Differences in urinary proteins related to surgical margin status after radical prostatectomy

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Abstract. Presented exploratory pilot study was aimed at evaluation of proteins present in urinary specimens collected from prostate cancer suffering subjects after radical prostatectomy, divided into two experimental cohorts: positive (n=15) and negative (n=15) surgical margins (PSM/NSM). The presence of PSM suggests inadequate cancer clearance and the possible need for additional treatment. Proper identification of these risk-patients is therefore of a paramount importance. Total protein profiles were firstly identified by using SDS-PAGE and compared by using partial least square discrimination analysis (PLS-DA), which revealed differences in molecular weights of 80-99 and 150-235 kDa between the experimental groups. For further identification of proteins, comparative proteomic technologies were employed. Two-dimensional gel electrophoresis with subsequent identification of protein spots by using MALDI-TOF mass fingerprinting revealed differential expression of proteins between NSM/PSM cohorts. Moreover, in PSM group, three uniquely identified proteins (cyclin-dependent kinase 6, galectin-3-binding protein and L-lactate dehydrogenase C chain) were found, which show tight connection with prostate cancer and presence of all of them was previously linked to certain aspects of prostate cancer. These proteins may be associated with the molecular mechanisms of prostate cancer development; hence, their identification may be helpful for the assessment of disease progression risk after radical prostatectomy, but also for possible early diagnosis.

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Introduction

Prostate cancer (PCa) is one of the most frequent malignant diseases of older men, whose incidence in Northern and Western Europe exceeds 200 new estimated cases/100,000 and is still growing, especially in the younger age group (35-64 years) (1). In European and North-American countries due to development of demography this disease becomes not only medical, but also an economical issue (2).

The cause of prostate cancer still remains unsolved and only few risk factors are known that affect development of malignant prostate disease. These include increasing age, the black race and hereditary factors (3,4). Not all forms of prostate cancer cause clinically significant disease. According to autopsy examinations 60-70% of older men die with prostate cancer, however only 3% of prostate cancer (5). Malignant disease is suspected on the basis of prostate-specific antigen (PSA) levels and palpation of the gland. Definitive diagnosis needs to be confirmed by transrectal needle biopsy.

Unfortunately, even with the sophisticated pre-operative staging techniques, an average of 28% of those undergoing radical prostatectomy are found to have positive surgical margins (6). A positive surgical margin (PSM) is defined as the presence of tumor at the inked surface of the resected specimen and as such implies incomplete excision of malignant tissue (7). These patients are at increased risk of biochemical relapse, 50-60% at 5 years, and subsequent clinical relapse, although by no means every patient will suffer eventual disease recurrence (8). Several explanations are given for why a PSM is not always associated with tumor recurrence. The surgery results in ischaemia and fibrosis, both of which may destroy small areas of residual carcinoma as the malignant tissue is unable to survive in its new environment. Alternatively, it may be a result of the desmoplastic response (6).

Evidence from randomized trials suggests that immediate secondary therapy is beneficial for patients with adverse pathology after surgery rather than watchful waiting (9). Since not all of the patients with PSM develop disease recurrence,

physicians face the challenge of advising an individual on the necessity of secondary therapy. Potential non-invasive monitoring of presence of PSM can be helpful as an auxiliary tool in this decision making process. Recent advances in 'omics'-based technologies have greatly facilitated the possibilities to reliably study various types of molecules, linked to different pathological states (10). Among these, proteomics allows the comprehensive identification of broad spectrum of disease-specific proteins, which are important for detailed description of certain diseases, such as PCa (11). In particular, urinary proteins can serve as an informative tool, simply obtainable in sufficient amounts in non-invasive way.

Our current objective is therefore dual. First, we want to distinguish patients with confirmed PSM or NSM after radical prostatectomy by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and its subsequent statistical processing by partial least squares discriminant analysis (PLS-DA). Secondly, we want to identify the major protein differences between the groups by using 2-D PAGE and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) a powerful tool for proteomics application. Overall, the disparities in expression of urinary proteins in PSM and NSM groups are highlighted.

Materials and methods

Chemical compounds. All reagents employed for study, as standards, and others were purchased from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity, unless noted otherwise.

Clinical urinary samples. For purpose of this study, urine samples from patients suffering from prostate cancer (n=30), obtained from University Hospital in Motol, Department of Urology, Prague, Czech Republic, were studied (Table I). All the samples were obtained 3 months after radical prostatectomy. Tested patients were divided into two experimental groups. The first one consisted of patients with negative surgical margins (NSM) (n=15). The second group consisted of patients with PSM (n=15). Enlistment of patients into realized clinical study was approved by the Ethics committee (reference EK-377/13).

Tumors were staged according to the 2002 TNM staging system. Extraprostatic extension (pT3a, pT3b) was defined as the extension of the tumor beyond the confines of the gland into the periprostatic soft tissue. A PSM was defined as the presence of tumor at the inked surface of the resected specimen. Histological Gleason grading was performed by a dedicated genitourinary pathologist. Level of serum PSA was measured 3 months after the surgery and this test was performed in a single hospital laboratory under standardized settings.

Determination of total protein in urinary samples. Total protein was quantified using the Skalab 600 M kit (Skalab, Svitavy, Czech Republic), according to the manufacturer's instructions. Measurements were carried out on automated spectrophotometer BS-400 (Mindray, Shenzhen, China).

Acetone precipitation of urinary proteins. Urinary samples (50 μl) were added to 200 μl of acetone. The mixtures were stored for 3 h at -20˚C and further were centrifuged using Microcentrifuge 5417R (Eppendorf AG, Hamburg, Germany) under 10,000 g at 4˚C for 15 min. The pellets were washed with diethylether and ethanol mixture (6:1) and centrifuged (10,000 g at 4˚C, 5 min), then solvent was removed and the pellet was dried on air.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Pellets were diluted in 5 μl of ACS water, mixed with protein loading buffer (PLB) in a ratio of 1:1 and incubated at 95˚C for 5 min in Thermomixer® R (Eppendorf AG). Further, samples were removed into the wells in the 12.5% polyacrylamide gel. Electrophoresis was performed in 1X Tris-glycine-SDS running buffer for 60 min in the electrophoretic bath (Bio-Rad, Berkeley, CA, USA) at 110 V. Gels were visualized following protocol of staining with Coomassie brilliant blue staining (12).

Data collection. The decision was made to describe each individual by series of values as curves extracted from electrphoreograms. This approach was chosen among other alternatives namely description of individuals by pairs of band positions and intensities due to its advantages in application of machine learning algorithms. A set of curves can be easily transformed into data matrix in comparison with other descriptions of individuals. The extraction of curves and estimation of molecular weight was made according to ref. 13. The data collection was facilitated by the MATLAB programming language.

Exploratory analysis. Exploratory analysis was performed by examining tables and plots of the observed data. The transformation was identified to evaluate the raw data on the basis of plots and knowledge of the expected scale of measured variables. Exploratory analysis was used to: i) identify missing and outlying values, ii), verify the quality of the data and find appropriate corrections, and iii) to determine the intervals of spectra used in the projection to latent structure discriminant analysis model relating outputs of experiments to predictors according to the experimental settings.

Statistical modeling. The standard projection to latent structure discriminant analysis models (PLS-DA) were used to find parts of curves differing in examined groups according to experimental settings. The standard PLS 1 algorithm was used to construct latent variables and ordinary least squares were used to determine the coefficients of classification model relating the latent variables to output of the experiment. The leave-one-out validation was used to assess the quality of different models and to choose the correct number of latent variables with respect to the performance of model on the validation data set. The fraction of explained variation R2 was also computed to provide more detailed evaluation of trained models. The interpretation of PLS-DA models was performed by the examination of loading plots and S plots. The interesting parts of curves in relation to the response were pointed out by introducing limits to denominated values in S plots.

2-D PAGE analysis. Pellets, collected after acetone precipitation were diluted in 125 μl of rehydration buffer (2 M urea, 7 M thiourea, 4% 3-[(3-Cholamidopropyl)dimethylammonio]-

Table I. Stratification of the patients from whom urinary samples were collected.

Patient	TNM stage	Surgical margin status	Gleason score	PSA (ng/ml) in 3rd month after the surgery
1	pT2a	Negative	3+3	0.009
2	pT2c	Negative	3+3	<0.005
3	pT2c	Negative	3+3	0.020
4	pT2c	Negative	3+4	<0.005
5	pT2c	Negative	3+3	0.031
6	pT3a	Negative	3+3	0.010
7	pT3a	Negative	3+3	0.011
8	pT2c	Negative	3+3	0.006
9	pT2c	Negative	3+3	<0.005
10	pT2c	Negative	3+3	0.012
11	pT2c	Negative	3+3	<0.005
12	pT2c	Negative	3+3	0.006
13	pT2c	Negative	3+3	0.019
14	pT2c	Negative	3+3	0.024
15	pT2a	Negative	3+3	0.005
16	pT2c	Positive	3+3	0.027
17	pT3a	Positive	3+4	0.032
18	pT3a	Positive	3+3	0.409
19	pT3a	Positive	3+5	0.748
20	pT2c	Positive	3+4	0.266
21	pT3a	Positive	3+2	0.044
22	pT3b	Positive	4+3	0.095
23	pT3a	Positive	3+3	0.009
24	pT2c	Positive	3+4	0.011
25	pT3b	Positive	3+4	0.008
26	pT3	Positive	4+3	0.008
27	pT2c	Positive	3+3	0.034
28	pT3a	Positive	3+3	<0.005
29	pT3a	Positive	4+3	0.19
30	pT2a	Positive	3+4	0.007

1-15 indicate the patients belonging to NSM group; 16-30 indicate the patients from PSM, both after radical prostatectomy.

1-propanesulfonate hydrate, 100 mM dithiothreitol, 0.2% ampholyte with pH 3.0-10.0, 0.001% bromophenol blue), and the solution was sonicated (8x2 sec). The resulting solution was employed for 12-h rehydration on 7-cm IPG strips (Bio-Rad). The IPG strips, after rehydration, were focused on Protean® IEF Cell (Bio-Rad) at 20˚C in 3 steps. In the first step, electric voltage was increased linearly over 20 min from 0 to 250 V. Second step comprised the voltage increase to 4,000 V (maintained for 2 h). In the third step the electrical parameters were set so that the total value reached 10 kVh. The electric current was limited to the value of 50 mA/strip. After isoelectric focusing, strips were incubated for 10 min at 25˚C on a shaker with the First equilibration buffer [6 M urea, 20% *v/v* glycerol, 2% *w/v* SDS, 0.375 M Tris-HCl (pH 8.8), 2% *w/v* dithiothreitol]. Thereafter, the solution was replaced and the strips were incubated with the second equilibration buffer [6 M urea, 20% *v/v* glycerol, 2% *w/v* SDS, 0.375 M Tris-HCl (pH 8.8), 2.5% *w/v* iodacetamide, 0.001% bromophenol blue] for 10 min. Strips, prepared in this manner were washed in Tris glycine-SDS running buffer. The strips were then placed on the back of the flatbed electrophoretic glass plate. Subsequently, the agarose was poured between the plates, where the strip was inserted. After solidification of agarose, the plates were inserted in the electrophoretic bath with Tris-glycine-SDS running buffer. Electrophoresis was set to 75 min at a voltage of 180 V. Gels were stained using Coomassie brilliant blue and silver (12).

In-gel tryptic digestion. For excision of the spots from 2D gels of urinary samples EXQuest™ SpotCutter (Bio-Rad) was utilized. The in-gel digestion with trypsin was performed according to a protocol of Shevchenko *et al* (14). The digests of proteins were further employed for peptide mass fingerprinting (PMF).

Matrix-assisted laser Desorption/Ionization time-of-flight (MALDI-TOF). The mass spectrometry experiments were performed on a MALDI-TOF mass spectrometer Bruker ultrafleXtreme (Bruker Daltonik GmbH, Bremen, Germany), using 2,5-dihydroxybenzoic acid as matrix. The saturated matrix solution was prepared in 30% acetonitrile and 0.1% trifluoroacetic acid. Mixture was thoroughly vortexed and ultrasonicated using Bandelin 152 Sonorex Digital 10P ultrasonic bath (Bandelin Electronic, Berlin, Germany) for 2 min at 50% of intensity at room temperature. For sample preparation the dried-droplet method was utilized, where solutions of digested proteins were mixed with matrix solution in volume ratio of 1:1. After obtaining a homogeneous solution, 2 μl was applied on the MTP 384 polished steel target plate (Bruker Daltonik GmbH) and dried under atmospheric pressure at 25˚C. All measurements were performed in the reflector positive mode in the *m/z* range 400-6,000 Da. The MS spectra were typically acquired by averaging 500 sub spectra from a total of 500 shots of the laser with laser power set 5% above the threshold.

Peptide mass fingerprinting. Peptide mass fingerprinting (PMF) was done using MASCOT server (Matrix Science, Boston, MA, USA) for comparing mass spectra with UniProt database. For database search the following parameters were used: trypsin was used as the enzyme, zero or one missed cleavage was allowed, taxonomy was set to *Homo sapiens*, oxidation of methionine or/and *N*-term acetylation was added as variable modification, peptide tolerance was set to ±0.5 Da, mass values were set as MH^+ and were obtained from monoisotopic peaks.

Results and Discussion

Urine is a specific filtrate of blood; the protein components of urine are qualitatively similar to those of blood but much more diluted (15). An advantage for urine over blood is that urinary proteins are stable and do not undergo significant proteolysis within several hours of collection. Hence, urinary proteomics

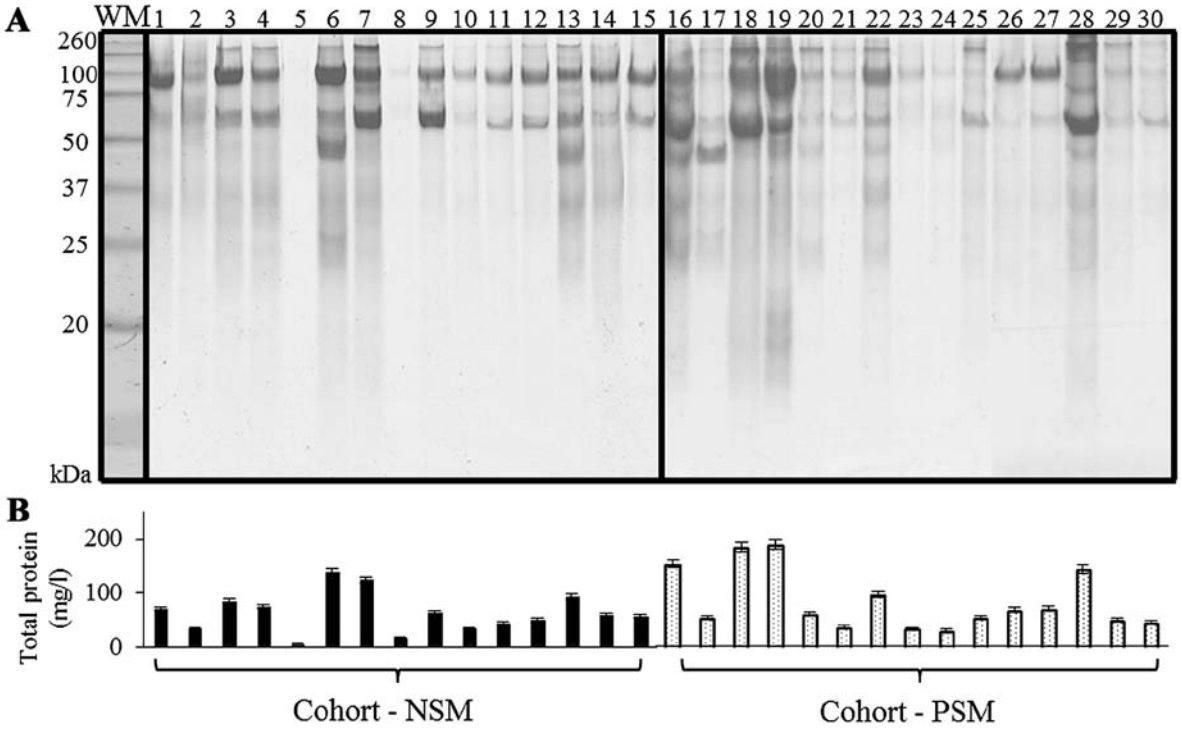

Figure 1. (A) SDS-PAGE of urinary proteins after acetone precipitation of both cohorts of patiens [lanes 1-15, patients with negative surgical margins (NSM); lanes 16-30, patients with positive surgical margins (PSM), both observed after radical prostatectomy]. WM, weight marker (kDa). Specific conditions of preparation of SDS-PAGE are given in 'Acetone precipitation of urinary proteins and SDS-PAGE' in Materials and methods. (B) Summary of total protein in individual samples (1-30), determined spectrophotometrically by using pyrogallol red method.

presents an attractive approach to cancer biomarker discovery, not only for urological malignancies (16), but for other systemic malignancies and evaluation of current health status (17). Many advantages favor the use of urine over blood and tissues samples, including the fact that urine-based tests are non-invasive, and urine is non-infectious for HIV and less infectious for many other pathogens (18).

Evaluation of total protein profiling by SDS-PAGE and its statistical processing. The migration of the molecular weight marker and urinary proteins of NSM, PSM patients on SDS-PAGE is presented in Fig. 1A. SDS-PAGE is a rapid and simple technique for protein pattern elucidation, quantification and determination of groups of proteins with similar molecular weight (M_r) (19). The visualization of NSM/PSM groups revealed proteins with relatively wide range of M_r that, according to previously published results, correspond to commonly present albumin (M_r 60-70 kDa); 80-110 kDa range belongs to transferrin and uromodulin (formerly Tamm-Horsfall protein), together with albumin polymeric complexes can be determined, in 160-200 kDa range the major band is commonly dedicated to IgG, IgA monomers and C_3. Less distinct bands at M_r 31 kDa were previously linked with carbonic anhydrase (20-22). For confirmation, total proteins in urinary specimens were quantified spectrophotometrically (Fig. 1B). Higher protein levels were determined in PSM cohort (85.3±55.9 mg/l) when compared to NSM one (mean 64.1±36.2 mg/l).

Since our first aim was the determination of NSM/PSM SDS-PAGE protein patterns we employed partial least square discriminant analysis (PLS-DA) model, which utilized curves (band intensities) extracted from SDS-PAGE gels. To facilitate the comparability of curves the linear interpolation of all curves was carried out to identical values of molecular weight. Carrying out the linear interpolation a pair of limits was introduced to exclude marginal parts of curves and the linearization of molecular weight was performed. The leave-one-out validation was carried out to assess the prediction quality of the model and to choose the number of latent variables according to the prediction accuracy criterion. The results of leave-one-out validation of PLS DA model are presented in Table II.

Table II. The evaluation of partial least square discriminant analysis (PLS-DA) model.

Model	Performance on the validation set	Fraction of explained variation	Estimated no. of latent variables
NSM/PSM	83.33%	50.20%	1

As seen in Fig. 2A, in our NSM/PSM model the proteins in mass ranges of 80-99 and 150-235 kDa were evaluated as the most significant in discrimination between examined groups. The quadrant expression based on comparison of the curves projection shows separate distribution of both NSM and PSM groups (Fig. 2B).

Obtained results revealed interesting disparities in protein patterns; however co-migration of proteins with similar

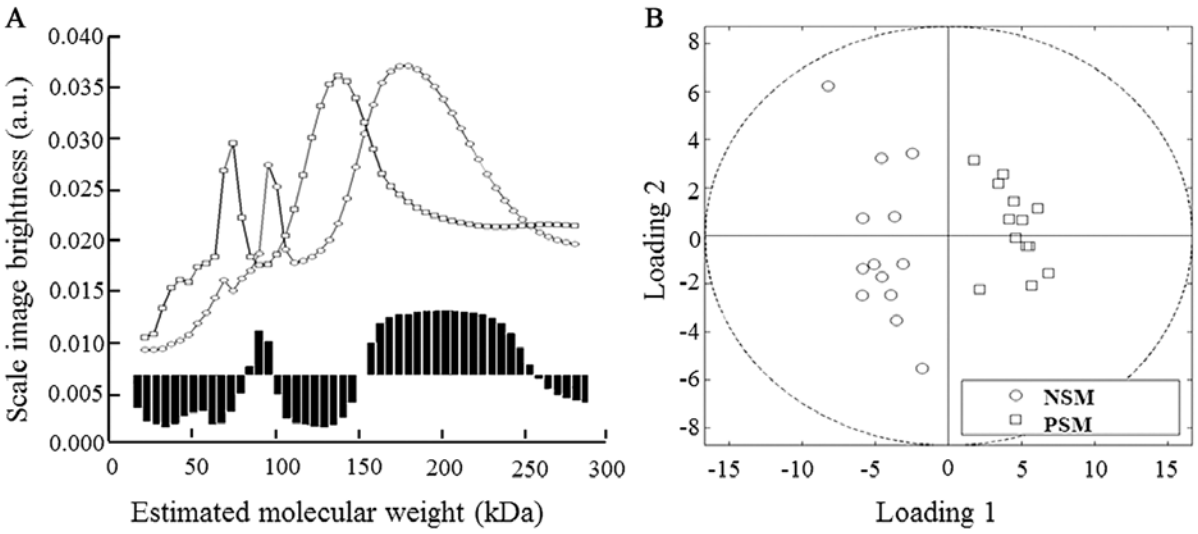

Figure 2. Statistical processing of SDS-PAGE of urinary samples expressed as PLS-DA results, where circle indicates subjects with NSM and square the subjects with PSM. Insert, the loading plot presentation of differences between curves of NSM and PSM patients. The highest bars (80-99 and 155-250 kDa) indicate the most effective parts, reflected in latent variable, showing the most significant differences in protein composition. (B) The quadrant expression of comparison of SDS-PAGE, obtained from NSM subjects (circle) and PSM subjects (square). One point in the graph represents the projection of the whole curve.

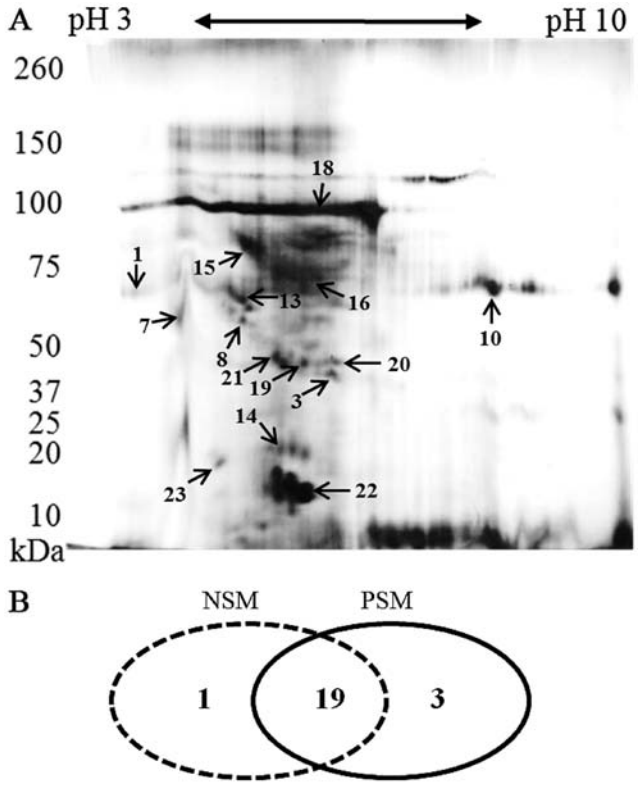

Figure 3. (A) Proteome map (subject 19 from the PSM group), obtained via 2-D electrophoresis. The protein separation was based on their differential pH value for the isoelectric point (pI; x-axis) and molecular weights (y-axis). The protein spots were excised and underwent in-gel tryptic digestion followed by MALDI-TOF MS. The number labeling in the figure corresponds to the numbers of proteins listed in Table III. (B) Diagram showing number of overlaping and unique proteins obtained from analyses of urinary samples of PSM and NSM cohorts.

mass decreased the separation yields and thus better separation method was required for further analyses of proteins of interest.

2-D electrophoresis and PMF. Because two-dimensional gel electrophoresis employs both isoelectric focusing of target molecule, its M_r and it is unlikely that two molecules will be similar in two distinct properties, higher separation resolution is provided (23). We analyzed urinary protein profiles of patients with NSM (n=15) and PSM (n=15) by using acetone precipitation for proteins isolation with subsequent 2-D electrophoresis and MALDI-TOF identification.

Following 2D-PAGE and staining, the most prominent spots were picked up for the MS analysis. Their positions in the 2-D map of representative subject belonging to PSM group are shown in Fig. 3A. Some proteins were presented as a horizontal row of multiple spots (with small changes in pI and molecular weight), likely caused by variable posttranslational modifications (24). The identified proteins and their characteristics are shown in Table III. It was revealed that majority of proteins (19 overlaping proteins) can be identified in both groups. In both, PSM and NSM groups, unique proteins were found (n=1 in NSM; n=3 in PSM) as depicted in Fig. 3B.

The identified proteins were further classified using the data from the UniProt Knowledgebase. As shown in Fig. 4A, belonging to PSM group, regarding to molecular functions, most of identified proteins (46%) have binding function, which was followed by catalytic activity, transport and organization of cellular components. Proteins (21%) were involved in response to stimuli, followed by cellular and metabolical processes (18%). One third of identified proteins are enzymes (35%) and most of proteins had extracellular region location (41%). The majority of extracellular proteins is related to close contact of urine with glands in the male urinary tract and fact that significant fraction of urinary proteins is derived from plasma (25). In NSM group (Fig. 4B), lower portion of binding proteins (37%) was observed with increase of transport proteins (21%) and proteins responsible for organization of cellular components (24%). Further, in NSM group, decreased number of enzymes was identified and in four

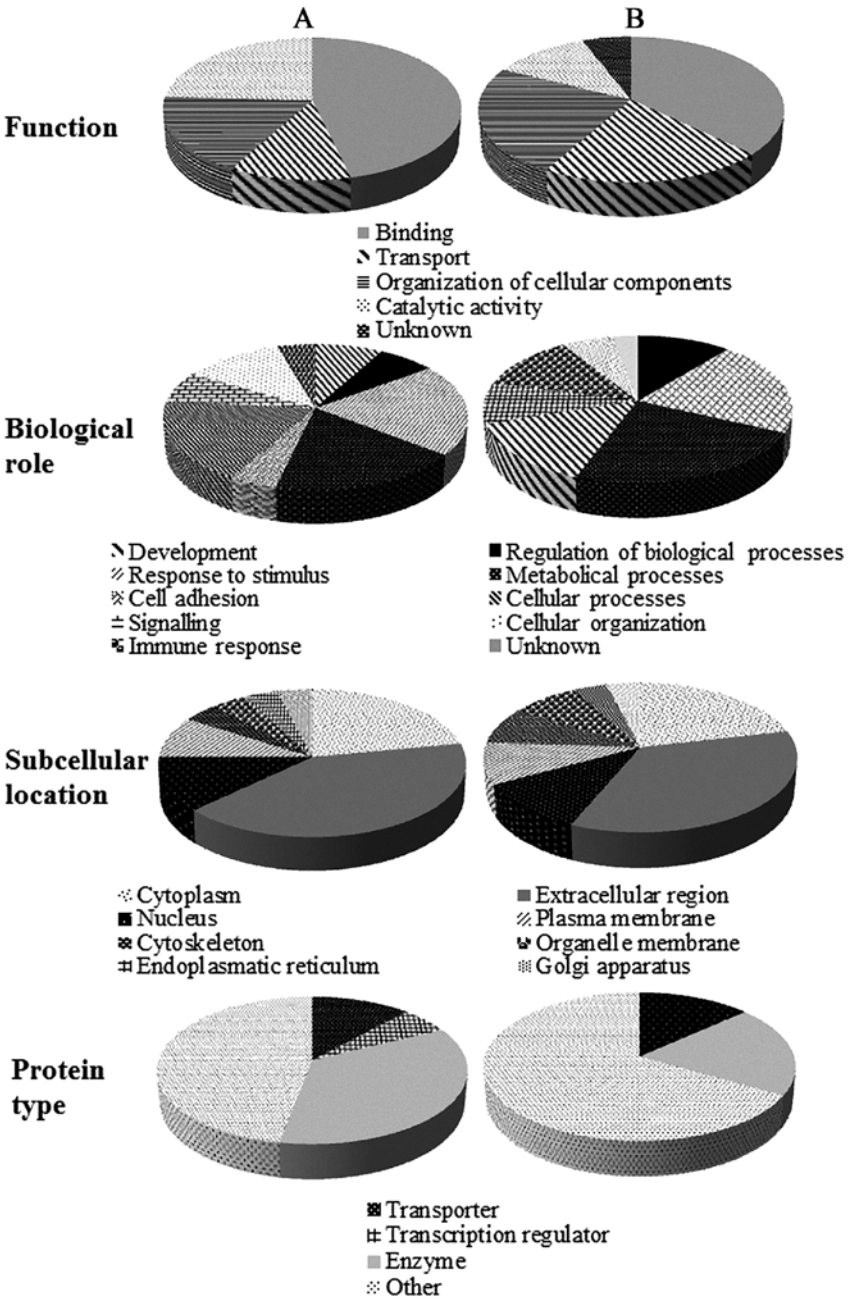

Figure 4. Diagrams showing classification of the identified proteins in urine of (A) PSM and (B) NSM cohorts. The molecular function, biological processes in which they are involved, subcellular location, and type of protein were evaluated by using UniProt database. All the proteins identified in each group were employed in the evaluation.

subjects, elevated expression of testis-expressed sequence 33 protein (TEX33_HUMAN), protein with unknown function and biological role, was determined. In NSM cohort, higher abundance of E-cadherin, calcium 2^{+}-dependent cell-adhesion protein, maintaining homeostasis (26), was determined. Several reports show that E-cadherin is tumor-invasive suppressor and decreased cadherin expression has been associated with more advanced tumor stage, grade and poor prognosis in PCa (27-29). Shimamura and coworkers demonstrated that patients suffering from pancreatic adenocarcinoma with downregulated E-cadherin had a tendency to have PSM (30). α fetoprotein, potential tumor biomarker (31), was identified (27%), in lower abundance in NSM group, which points at possible higher relapse potential of PSM subjects. Another interesting finding was the disparity in urinary prostatic acid phosphatase (Table III). In 1936 Gutman and coworkers observed that serum activity of this enzyme is significantly higher in PCa patients, especially suffering from osteoplastic metastases (32), and it was extensively used for PCa diagnosis prior to introduction of PSA (33). Our results demonstrate that PSM cohort comprises more subjects, whose urinary specimens contain detectable levels (73%) of prostatic acid phosphatase when compared to NSM group (37%). In NSM cohort, also higher abundance of plasminogen (angiostatin), potent inhibitor of angiogenesis (34), was determined.

From all identified proteins, 3 unique proteins have been determined in PSM group, whose expression in NSM group is downregulated to undetectable levels. All of them (cyclin-

Table III. Expression of basic characteristics and abundance of proteins (%), identified in both groups (NSM, n=15, PSM, n=15) after in-gel tryptic digestion and MALDI-TOF identification.

No.	Protein	UniProt accession entry	Predicted mass (M_r)	pI	Approx. MASCOT score	NSM (%)	PSM (%)
1	α-fetoprotein	FETA_HUMAN	70.1	4.57	402	27	53
2	AT-rich interactive domain-containing protein 1A	ARI1A_HUMAN	242.0	6.08	367	67	27
3	Cyclin-dependent kinase 6	CDK6_HUMAN	36.9	6.02	358	-	40
4	E-cadherin	CADH1_HUMAN	99.1	4.50	211	87	34
5	Fascin	FSCN1_HUMAN	54.5	5.50	663	73	53
6	Fatty acid-binding protein, intestinal	FABPI_HUMAN	15.2	5.30	258	27	40
7	Galectin-3-binding protein	LG3BP_HUMAN	65.3	4.90	559	-	53
8	Keratin, type I cytoskeletal 10	K1C10_HUMAN	58.8	5.10	398	67	87
9	Keratin, type I cytoskeletal 9	K1C9_HUMAN	62.0	4.90	157	94	53
10	Keratin, type II cytoskeletal 1	K2C1_HUMAN	66.0	8.10	304	73	67
11	L-lactate dehydrogenase C chain	LDHC_HUMAN	36.2	7.08	456	-	27
12	Plasminogen	PLMN_HUMAN	93.2	6.20	198	67	40
13	Protein disulfide-isomerase A4	PDIA4_HUMAN	72.9	5.16	264	60	73
14	Retinol-binding protein 1	RET4_HUMAN	20.5	5.76	441	73	67
15	Transferrin	TRFE_HUMAN	79.3	5.20	280	87	94
16	Serum albumin	ALBU_HUMAN	69.3	4.70	360	100	100
17	Testis-expressed sequence 33 protein	TEX33_HUMAN	30.7	5.65	256	27	-
18	Uromodulin	UROM_HUMAN	95.0	5.05	762	100	94
19	Acid phosphatase, prostate	PPAP_HUMAN	44.5	5.89	256	37	73
20	Guanine nucleotide binding protein	GBLP_HUMAN	40.4	5.69	401	53	67
21	Serine proteinase inhibitor, clade A	A0A024R6N9_HUMAN	46.6	5.42	301	34	53
22	CD59 glycoprotein	CD59_HUMAN	14.2	6.02	299	53	87
23	Lithostathine-1-α	REG1A_HUMAN	18.7	5.00	178	87	73

The identified proteins including the UniProt identification number, predicted pI and the molecular weight (kDa) are summarized. All of the proteins showed on Fig. 3 are in the expected range of pI and molecular weight.

Table IV. Characterization of functions of the 3 unique proteins found only in the PSM group.

Protein	Gene	Subcellular location	Biological function	Described linkage to PCa	(Ref.)
Cyclin-dependent kinase 6	*CDK6*	Nucleus, cytoplasm	Control of cell cycle and differentiation, promotes G_1/S transition	Yes	(36)
Galectin-3-binding protein	*LGALS3BP*	Extracellular region	Promotes integrin-mediated cell adhesion, stimulate host defense against tumor cells	Yes	(37)
L-lactate dehydrogenase C chain	*LDHC*	Cytoplasm	Possible role in sperm motility, conversion of L-lactate and NAD to pyruvate and NADH in anaerobic glycolysis	Yes	(38)

dependent kinase 6, L-lactate dehydrogenase C chain and retinol-binding protein) were determined in more than one case (27-67% abundance), which points to their possible connection with presence of residual tumor tissue. The list of these proteins with a description of their biological role is shown in Table IV.

The identified proteins, unique for PSM cohort were previously described in certain aspects of prostate cancer development. Cyclin-dependent kinase 6 (CDK6) binds to and is activated by cyclin D1 and thereby enhances the transition of cells through the G_1 phase (35). The regulation is performed through regulation of the phosphorylation state of retinoblastoma protein (pRb). When hyperphosphorylation of pRb occurs, it leads to release of transcription factors, which enhance progression of the cell cycle (36). Since the dysregulation of the cell cycle is one of the defined hallmarks of cancer (37), Palbociclib, oral inhibitor of CDK4/6, was developed to manage ER^+ and HER2 amplified breast tumors (38). In prostate cancer only slight evidence exists pointing to a role of CDK6. Lim *et al* (35) demonstrated that CDK6 can easily bind to the androgen receptors (AR), which play a pivotal role in prostate cancer (39), and it was revealed that CDK6/AR binding stimulates transcriptional activity in presence of dihydrotestosterone (35). Moreover, they have shown that androgen-sensitive LNCaP PCa cells, engineered to stably overexpress CDK6 display increased elevated PSA expression and enhanced growth attributes. The same group indicates that CDK6 is overexpressed in 44% of PCa; hence presence of this protein in urine could be clinically interesting for evaluation of post-prostatectomy status.

Another uniquely identified protein, connected with PSM status was galectin-3-binding protein (also named Mac-2BP or tumor-associated antigen 90K) that is a highly glycosylated secreted protein, capable of inducing the expression of number of cytokines (IL-1, IF-2 and IL-6) (40). Previous studies have indicated that galectin-3-binding protein promotes tumor metastasis and that the tumor promotion mechanism in metastasis is associated with galectins (41). Furthermore, it was revealed that galectin-3-binding proteins enhance tumor cell adhesion, which may aid tumor cells to avoid apoptosis and is thus highly expressed in PCa samples (42). Although the potential of serum galectin-3-binding protein was discussed in several reports (40,42,43), to our knowledge, this is the first report, showing its possible diagnostic utilization in analysis of urinary specimens.

L-lactate dehydrogenase C chain or the cancer/testis antigen 32 is typically expressed in normal male germ cells but are silent in normal somatic cells. Nevertheless, they are aberrantly expressed in several types of cancer, including PCa (44). Several studies shown that the expression of L-lactate dehydrogenase C chain is frequently associated with higher grade lesions and advanced disease with a poorer outcome (45,46), which is consistent with the general fact that PSM is more often linked with higher Gleason score and higher pathologic stage (47) (higher TNM and GS of PSM subjects are shown in Table I).

In conclusion, our examination of urinary proteomes of human PCa specimens identified differences between groups of patients with positive and negative surgical margins after radical prostatectomy. The significance of these findings in the context of presence of PSM is represented by molecular changes leading to alterations in biochemical pathways, hopefully related to the presence of residual tumor after surgical treatment failure. Further evaluation of proteomes of PSM/NSM patients and observation of their biochemical and clinical recurrence will be needed to address a potential significance of urinary proteins in this phenomenon. Ultimately, development of combination of genomic-proteomic approaches for monitoring of biological processes will be fundamental in further endeavors to understand the PCa and PCa-related statuses in detail.

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