HUMAN SERUM LOW MOLECULAR MASS PROSTATE-SPECIFIC ANTIGEN AS BIOMARKER

SPECIFIČAN ANTIGEN PROSTATE MALE MOLEKULSKE MASE KAO BIOMARKER U SERUMU ČOVEKA

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Summary

Background: Prostate-specific antigen (PSA) is a glycoprotein tumor marker known to exist as numerous glycospecies. Investigations on its glycobiochemical properties aimed at their use in the preparation of adjuncts in determining PSA concentration for clinical purposes have accumulated a lot of data on its structural properties. In this study, we reconsidered unexplored ubiquitously present low molecular mass species of PSA regarding to molecular mass, origin and pathophysiological source specificity in order to evaluate them as biomarkers.
Methods: Data on low molecular mass PSA-immunoreactive species from sera of subjects with prostate cancer (PCa), benign prostatic hyperplasia (BPH), breast cancer (BCa), and urine of healthy males obtained by on-chip immunoaffinity chromatography combined with mass spectrometry were analyzed.
Results: The results obtained indicated PSA species common to BCa, PCa, and BPH at 12–13 kDa, 17–19 kDa and 21–24 kDa. The striking difference in predominant frequencies made the profile characteristic in each examined pathophysiological condition. On the other hand, paired groups of prostatic and extraprostatic PSA contained rare species with small differences among groups concerning individual species. Low molecular mass PSA also included rare species unique for each group of samples.
Conclusions: The results obtained revealed that uniformity of low molecular mass PSA-immunoreactive species in sera prevails over diversity related to cancer and non-cancer conditions, but at the same time some of them are molecules with biomarker potential for BPH detection.

Keywords: benign prostatic hyperplasia; breast cancer; low molecular mass species; prostate cancer; prostate-specific antigen

Kratak sadržaj

Uvod: Specifičan antigen prostate (PSA) je glikoproteinski tumorski marker koji postoji u obliku brojnih molekulskih vrsta. Podaci o njihovim glikobiohemijskim osobinama su dobijeni u studijama vršenim u cilju ispitivanja mogućnosti njihovog korišćenja kao dodatnog kliničkog parametra. U ovom radu je analiziran biomarkerski potencijal PSA vrsta koje imaju malu molekulsku masu a koje, do sada, bez obzira na njihovo univerzalno prisustvo, nisu ispitivane u smislu specifične povezanosti sa različitim patofiziološkim stanjima.
Metode: Analizirani su podaci o PSA-imunoreaktivnim vrstama male molekulske mase u serumima osoba sa kancerom prostate (PCa), benignom hiperplazijom prostate (BPH), kancerom dojke (BCa) i urinima zdravih muških osoba koji su dobijeni imunoafinitetnom hromatografijom na čipu u kombinaciji sa masenom spektrometrijom.
Rezultati: Dobijeni rezultati su ukazali na predominantne PSA vrste male molekulske mase u regionima 12–13 kDa, 17–19 kDa i 21–24 kDa koje su bile zajedničke za sve ispitivane grupe uzoraka. U zavisnosti od patofiziološkog stanja, njihova zastupljenost se, međutim, izrazito razlikovala između datih regiona. Pored ovih, detektovane su i retke molekulske vrste zajedničke za prostatični i ekstraprostatični PSA, kao i retke vrste karakteristične za svaku od ispitivanih grupa uzoraka.
Zaključak: Uprkos tome što sličnost PSA vrsta male molekulske mase preovladava nad raznovrsnošću vezanom za maligno ili benigno fiziološko stanje, postoje one koje imaju biomarkerski potencijal za detekciju BHP.

Ključne reči: benigna hiperplazija prostate; kancer dojke; molekulske vrste male mase; kancer prostate; specifičan antigen prostate

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Introduction

As a heterogeneous disease, blood-based diagnostics of prostate cancer (PCa) is very demanding and, in spite of many efforts, finding suitable biomarkers is still a challenging task (1, 2). In the 1980s introduction of prostate-specific antigen (PSA), an enzyme produced by prostate epithelial cells, as an organ-specific marker had a favorable impact on monitoring the progression or recurrence of PCa (3). However, due to lack of specificity and sensitivity, PSA did not meet the greatest clinical screening need, *i.e.* early detection and differentiation between benign and malignant prostatic pathologies. Thus, although PSA is the most commonly used marker for cancer, the cost to benefit ratio is questionable and an issue of disparate opinions. To improve the diagnostic potential of the PSA test, several other analyses have been introduced, such as determination of PSA density, PSA velocity and doubling time, as well as measurement of free and complexed PSA forms (3–5). PSA is known to exist as numerous molecular species differing in activity and molecular mass, depending on both peptide and oligosaccharide moieties and these changes under pathophysiological conditions (6–9). They result from diverse routes of PSA processing, alternative splicing and glycosylation, reflecting normal prostate biology (10, 11). Investigations of the glycobiochemical properties of PSA aimed at their use in the preparation of adjuncts to tests for PSA concentration have accumulated a large number of data (9, 12–14). Different PSA sources, such as seminal plasma, serum, urine and prostate tissue, have been examined using classical as well as advanced separation and enrichment methodologies (6, 8,15–17). Proteomic and glycomic approaches that open new possibilities in the field of biomarkers in general, provide an opportunity to take advantage of the structural heterogeneity of the PSA molecule in the search for better diagnostic tools for PCa (13, 18–20). Signatures of molecular species of PSA that might suggest a cancerous or non-cancerous origin await full clinical exploration and depend on the development of specific capturing reagents, like specific antibodies, and the advancement of technological platforms (21–23). The lack of an ideal tumor marker and the necessity to combine different markers rather than using a single one, provide room for applying PSA molecular species in their full context.

This study deals with ubiquitously present low molecular mass PSA species, *i.e.* those below 28.4 kDa. This is the mass of mature full length glycosylated PSA as the established standard (24). Regardless of the methods and sources used, such species have been observed in a broad molecular mass range down to 3 kDa, but their origin is not explained (6, 20, 25). Some of them may result from degradation in the circulation or enter it as fragments originating from a tumor environment, suggesting the involvement of different proteases in their formation. Thus,

low molecular mass free PSA-immunoreactive species were also observed using on-chip immunoaffinity profiling of human sera from subjects with PCa, benign prostatic hyperplasia (BPH) and breast cancer (BCa), but were not thoroughly considered (20, 26–28).

In this study, unexplored ubiquitously present low molecular mass free PSA-immunoreactive species were reconsidered in order to compound intrinsically heterogeneous PSA molecules regarding their molecular mass, origin and pathophysiological source specificity. Human serum low molecular mass free PSA-immunoreactive species from subjects with PCa, BPH and BCa were revisited and existing data complemented with urine profiles from healthy subjects, as a source of low molecular mass PSA species generated under normal physiological conditions.

Materials and Methods

Chemicals and reagents

Monoclonal anti-free PSA antibody, isotype IgG2a, clone 8A6 (specific for conformational epitope I), was purchased from Hy Test (Turku, Finland). ProteinChip PS20 (preactivated surface), sinapinic acid and ProteinChip all-in-one protein standards II were from BioRad (Hercules, CA, USA).

Concentrations of total and free PSA were determined using appropriate commercially available immunoradiometric assays (Institute for the Application of Nuclear Energy, Serbia). The PSA assays were calibrated against standards from the National Institute for Biological Standards and Control (Hertfordshire, United Kingdom): total PSA:PSA (90:10) first International standard NIBSC Code 96/670 and PSA (free) first International standard NIBSC Code 96/668. All other chemicals were reagent grade.

Biological samples

Leftover serum samples from de-identified subjects seen at the Institute for the Application of Nuclear Energy, Zemun, for determination of tumor markers as part of follow-up were examined. Using of existing human specimens that is not considered as human subjects' research was approved by the local Ethical committee.

Samples of PCa sera were from patients diagnosed with locally advanced and advanced cancers, and samples of BCa sera had cancer antigen 15-3 (BCa tumor marker) concentrations from 40 IU/L to 669 IU/L. Serum PSA concentrations ranged from 0.4–6.8 µg/L for BPH samples (n = 12), 28.1–228.4 µg/L for PCa samples (n = 12), and 0.03–0.50 µg/L for BCa samples (n = 10). Sera were used immediately or stored in aliquots at –20 °C until processed.

Urines (n = 7) with normal biochemical parameters were obtained from male volunteers (laboratory personal). Samples were centrifuged at 900 × g for 20 min, and used immediately or stored at –20 °C until processed.

Chemical release of prostate-specific antigen (PSA) from the complex with α_1-antichymotrypsin (ACT)

The PSA-ACT complex was cleaved according to Peter et al. (29). Ethanolamine solution (0.2 mol/L; pH 10.3; 100 µL) was added to 100 µL of serum followed by incubation at 25 °C for 24 h. The samples were then neutralized with 0.5 mol/L HCl and analyzed without further treatment by on-chip mass spectrometry.

PSA profiling using on-chip mass spectrometry

Samples were analyzed by on-chip mass spectrometry as previously described (20). Anti-fPSA monoclonal antibody (5 µL) was immobilized on each spot of the preactivated-surface protein chip array (8 spots) by chemical adsorption overnight at 4 °C. Spots were washed thrice with 5 µL of 0.05 mol/L phosphate buffered saline (pH 7.2) for 1 min at room temperature (RT), and subsequently blocked with 0.5 mol/L Tris-HCl buffer (pH 8.0), for 1 h in a humid chamber at RT. The same washing procedure was repeated followed by addition of the test sample (5 µL of serum or urine) to each spot. Incubation proceeded for 2 h in a humid chamber at RT, and the spots were rinsed again thrice with 0.05 mol/L phosphate buffered saline (pH 7.2) and twice with deionized water. After drying, 1 µL of 50% sinapinic acid (in acetonitrile/dH$_2$O/trifluoroacetic acid [50%/49.9%/0.1%]) was added to each spot, dried and then reapplied. All procedures (antibody coupling, washing steps, blocking and sample binding) included shaking (150 rpm).

Non-specific binding was checked using 0.05 mol/L phosphate buffered saline (pH 7.2) instead of a serum sample. Peaks detected on the control chip with immobilized anti-fPSA antibody were not considered.

All spectra were obtained by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry on a ProteinChip reader, series 4000, personal edition (BioRad), using Ciphergen Express software 3.0 (BioRad, Hercules, CA, USA). The working parameters were: 25 kV positive ion acquisition mode, mass range 2.5–50 kDa, laser energy 6000 nJ with 8 815 laser shots per spot. Mass calibration was done using ProteinChip all-in-one protein standards II.

Results

A comparative survey of fPSA-immunoreactive species from previously characterized sera of males and females and from the newly profiled male urines of healthy subjects is shown in *Table I*. These were separated by on-chip immunoaffinity capture combined with mass spectrometry detection (*Figure 1A, B*).

Molecular species at 28 kDa (peak frequency 92–100%) corresponding to full length glycosylated PSA were present in all examined sera samples. The numerous low molecular mass PSA detected at 12–28 kDa exhibited striking differences concerning occurrence and frequency of individual species (peak frequency 17–92%). The pattern of urinary fPSA-immunoreactive species differed noticeably from

Table I A comparative survey of fPSA-immunoreactive species in serum and urine.

Region	BCa serum[1]		PCa serum[1,2]		BPH serum[1,2]		Urine	
	Species number	Peak frequency (%)	Species number	Peak frequency (%)	Species number	Peak frequency (%)	Species number	Peak frequency (%)
12–13 kDa	3	20–60	3	33–42	2	50–58	0	0
13–17 kDa	5	20–90	5	17–42	6	17–42	3	43–86
17–19 kDa	3	20–40	6	17–92	5	17–75	3	43
19–21 kDa	1	60	1	25	2	17–42	1	71
21–24 kDa	5	20–70	2	33	4	17–50	1	29
24–28 kDa	2	30–70	5	25–58	5	25–67	2	71
28–30 kDa	6	30–100	3	100	3	92–100	0	0

BCa – breast cancer; PCa – prostate cancer; BPH – benign prostatic hyperplasia
[1] Ref. (27)
[2] Ref. (20)
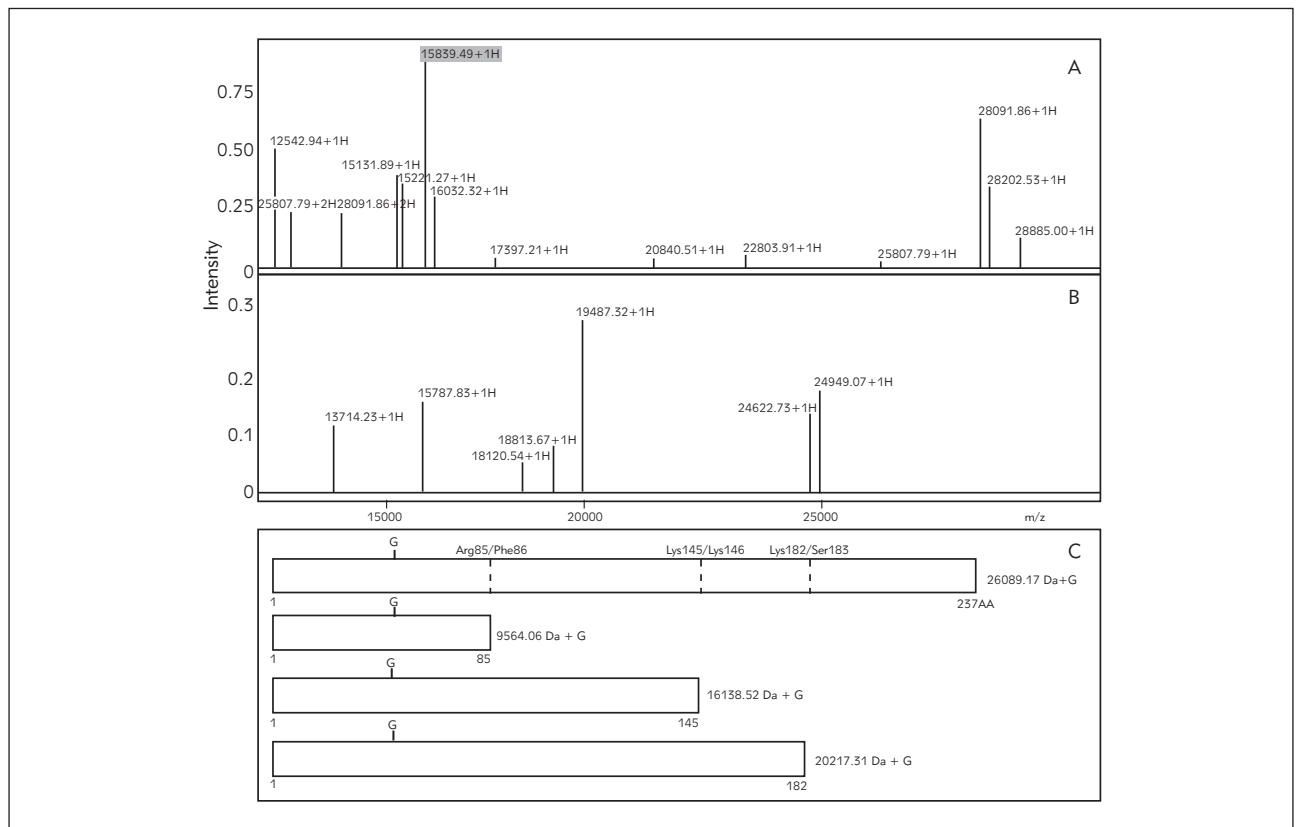
Figure 1 Representative spectra of PSA-immunoreactive species separated by on-chip immunoaffinity capture

A) PSA-immunoreactive species in PCa-serum subjected to PSA-ACT cleavage
Highlighted peaks represent PSA species enriched after chemical cleavage of the PSA-ACT complex (autoproteolytic fragments) in comparison to untreated PCa-serum.

B) PSA-immunoreactive species in healthy male urine
m/z – mass-to-charge ratio

C) Schematic diagram of PSA molecule
Arg85/Phe86, Lys145/Lys146 and Lys182/Ser183 represent position of internal cleavage sites; AA – amino acid sequence; G – glycan (estimated mass of complete biantennary N-glycan, 2352.16 Da, Ref.(24)

Table II Low molecular mass fPSA-immunoreactive species common to BCa-, PCa- and BPH-sera.

BCa		PCa		BPH	
Mean±SD m/z	Peak frequency (%)	Mean±SD m/z	Peak frequency (%)	Mean±SD m/z	Peak frequency (%)
12667.64±6.38	60	12703.16±3.09	33	12698.87±3.40	50
13536.04±5.35	90	13587.15±28.40	42	13578.59±37.04	25
17258.05±0.63	20	**17235.27±6.23**	**92**	17247.63±11.21	66
17397.76±36.77	40	**17387.61±6.86**	**92**	**17377.09±12.72**	**75**
20047.23±8.00	60	20020.90±9.92	25	19879.00±13.68	42
20971.82±135.27	70	20977.66±71.32	33	20969.63±48.39	25
23920.09±342.49	30	24031.44±80.94	25	24042.98±45.79	25
25845.34±40.85	70	25792.94±17.91	58	**25782.46±26.18**	**67**

BCa – breast cancer ; PCa – prostate cancer; BPH – benign prostatic hyperplasia
m/z – mass-to-charge ratio; SD – standard deviation
PSA species with the highest peak frequency are bolded

Table III Low molecular mass fPSA-immunoreactive species common to BCa/PCa-, PCa/BPH- and BCa/BPH-sera.

Mean±SD m/z	Peak frequency (%)	Mean±SD m/z	Peak frequency (%)
BCa		PCa	
12561.26±3.61	30	12574.68±7.13	33
15844.54±20.21*,#	30	15838.68±11.25*,#	42
17824.65±4.16	30	17889.24±7.83	42
23221.06±52.76	20	23229.81±76.06	33
PCa		BPH	
15448.08±3.27	17	15443.83±2.21	17
17145.83±6.23	42	17163.93±39.98	33
24534.99±93.42*	42	24620.61±31.77*	25
25000.42±37.52*	33	25054.01±53.35*	33
BCa		BPH	
16329.53±48.63	30	16373.08±15.82	33
23098.17±9.50	60	23137.69±49.73	33

BCa – breast cancer; PCa – prostate cancer; BPH – benign prostatic hyperplasia
m/z – mass-to-charge ratio; SD – standard deviation
*PSA species present in urine
#PSA species enriched after cleavage of the PSA-ACT complex (autoproteolytic fragments)

Table IV Low molecular mass fPSA-immunoreactive species unique to BCa-, PCa- and BPH-sera.

BCa		PCa		BPH	
Mean±SD m/z	Peak frequency (%)	Mean±SD m/z	Peak frequency (%)	Mean±SD	Peak frequency
12086.70±6.84	20	17987.83±96.15	17	14391.48±90.43	17
16579.04±1.30	20	13862.16±9.65	25	15370.10±7.57	17
23306.57±3.24	40	27550.19±56.79	25	20515.98±5.89	17
16759.61±23.65	**50**	18616.28±21.14	33	21994.77±12.94	17
22943.83±15.08	**60**	12879.39±14.13	42	17569.31±15.90	17
		15132.85±29.92#	42	16210.89±4.37	42
				22431.18±6.64	**50**
				12269.70±12.92	**58**
				18126.24±8.14	**67**
				26648.83±11.31	**67**

BCa – breast cancer; PCa – prostate cancer; BPH – benign prostatic hyperplasia
m/z – mass-to-charge ratio; SD – standard deviation
#PSA species enriched after cleavage of the PSA-ACT complex (autoproteolytic fragments)
PSA species with peak frequency ≥ 50% are bolded

those observed in serum samples in respect to distinct species at specified molecular mass ranges but not to the mass ranges themselves. In urine, which is supposed to contain enriched auto- and proteolytic degradation fragments of PSA, mature species were minor in comparison to serum samples. Thus, the most frequent species were observed at 15 kDa (peak frequency 86%) and 19 kDa–24.9 kDa (peak frequency 71%).

Some of the low molecular mass species detected at 12–13 kDa, 17–19 kDa and 21–24 kDa may be tentatively annotated as distinct fragments of full length glycosylated PSA with internal cleavage sites, due to breakage of disulphide bonds holding the PSA polypeptide together (*Figure 1C*). Under the experimental conditions used, they were predominantly found in serum, whereas other low molecular mass species that could not be exactly matched to any nick position in PSA were present in both serum and urine. However, regardless of this, low molecular mass fPSA-immunoreactive species could be grouped as those common to BCa, PCa and BPH (*Table II*), those common to BCa/ PCa, PCa/BPH, BCa/BPH (*Table III*) and as species unique to BCa or PCa or BPH (*Table IV*).

Thus, the main clusters at 12–14 kDa, 17–20 kDa and 21–26 kDa comprised molecular species found in BCa-, PCa- and BPH-samples, but at striking diversity in frequency within particular clusters (*Table II*). Among them, non-overlapping species at 12–14 kDa were most frequent in BCa and at 17–20 kDa or/and at 21–26 kDa in PCa and BPH, respectively.

PSA species common to paired groups of prostatic and extraprostatic PSA are shown in *Table III*. BCa/PCa groups exhibited similar patterns of rare species (peak frequency less than 50%), across the observed range of molecular masses. Among them, the species at 15.8 kDa may be a product of autoproteolytic activity of PSA, as it is one of those that characteristically appear after cleavage of the PSA-ACT complex, i.e. releasing an enzymatically active PSA molecule. It was, also, the most common PSA species in urine (peak frequency 86%). In the PCa/BPH groups, patterns of rare species (peak frequency less than 50%) were similar. Specifically, species at 24 kDa –25 kDa might also be auto/proteolytic fragments, since they were often detected in urine (peak frequency 71%).

The BCa/BPH groups shared species at 23 kDa, commonly observed in BCa (peak frequency 60%) and species at 16 kDa found at similar low frequencies in both BCa and BPH.

PSA species unique for each group of samples are shown in *Table IV*. BCa-samples were characterized by two clusters, one at 12.0 kDa, 16.5 kDa and 23.3 kDa (peak frequency less than 50%), and the other comprising 16.7 kDa and 22.9 kDa species (peak frequency equal or higher than 50%).

PCa-samples contain one cluster comprising 12.8 kDa, 13.8 kDa, 15.1 kDa, 17.9 kDa, 18.6 kDa and 27.5 kDa species (peak frequency less than 50%). PSA species at 15.1 kDa could be considered as an autoproteolytic PSA fragment, since it was associated with the species at 15.8 kDa, both being enriched after cleavage of serum PSA-ACT complex.

In BPH-samples, two clusters were observed, the first comprising 14.3 kDa, 15.3 kDa, 16.2 kDa, 17.5 kDa, 20.5 kDa and 21.9 kDa species (peak frequency less than 50%), while the second contained 12.2 kDa, 18.1 kDa, 22.4 kDa and 26.6 kDa species (peak frequency equal or higher than 50%).

Discussion

Driven by accumulated evidence for the existence of low molecular mass PSA and the absence of their definition in any principled way, our new look at them revealed putative relational rules in respect to distinct physiological conditions. Method-induced PSA fragmentation which could occur at nicks due to breakage of disulphide bonds during a matrix-assisted ionization event (30), or at proteolysis-sensitive sites in the polypeptide moiety, *in vivo* (11), were observed as clusters of free PSA-immunoreactive molecular species or individual peaks at specified molecular masses. PSA species were numerous in all examined groups, and detected in more or less the same number in each of the specified molecular mass ranges, but most frequently at 13–17 kDa and 24–28 kDa in BCa, 17–19 kDa in PCa and at 17–19 kDa and 24–28 kDa in BPH. In general, changes in the occurrence of nicked PSA molecules in a particular pathological condition might be due to differences in basic routes of PSA synthesis. On the other hand, changes in the profile of degraded PSA species might be due to differences in the presence and activity of distinct proteolytic enzymes in serum, urine or tissue, altered stability of PSA due to changes in glycosylation or autoproteolytic activity (31). Patterns of low molecular mass PSA species included those common to all examined groups as well as to BCa and PCa only, PCa and BPH only and BCa and BPH only, indicating prevalence of uniformity over diversity. When considering PSA species common to all groups examined, differences in their frequency made some predominant, and in this way characteristic for each of them. These findings confirmed previous data indicating a relation of various pathophysiological conditions of the prostate to changes in the ratio of PSA species containing distinct structural/glycan moieties, and consequently existing as various species rather than to the occurrence or lack of unique particular species themselves (6, 12, 25, 32, 33). Some of the remaining low molecular mass PSA species could be grouped as those found in BCa and PCa only (4 species), in PCa and BPH only (4 species), and in BCa and BPH only (2 species). They were rare and did not differ much between groups in respect to a single species, as was the case for those common to all examined groups of samples. In particular, species common to PCa and BPH showed no marked diversity in mutual peak frequencies, whereas species common to BCa and BPH were more frequent in BCa.

In the BCa/PCa and PCa/BPH groups, distinct auto/proteolytic species were present. Their origin was deduced from the pattern of enzymatically active PSA released from its complex with the protease-inhibitor ACT and pattern of PSA in urine as source of low molecular mass species generated under normal physiological conditions (29, 34). Taken together, this suggests autoproteolytic fragments common to cancer samples, *i.e.* both BCa and PCa, different from auto/proteolytic fragments common to prostatic PSA from any source (serum and urine) or physiological condition examined (PCa, BPH, healthy subjects).

The results obtained for paired groups could not be unambiguously related to available data about free PSA in the examined physiological conditions, which are mainly related to its serum concentrations. Thus, it has been shown that free PSA is the predominant form in BCa-sera (35), whereas the ratio of total PSA/free PSA in the gray concentration zone of 4–10 ng/mL is lower in PCa than in BPH (36). In this study we evaluated samples with high PSA concentrations from subjects with locally advanced and advanced PCa and samples with significantly lower concentrations from subjects with BCa (having elevated CA15-3 as a marker of cancer progression) and BPH as a non-cancer condition. In spite of this, the patterns obtained revealed certain community/uniformity in relation to a cancer origin of PSA species, which could support existing data on similarity/homology of prostate and breast cancer (37). Moreover, frequent molecular species associated with prostatic PSA but not with extraprostatic PSA, *i.e.* found only in male sera also emerged (27).

Besides common species, there were also unique low molecular mass PSA species observed only in one group of samples. Their existence might be of clinical relevance in the case of BPH and PCa, since PSA concentrations alone cannot discriminate between these conditions. Much effort has already been made to reach this endpoint using various approaches but with no definitive success. The results obtained indicate that the frequency of PSA species unique for PCa is low, and, therefore, less useful for discrimination between these pathological conditions than common high but differently frequent species.

Basically, they might occur more often due to altered activity of specific proteases in cancer acting on ubiquitous/common mature PSA species rather than on cancer-specific mature species. On the other hand, unique low molecular mass species were more numerous and some of them very common in BPH, directing efforts to explore them for clinical purposes rather than cancer-specific species. Although this finding refers to low molecular mass PSA, it agrees with data on the existence of BPH-specific PSA, named BPSA, which was identified as full length PSA having two internal cleavage sites (25).

Conclusions

As a posttranslational modification, proteolytic degradation is an important source of diversity in the proteome (38). It can produce many distinct low molecular mass species from a particular higher molecular mass protein. The fragmentation pattern may reflect different pathogenic processes, and protein fragments have already been recognized as diagnostic and prognostic biomarkers in different types of cancer, renal and connective tissue diseases, *etc* (34, 39, 40). Bearing in mind many limitations of this study regarding selection of fPSA-immunoreactive fragments as well as sample heterogeneity in distinct groups, the results obtained add new value to low molecular mass PSA-immunoreactive species in sera. They revealed community/uniformity in the diversity without respect to the pathophysiological condition examined, but at the same time allocated some species as having biomarker potential to differentiate between prostate cancer and benign prostatic hyperplasia.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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