hierarchical clustering analysis using the J Express

Table I. Clinical and pathological characteristics of the samples analyzed.

No.	Sample	Age	Diagnosis	Staging	Gleason[a]	Rx
1	UP-02	76	BPH	-		TURP
2	UP-04	72	ADC prostate	T3aN0M0	7 (3+4)	Radical prostatectomy
3	UP-05	75	BPH	-	-	TURP
4	UP-06	65	BPH	-	-	TURP
5	UP-07	74	BPH	-		TURP
6	UP-08	73	BPH	-	-	TURP
7	UP-09	71	ADC prostate	T2N0M0	5 (2+3)	Radical prostatectomy
8	UP-11	65	BPH	-	-	TURP
9	UP-12	62	BPH	-	-	TURP
10	UP-13	77	BPH	-	TURP	TURP
11	UP-15	71	BPH	-	-	TURP
12	UP-16	55	Ca prostate	T2N0M0	7 (3+4)	Radical radiotherapy + channel TURP
13	UP-17	85	BPH		-	TURP
14	UP-18	64	ADC prostate	T2N0M0	7 (3+4)	Retropubic prostatectomy
15	UP-19	62	BPH	-		TURP
16	UP-20	75	BPH	-	-	TURP
17	UP-22	80	BPH	-	-	TURP
18	UP-23	58	ADC prostate	T3cN0M0	7 (4+3)	Retropubic prostatectomy
19	UP-24	63	BPH	-		TURP
20	UP-25	82	BPH	-	-	TURP
21	UP-27	80	BPH	-	-	TURP
22	UP-28	73	ADC prostate	T2N0M0	5 (2+3)	Radical prostatectomy
23	UP-33	66	Ca prostate	T3aN0M0	5 (2+3)	Radical prostatectomy
24	UP-34	72	Ca prostate	T3aN0M0	5 (2+3)	Radical prostatectomy

BPH, benign prostate hyperplasia; ADC, adenocarcinoma; TURP, trans urethral resection of prostate. [a]The old Gleason scores were applied in this study as against the modified Gleason system reported after the Gleason consensus conference in 2005 (22).

Pro V1.1 software (java.sun.com) as previously described (18,19).

Protein identification by in gel-digestion and MALDI-TOF-MS analysis. Approximately 500-750 μg protein was loaded for micro preparative runs for peptide mass fingerprinting (PMF). The observed differentially expressed protein spots on 2D gels were selected and systematically cut/picked from Instant Blue-stained gels (Expedeon™) by robotic Proteome Works Plus Spot Cutter (Bio-Rad, Hercules, CA). The gel plugs were deposited into a 96-well micro-titer plate with ~1-5 gel pieces per well for further in-gel protein digestion. Automated digestion was performed essentially as described in the old Mass PREP Station Digestion protocol 5.7S (Micro Mass UK) with minor modifications. The gel plugs were washed with destaining solution (100 mM Ammonium bicarbonate in water) at 37˚C. After two destaining steps, the buffer was removed, and the gel plugs were dehydrated by adding acetonitrile. Reduction and alkylation steps were done using freshly prepared DTT and Iodoacetamide respectively. Sequencing grade porcine trypsin (Promega) solution (20 μg/ml stock, diluted to 0.0375 mg/ml in 25 mM NH_4HCO_3 buffer) was then added to restore the gel plug to its original volume. Digestion was carried out at 37˚C for 4.5 h. Resulting peptides in the gel plugs were extracted once using formic acid. The extracted peptides (0.8 μl) were spotted with (0.8 μl)-cyano-4-hydroxycinnamic acid matrix, 10 mg/ml (1/1v/v ACN/0.1% aqueous TFA) onto MALDI Target plate. The whole process was performed using the Janus™ Automated Mass Prep Station (Perkin-Elmer). Peptide mass fingerprinting MS spectra were acquired on a bench top MALDT-TOF Micro Mx (Waters, Manchester, UK). Each spot was analyzed in positive ion reflector mode, by accumulating signal with up to 1000 laser shots (20 sub-spectra of 50 shots) over the m/z range of 800-3000 Da. ACTH (Sigma, St. Louis, MO); [MH]+ = 2465.199 Da was used for lock mass correction for every acquisition at a concentration of 500 fmol spotted on target with-cyano-4-hydroxycinnamic acid as matrix. A standard digest of Enolase or alcohol dehydrogenase (ADH) at concentration of 250-500 fmol was spotted on target as multi-point external calibrant. Mass Lynx Global Mass-Informatics v4.0 (Waters) was used for all automated data acquisition. The acquired MS data were background subtracted, smoothed and de-isotoped at medium threshold. Protein Lynx Global Server (PLGS) 2.2 (Waters) was used for all automated data processing

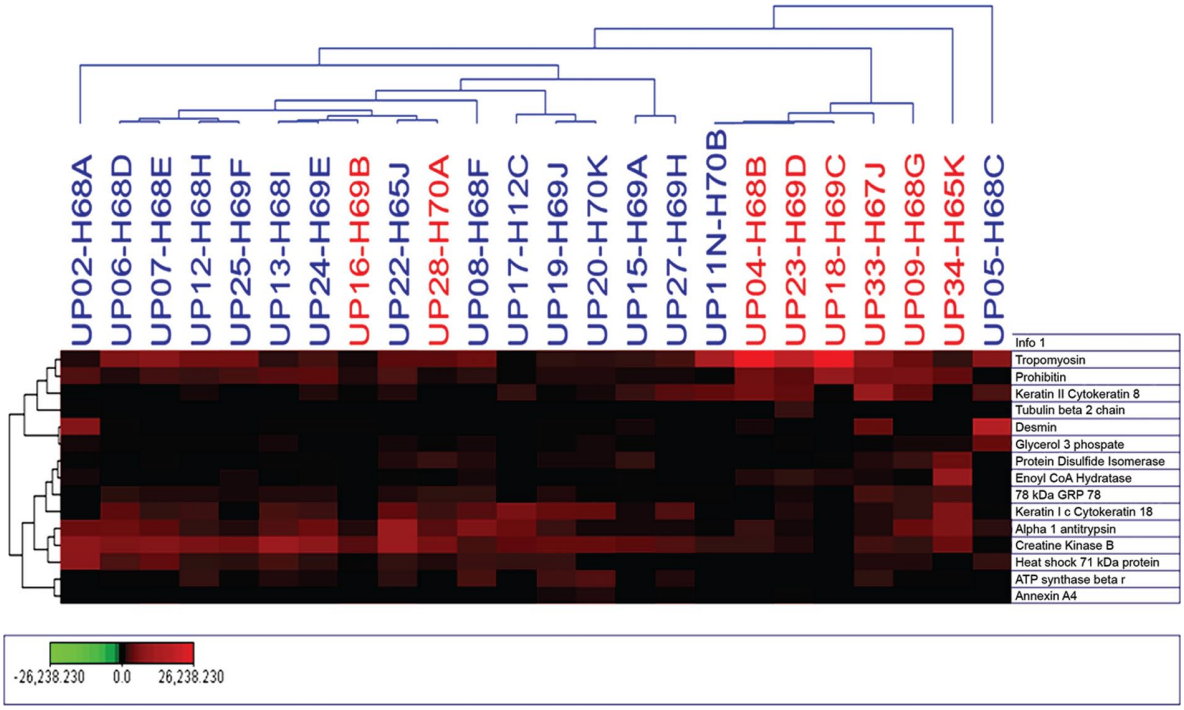

Figure 1. Hierarchical Cluster analysis using the expression patterns of 15 proteins that have been identified both in this study and previously published studies between benign prostate hyperplasia (blue) and prostate carcinoma (red). The dendrogram was generated using the Pearson Correlation distance metric and an average linkage clustering method from the J-Express software.

and database searching. The generated peptide masses were searched against a protein sequence database (Swiss-Prot) using the PLGS 2.2 for protein identification (Waters).

The databank search query parameters includes taxonomy as *Homo sapiens*, peptide tolerance of 100 ppm, estimated calibration error of 0.025 Da, molecular weight/pI range of 0-200000 Da/0-14, three (3) minimum peptides to match, trypsin as primary digest reagent, 1 missed cleavages, fixed modifications-carbamidomethyl cysteine and variable modifications of oxidation methionine. A MASCOT protein scores for combined MS spectra of >60 were considered statistically significant ($p<0.05$). In addition to other parameters, (predicted/observed pI/Mr, probability, scores etc.) for confirmation of identified proteins after database search, the digested peptides are analyzed 2-3 times for PMF on different MALDI plates and at different runs. Only protein identifications that give the same result in all the runs for each spots are considered as unambiguous protein identification.

Results

Protein expression in benign and malignant tumor tissues. We have characterized the expression of multiple proteins in human prostate carcinoma and in BPH using the technique of 2-DE. Tumor cells were collected from 16 patients diagnosed with BPH and eight prostate carcinoma patients (Table I).

The method reproducibility was evaluated by running two separate samples more than once and showed an average correlation coefficient of 0.88 as previously described for breast, ovarian and prostate tumors (12,15,16,20). Thereafter each sample was, run once and individual sample of the same diagnosis was considered as replicate under a cohort of analysis group (biological replicate for PBH vs. biological replicate for PCa). The average expression levels were taken for comparison between two sample groups being analyzed.

An average 1175 spots were resolved on 25x20 cm 2-DE large format gels, and >75% of the spots were successfully matched among all gels.

We observed statistical changes in the expression of 47 proteins between BPH and prostate carcinomas using Student's t-test analysis ($p<0.05$, 98%CI). Only 22 of the 47 protein spots were successfully identified using MALDI-TOF MS analysis. Among the proteins that are highly expressed in prostate cancer than BPH are protein disulfide isomerase, 14-3-3 protein, Enoyl CoA hydrase, prohibitin and Tubulin-β-2. Proteins that show increased levels of expression in BPH compared to cancer are Keratin II, Desmin, HSP-71 kDa, ATP synthase β-chain and Creatine kinase β-chain.

Validation of differentially expressed for tumor classification. Fifteen of the 22 identified proteins were previously been reported to differ in expression between BPH and prostate carcinomas (11-13). We used the expression patterns of these biomarkers for clustering analysis of BPH and prostate adenocarcinoma and almost all samples were correctly classified, as shown in Fig. 1. The locations of some of the identified proteins on the 2-DE gel are shown in Fig. 2 and names and other characteristics of the protein spots are listed in Table II.

From the above result, it is likely that malignant potential, both stage and grade, influences the discriminating power of the protein dataset. This was apparent when all the features of the samples defined as benign hyperplasia and different malignant variables was used for the clustering analysis. We therefore sought to determine whether the subset of 15 proteins

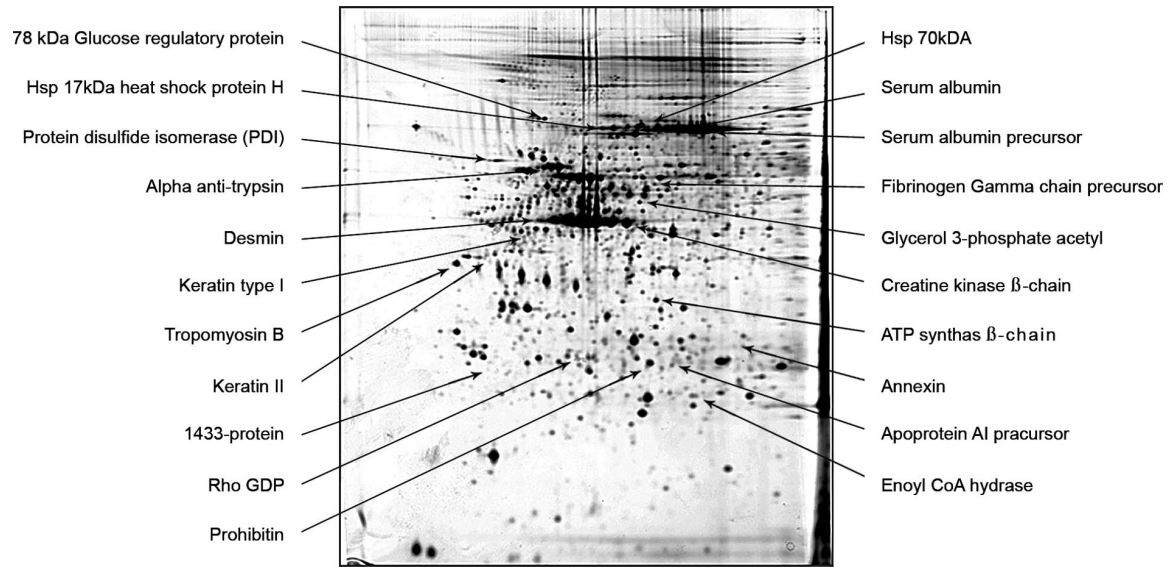

Figure 2. A representative 2-DE gel image of a prostate tumor showing some of the identified differentially expressed proteins between benign hyperplasia and different grades of prostate carcinomas.

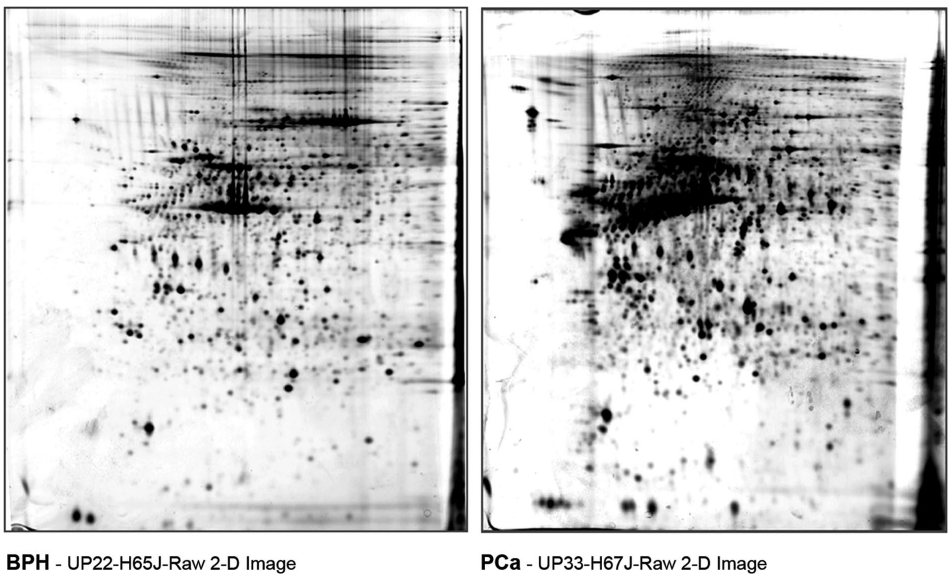

Figure 3. Representative 2-DE gels derived from one of the prostate hyperplasia and prostate cancer samples.

identified in this study, as well as in previously published studies, could be used for clustering according to Gleason grade classification of malignant prostate cancer samples as described below.

Protein expression in malignant tumors of different grades. A set of eight prostate cancer tissues of different histological Gleason scores were analyzed. Four of the eight samples had Gleason scores of 5, while the remaining four samples had scores of 7. Because of the small sample size, the Gleason 5 samples in this analysis will be referred to as (low malignant) and Gleason 7 as (high malignant tumors) as adapted from the previously described Gleason scoring system (21). Furthermore, the old Gleason scores was applied in this study as against the modified Gleason system reported after the Gleason consensus conference in 2005 (22).

The expression levels of 20 protein spots were significantly differentially expressed between Gleason 5 and 7 samples ($p<0.05$, 98% CI) using Student's t-test. This dataset of 20 protein spots were used in the cluster analysis of malignant tumors with different Gleason grading scores and all samples were correctly classified (Fig. 4A and C). Only eight of the 20 spots were among the 15 identified proteins that have been previously reported to differ in their expression levels between benign and malignant prostate tumors by other laboratories. We then used the expression patterns of the same 15 biomarkers for clustering analysis of malignant tumors of low and high Gleason scores and all samples were correctly classified as

Table II. Identified differentially expressed proteins between benign prostate hyperplasia and prostate cancer.

Gel SSP no.	Accession no.	Description	MW	pI	Score	Probability[c] (%)	Coverage (%)
1211	P06468	Tropomyosin β chain fibroblast and epithelial[a]	32969	4.4357	10.4396	32.77	32.3944
1226	P05787	Keratin type II cytoskeletal 8 Cytokeratin 8 K[a,b]	53510	5.3399	11.5553	100	59.751
2101	P35214	14-3-3 protein γ protein kinase C inhibitor p	28153	4.606	10.9851	56.54	47.1545
2506	P07237	Protein disulfide isomerase precursor PDI EC 5[a]	57080	4.5643	11.5552	99.99	47.4409
3303	P08727	Keratin type I cytoskeletal 19 Cytokeratin 19[b]	44079	4.8591	11.5553	100	73.25
3305	Q25472	Actin muscle type A2	42235	4.9501	10.1042	23.43	50.5291
3424	P17661	Desmin[a,b]	53372	5.0295	11.0741	61.81	50.3198
3510	P01009	α1 antitrypsin precursor[a,b]	46707	5.2405	11.5474	99.22	63.1579
3529	P05217	Tubulin β2 chain[a]	49799	4.602	11.2292	72.18	44.9438
3603	P11021	78 kDa glucose regulated protein prec GRP 78[a,b]	72288	4.8748	11.3436	80.92	56.1162
5117	P52565	Rho GDP dissociation inhibitor 1[b]	23192	4.8171	11.5198	96.52	29.902
6306	P12277	Creatine kinase B chain EC 2 7 3 2 B CK[a,b]	42617	5.2167	11.5473	99.21	53.0184
6535	P02679	Fibrinogen γ chain precursor[b]	51463	5.1561	11.5547	99.94	53.6424
6613	P08109	Heat shock cognate 71 kDa protein[a]	70827	5.1998	10.5211	35.55	51.548
6632	P38646	Stress 70 protein mitochondrial precursor 75 kDa	73733	5.8975	11.5356	98.05	20.9131
6632	P08107	Heat shock 70 kDa protein 1 HSP70 1[a,b]	70009	5.3187	11.3633	82.53	36.5055
7005	P30084	Enoyl CoA hydratase mitochprecursor EC 4[a,b]	31351	8.0728	11.5552	99.99	37.5862
7101	P35232	Prohibitin[a,b]	29785	5.4293	11.5269	97.2	25.3676
7110	P02647	Apolipoprotein A I precursor Apo AI[b]	30758	5.4309	11.5519	99.66	67.4157
7212	P10719	ATP synthase β chain mitochondrial precursor[a,b]	56318	5.0198	10.819	47.89	45.7467
7304	O75643	Small nuclear ribonucleoprotein 200 kDa helicas	194354	6.2509	11.0926	62.96	23.5744
7403	Q9HXW7	Glycerol 3 phosphate acyltransferase EC 2 3 1 15	94757	9.5317	11.0348	59.42	23.2614
7521	P05783	Keratin type I cytoskeletal 18 Cytokeratin 18[a,b]	47897	5.1674	11.5553	100	43.8228
7623	P02768	Serum albumin precursor	69321	5.8601	11.5553	100	47.7833
8106	P09525	Annexin A4 Lipocortin IV Endonexin[a,b]	35729	5.7268	11.5406	98.54	43.0818
8603	P02768	Serum albumin precursor[b]	69321	5.8601	11.5553	100	49.2611
3622	P10809	60 kDa heat shock protein mitochondrial precursor[b]	61016	5.5503	10.7736	45.76	61.4311

[a]Proteins that have also been identified in previously published studies, Lexander *et al* (12,23). [b]Proteins that have been described/annotated with functional links to different signaling pathways in Ingenuity Pathway Analysis (IPA) database as described in Table III and Fig. 6). [c]PMF analyzed 2-3 times at different runs and only concurrent results in all the runs are considered as unambiguous protein identification.

shown in Fig. 4B. The differential expression changes of the subset of 15-biomarker proteins based on Gleason scores is shown in Fig. 5.

Similarities in the proteome of prostate tumors of different ethnic groups. We compared our results with previously published studies on prostate tumors (11-13) and to our knowledge; these samples were obtained from Swedish patients, while samples analyzed in this study were primarily from Saudi men diagnosed with prostate tumor.

We observed a high degree of similarity between our generated 2-DE maps and some of the previously published 2-DE images of prostate tumor tissues. Although we did not have access to high-resolution digital copies of the published gel images, we used computer-assisted image analysis for the comparisons. Despite this caveat, using the low-resolution image of one of the published 2-DE-gels, we successfully matched >50% of the gel spots from our gel with the published image (data not shown).

In this study, we used IPG strips of similar pH gradient (4.0-7.0 non-linear) for the first dimension iso-electric focusing as in other reported studies. However, our 2nd dimension SDS-polyacrylamide gels consist of 12% homogeneous gels as opposed to the linear gradient (10-13%) gels that were used in the others studies (12,23,24) and our previously published data (10,11). Representative 2-DE gels derived from one of the prostate hyperplasia and prostate cancer samples are shown in Fig. 3. Apart from a high degree of similarity in the gel images derived from these sample cohorts, we also observed similar expression changes between different tumor groups and different malignant grades. More than 60% (15/22) of the identified differentially expressed proteins between BPH and prostate cancers in this present study have also been previously reported (Table II and Fig. 2). Interestingly, a similar observation

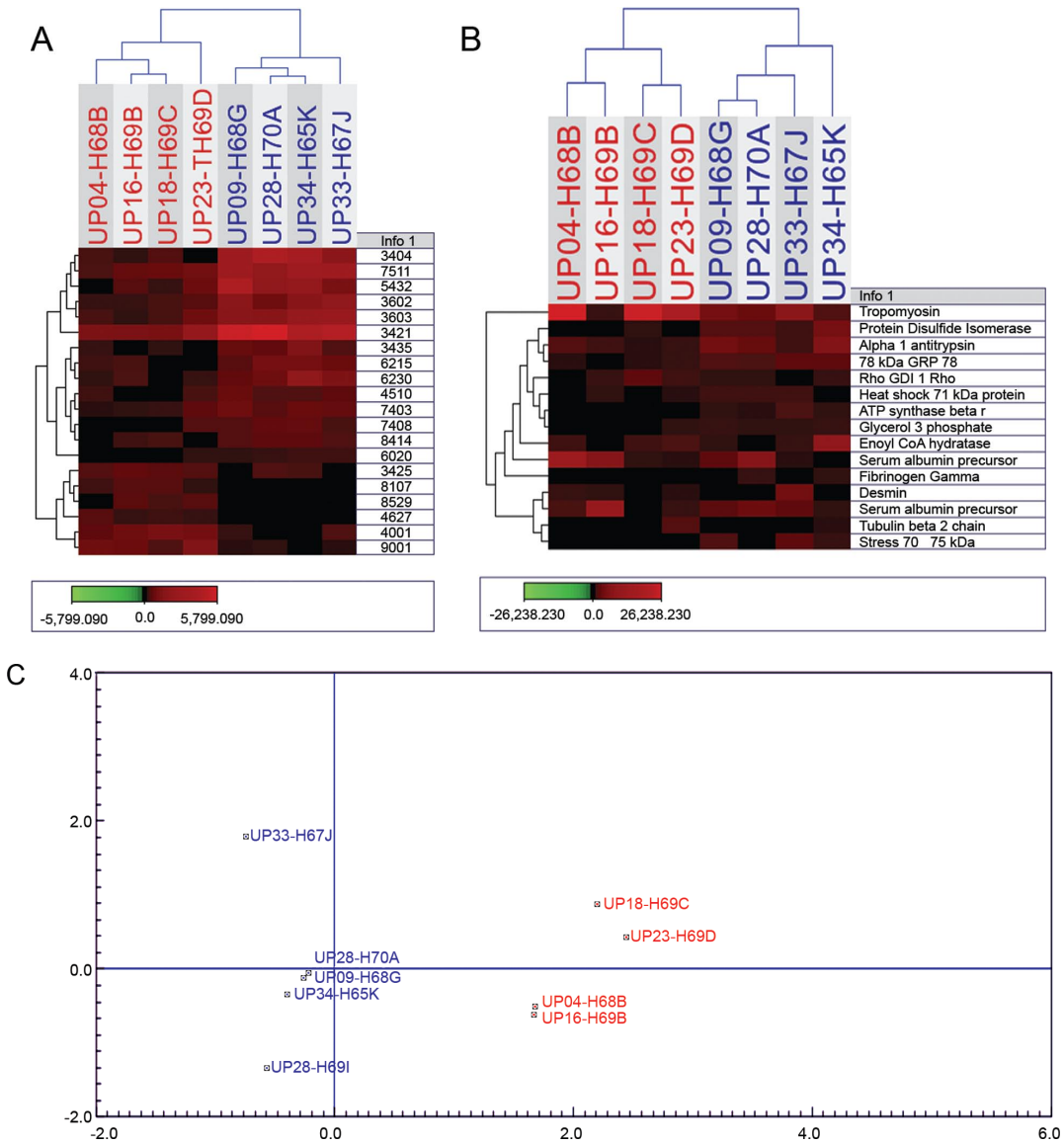

Figure 4. (A) Hierarchical Cluster analysis using the expression patterns of 20 proteins that are differentially expressed between Gleason 5 (low grade) and Gleason 7 (high grade) malignant prostate tumors. (B) Hierarchical Cluster analysis using the expression patterns of 15 proteins that have been identified both in this study and in previously published studies. The names of several of the identified proteins are indicated in the dendrogram (red, high grade Gleason 7; and blue, low-grade Gleason 5 cancers). The dendrogram was generated using the Bray Curtis distance metric and an average linkage clustering method from the J-Express software. (C) The correspondence analysis of the same dataset.

was found among identified proteins in this study between malignant tumors of different stage and Gleason scores and those reported by other studies. These results indicate; that inter laboratory comparison of 2-DE maps is possible and that 2-DE findings are reproducible with adequate experimental settings. Therefore, the proteins identified in this study are considered as potential markers for prostate cancer and BPH.

Discussion

Several studies have described marked alterations in prostate tissue and serum samples from prostate cancer patients using methods including immunohistochemistry, tissue micro-arrays and SELDI protein-chips (25-27). However, very few studies have analyzed human prostate tissues using 2-DE based protein expression analysis. It is necessary that potential protein markers, identified by proteome analysis, be verified by other methods such as immunohistochemistry using large numbers of representative tumor materials. The obvious limitation of such validation studies is lack of commercially available antibodies to most of the described proteins. An alternate form of validation should be considered, if independent investigators, using similar disease samples can reproducibly obtain similar results.

This discussion will focus on the reproducibility of published 2-DE data on prostate tumors, and the use of data derived from this study as a validation and as a proof of concept for the discovery of biomarkers for prostate tumors.

Studies of breast, ovarian and prostate tumors have shown that 2-DE gels are very reproducible when protocols are carefully developed (12,15,16,20). In this present study, analyzed samples were procured by either TURP (all BPH) or radical prostatectomy (all prostate cancer samples) and immediately processed for 2-DE as previously described

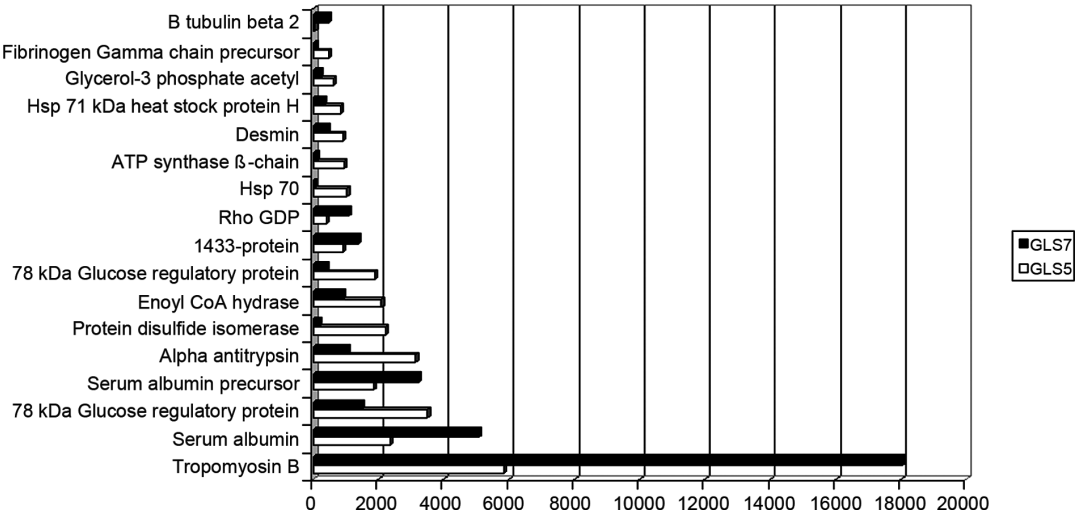

Figure 5. Bar chart of the differentially expressed proteins between Gleason scores 5 and 7 (black-filled, Gleason 7; and white-empty, Gleason 5 tumors).

(10,11,15). Even though the BPH samples were taken from the central zone of the prostate since they are procured by TURP, while the prostate cancer samples are typically derived from the peripheral region of the prostate gland, the resolutions of total separated proteins spots from our two sample procurement methods were very similar. Differences in protein expression patterns between benign and malignant tumors could be attributed to either true biological changes or changes due to differences in sample collection methods as previously reported (23). The latter possibility was seriously considered; therefore, the focus of the analysis in this study is directed at changes in protein expression between malignant tumors of different grades that were procured by same collection method (radical prostatectomy).

We observed changes in the expression of 47 proteins between BPH and prostate carcinomas samples. The expression levels of these protein spots allowed discrimination between benign hyperplasia and prostate carcinomas (data not shown). Previously published results, using fresh prostate tumor samples (10-12,24), reported similar findings to that of this study and also highlighted the possibility of disease classification using an artificial learning model of multiple polypeptides (13,18,19). Fifteen of the 22 (68%) proteins identified in this study have been previously described to differ in expression between benign hyperplasia and prostate carcinomas (12,13,23,24). This observation validates previously reported protein changes in prostate tumors (12,13,23,24). The fact that these datasets can be largely reproduced at different laboratories is encouraging.

Tumor staging and Gleason grading system remains one of the most powerful prognostic predictors in prostate cancer (28,29). However, the use of these parameters in combination with measurement of serum PSA pre- and post-radical prostatectomy can not conclusively predict recurrence or progression of the disease (30). This has drawn caution on the prognostic evaluation of prostate tumors procured by radical prostatectomy and led to a call for the discovery of more objective quantitative biomarkers. The 'low malignant-Gleason 5 cancers' showed >2-fold differences in the expression of 78 kDa glucose regulatory protein, α antitrypsin, protein disulfide isomerase, enoyl CoA-hydrase, Hsp-70, ATP synthase β-chain, desmin, Hsp-71 kDa, glycerol-3 phosphate acetyl, and fibrinogen γ chain precursor than the 'high malignant cancers with a Gleason score of 7'. In addition, these Gleason 5 cancers showed <2-fold decrease in the level of expression of Rho-GDP, tropomyosin-B and Tubulin-β-2. Even though the sample size in this study is small, our findings are similar to, and support, those described in other studies with larger sample sizes (13). Furthermore, 12 of 23 proteins identified in another previous study that differs significantly between BPH and prostate cancer are among the 15 proteins validated in this study, although their usefulness for discriminating tumors of different Gleason grades was not evaluated (13).

The results of this study can offer alternative complementary approach to the classical Gleason scoring system. The Gleason grading system from tissue samples can be mirrored into proteomics quantitative data for more objective assessment of disease status. The potential of using multiple markers for disease classification against the use of single markers has been advocated. The expression levels of 190 protein spots from 2-DE gels were able to discriminate between ovarian tumors of low malignant potential from ovarian carcinomas (19). Similarly, we are among the first to discriminate BPH from carcinoma based on the expression levels of only nine proteins (10,13). As shown in Fig. 4, the expression levels of 20 protein spots was able to discriminate between G5 and G7 prostate tumors using unsupervised hierarchical cluster analysis. This implies that it will be difficult to define a universal protein panel for BPH and prostate carcinoma. Therefore, specific marker sets need to be defined for different malignant tumor sub-types.

The majority of molecules identified in this study are involved in various signaling pathways and mostly regulates among others, proliferation, survival, cell cycle progression and apoptosis and these molecules are located mostly in the cytoplasm and only a few are located in the nucleus and extracellular space. While some of these molecules act as transporters, others act as enzymes and transcription regulators as presented in Table III and Fig. 6.

Table III. Functional characteristic of some of the identified proteins between prostate hyperplasia and prostate carcinoma.

Accession no.	Ing. symbol	Entrez gene name	Cellular location	Family/type(s)	Gl5-Gl7[a]	T2-T3[a]	Biomarker application(s)
P02768	ALB	Albumin	Extracellular space	Transporter	-2.155	2.684	Diagnosis, myocardial necrosis, Crohn's disease, ulcerative colitis
P09525	ANXA4	Annexin A4	Plasma membrane	Other	1.000	1.000	Almost exclusively expressed in epithelial cells
P02647	APOA1	Apolipoprotein A-I	Extracellular space	Transporter	2.691	2.691	Diagnosis of breast cancer and atherosclerosis
P52565	ARHGDIA	Rho GDP dissociation inhibitor (GDI) α	Cytoplasm	Other	-2.571	-2.165	Unknown, but implicated in proteinuria, colorectal cancer
P10719	ATP5B	ATP synthase, H^+ transporting, mitochondrial F1 complex, β polypeptide	Cytoplasm	Transporter	7.440	1.000	Unknown, cell growth, HIV-1, Alzheimer's disease
P12277	CKB	Creatine kinase, brain	Cytoplasm	Kinase	1.000	-	Implicated in prostatic carcinoma, lung cancer, chronic renal failure, acute myocardial infarction
P17661	DES	Desmin	Cytoplasm	Other	2.059	-8.929	Unknown, implicated in dilated cardiomyopathy, hypertrophy
P30084	ECHS1	Enoyl CoA hydratase, short chain, 1, mitochondrial	Cytoplasm	Enzyme	2.335	-7.407	Metabolic process, implicated in colorectal cancer
P02679	FGG	Fibrinogen γ chain	Extracellular space	Other	7.634	1.000	Non-small cell lung carcinoma, ischemic cardiomyopathy, myocardial infarction
P11021	HSPA5	Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	Cytoplasm	Other	2.339	-1.613	Hyperplasia, hypertrophy, neurodegeneration, liver cancer, head and neck cancer
P10809	HSPD1	Heat shock 60 kDa protein 1 (chaperonin)	Cytoplasm	Enzyme	1.000		Adenocarcinoma, Alzheimer's disease, rheumatoid arthritis
P05783	KRT18	Keratin 18	Cytoplasm	Other	-3.322	-3.322	Head and neck cancer, colorectal cancer
P08727	KRT19	Keratin 19	Cytoplasm	Other	-4.950	-4.950	Diagnosis of cervical, ovarian, breast cancer, head and neck cancer, disease progression, prognosis
P05787	KRT8	Keratin 8	Cytoplasm	Other	-4.950	-4.950	Prognosis of breast cancer, dysplasia, cryptogenic cirrhosis, celiac disease, endometriosis, fibrosis
P07237	P4HB	Prolyl 4-hydroxylase, β polypeptide	Cytoplasm	Enzyme	11.540	-	Promising biomarker for diagnosis of gastric cancer. follicular adenoma, breast cancer, tumorigenesis
P35232	PHB	Prohibitin	Nucleus	Transcription regulator	1.000	-	Role in cellular apoptosis, growth, proliferation, colony formation, migration, cell cycle progression
P01009	SERPINA1	Serpin peptidase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 1	Extracellular space	Other	2.992	-	Disease biomarker for progression of renal cancer

[a]Fold change in expression levels between sample groups: Gl5, Gleason score 5; Gl7, Gleason score 7; T2-T3, T2 and T3 tumors. The data were partly generated from Ingenuity Pathway Analysis, Ingenuity IPA V8.7 (Ingenuity Systems, www.Ingenuity.com).

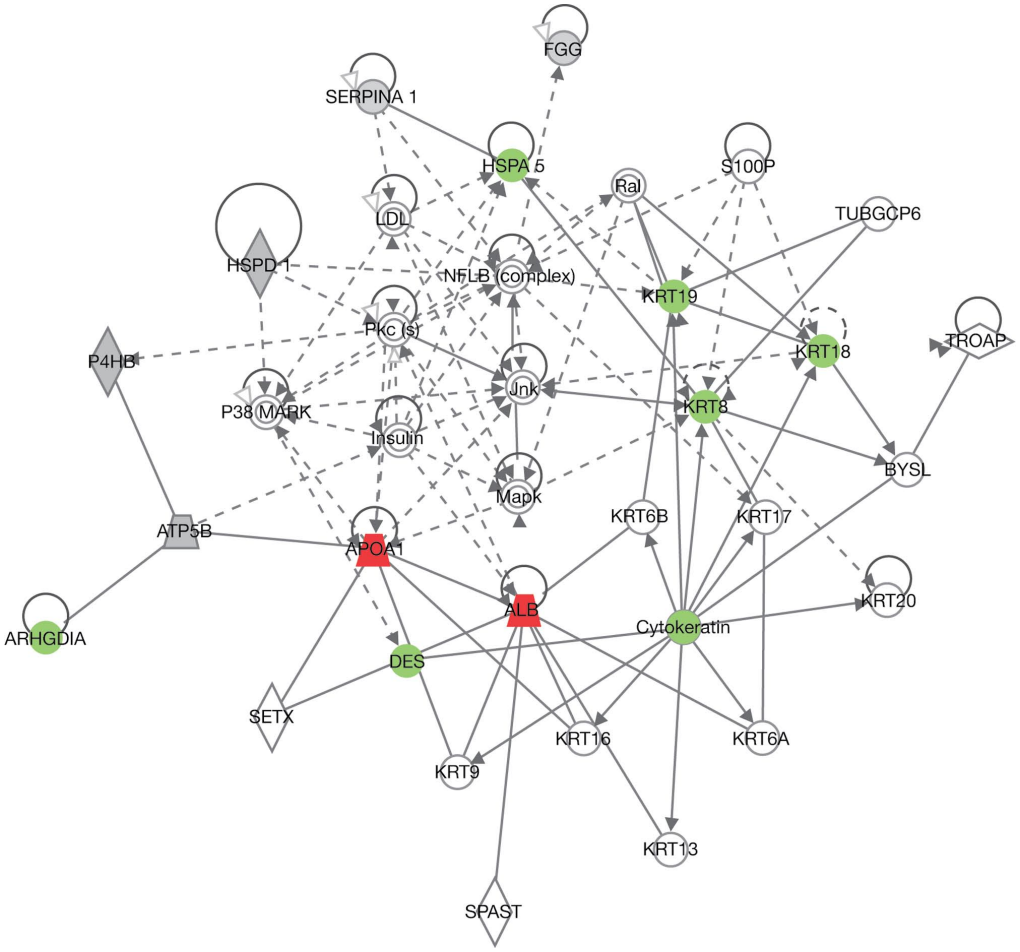

Figure 6. Canonical pathway analysis of network signaling of identified proteins in prostate cancer. Some of the identified proteins were mapped in Ingenuity database and represented in multiple sub-signaling networks and mostly regulates among others: proliferation, survival, cell cycle progression and apoptosis and these molecules are located mostly in the cytoplasm and only a few are located in the nucleus and extracellular space. While some of these molecules act as transporters, others act as enzymes and transcription regulators. The connection and the expression profile of some of the proteins are as indicated. Red indicates an up-regulated protein, and green color is indicative of down-regulation, a direct connection is by solid line and broken lines indicates an indirect interaction between different molecules ALB, albumin; APOA1, apolipoprotein A-I; ARHGDIA, Rho GDP dissociation inhibitor (GDI) α; DES, desmin; HSPA5, heat shock 70-kDa protein 5 (glucose-regulated protein, 78 kDa); KRT18, Keratin 18; KRT19, Keratin 19; KRT8, Keratin 8. The network analysis was done in Ingenuity Pathway Analysis program (IPA V8.7).

The limitation of this study is the small sample size however; the reproducibility of the results in line with previously published data on prostate cancer is very encouraging. The majority of analyzed samples in this study have had a follow-up of approximately 2-3 years and all patients are alive as at the time of this study. It is anticipated that in the near future, the prognostic evaluation based on using multivariate analysis of these proteins will be further validated when other clinical parameters such as residual free survival and patient outcome becomes available.

Previous studies have used tumors from Swedish patients (personal communication), while all samples reported in this study were obtained from Saudi men diagnosed with prostate tumors. The prevalence of prostate cancer is higher in Sweden than in Saudi Arabia. For example, the 1 year prevalence rates for Sweden and Saudi Arabia are 7346 and 377, while the 5-year prevalence rates are 28082 and 1236 respectively (31). It is interesting that the differences in protein expression during malignant progression are so similar in tumors from patients with different ethnical origin and with different disease prevalence. This study is the first attempt to characterize protein expressions in prostate tumors from patients of diverse ethnic backgrounds.

This study is the first of its kind to validate panel of proteins for objective classification and potential prognostic monitoring of prostate adenocarcinoma. These markers are found in prostate tumors in populations that differ with regard to the prevalence of prostate cancer (i.e., high in Sweden, low in Saudi Arabia); indicating that BPH and prostate cancers are biologically 'homogeneous' in their protein expression patterns between different ethnic populations.

These validated marker sets could be useful for accurate diagnosis of prostate cancer and prognostic monitoring. However, further characterization and validation on clinical significance of the identified potential protein markers on larger sample size of prostate adenocarcinoma is warranted.

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