Identification of Precursor Forms of Free Prostate-specific Antigen in Serum of Prostate Cancer Patients by Immunosorption and Mass Spectrometry

Jochen Peter,1 Carlo Unverzagt,2 Thomas N. Krogh, Ole Vorm, and Wolfgang Hoesel3


ABSTRACT

The structural features of the free prostate-specific antigen (F-PSA) present in human blood have not been clarified up to now, and it is, therefore, not known why F-PSA is not complexed by the protease inhibitors that are present in human blood in large amounts. This lack of information is mainly attributable to the low amount of F-PSA in serum, which makes the isolation and structural characterization very difficult, especially when only limited amounts of individual sera are available. It has now been demonstrated that F-PSA occurs as a mixture of different pro-PSA forms (zymogen forms) in the sera of prostate cancer patients, and that, in some of these sera, a form with the regular NH2 terminus of PSA is present as well. Among the five serum samples investigated, all contained the (−7), (−5), and (−4) pro-PSA forms, whereas the (−1) and (−2) forms were only present in three of them. These three samples also contained the form with the regular NH2 terminus. The (−3) and (−6) pro-PSA forms have not been detected thus far. The F-PSA has been isolated by immunoabsorption from the individual sera using streptavidin-coated magnetic beads. The pro-PSA forms were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry after producing peptides by endoproteinase from Lysobacter enzymogenes digestion of the SDS-PAGE-separated F-PSA band. The structural identity of the (−7)pro-PSA form was further proven by sequencing of that particular peptide using electrospray ionization quadrupole time-of-flight mass spectrometry.

INTRODUCTION

PSA4 is, because of its sensitivity and organ specificity, a very valuable protein marker in human blood for population screening, diagnosis, and monitoring of patients with PCa (1–5). There are, however, some limitations on the use of PSA in PCa diagnosis. Patients with BPH also show PSA concentrations up to about 15 ng/ml in serum. Therefore, PSA concentrations below 15 ng/ml cannot be used to distinguish between PCa and BPH (3). The determination of the ratio of noncomplexed F-PSA:T-PSA increases the specificity in the range of 4–15 ng/ml T-PSA to some extent, but there is still much need for improvement (3, 6, 7). This could be accomplished by looking for structural differences between the PSA from PCa and BPH patients. If differences were to be found, they could be used to develop better diagnostic means for the differentiation between these two conditions. An interesting target for that purpose poses the structural analysis of F-PSA from serum, although the low concentration of this analyte in human serum (8) turns this approach into a challenging task.

The reason for the presence of F-PSA in human blood is not yet clear, but this F-PSA should be a proteolytically inactive form, otherwise it would be complexed by protease inhibitors (e.g., α1-ACT and α2-macroglobulin), which are present in the blood in large amounts (9–11). Two different explanations have been proposed thus far for this finding: (a) the F-PSA could exist as an inactive form resulting from a nick in the PSA sequence, probably at the lysine at position 145; and (b) alternatively pro-PSA forms (zymogen forms) could be present that still carry all or parts of the signal sequence (12, 13). Experimental evidence for the presence of a nicked form was presented by Noldus et al. (14), who isolated partially purified F-PSA from a 230-ml pool of 59 PCa sera (T-PSA, >2000 ng/ml). Gas phase sequencing provided evidence for the presence of a form with the regular NH2 terminus and a nicked form. There was no indication for the presence of pro-PSA forms, but the F-PSA yield of their preparation was only about 25%, and it still contained impurities. Thus, the authors might have missed some minor PSA forms that could also have been present. On the other hand, Mikolajczyk et al. (15) found evidence for the presence of PSA forms larger than the F-PSA from seminal fluid when they separated the PSA from 75 ml of pooled PCa sera (containing 50–100 ng/ml T-PSA) by a combination of immunosorption and hydrophobic interaction liquid chromatography. The HIC high-performance liquid chromatography showed that about 25% of the F-PSA eluted at a similar position as the (−4) pro-PSA form from cultured prostate cells, but there was no further characterization. The authors, using Western blotting techniques after SDS-PAGE separation, did not find clear evidence for the presence of nicked F-PSA.

Different pro-PSA forms have been produced and clearly identified (mostly by NH2-terminal sequencing) in several cell culture systems after the cloning of the prepro-PSA gene and isolation of the expressed PSA forms. In a Syrian hamster tumor cell line, pro-PSA forms with seven and five amino acids in the precursor part were identified by Kumar et al. (16). Lövgren et al. (17) identified the same forms after cloning and expression of prepro-PSA in BHK cells as well as in a baculovirus system. In the latter system, a form with three amino acids corresponding to the precursor part was identified as well. Additionally a (−7)pro-PSA form was expressed and characterized in Escherichia coli cells (18).

Here we describe the unequivocal identification of different pro-PSA forms in the serum from an individual PCa patient. The F-PSA was isolated by immunosorption, and the intact molecule and its peptides were analyzed by MALDI-TOF MS. Moreover, the relative quantities of the pro-PSA forms and the F-PSA form with the regular NH2 terminus were investigated in four other PCa sera samples.

MATERIALS AND METHODS

Serum A (50 ml) from a patient suffering from PCa (T-PSA, 1890 ng/ml; F-PSA, 180 ng/ml) and reference serum were obtained from Bioclinical Partners Inc, Franklin, MA. The other four PCa sera (S1, S5, S6 and S9, 2 ml each) were from the sera collection of Roche Diagnostics GmbH (Penzberg, Germany). These sera contained between 6900 and 8500 ng/ml of T-PSA and...
between 640 and 960 ng/ml of F-PSA. PSA from seminal fluid was a product of Scripps Laboratories (San Diego, CA). The monoclonal antibodies used were from the protein chemistry department of Roche Laboratory Diagnostics. Streptavidin-coated magnetic beads of 2.5-μm diameter were the same as those used in the Roche Laboratory Diagnostics automatic immunanalyzer ELECSYS.

All of the other products used were either from Roche Molecular Biochemicals or from Merck GmbH (Darmstadt, Germany) if not indicated otherwise. Among these products were PBS, 50 mM potassium phosphate, and 150 mM sodium chloride (pH 7.4).

Isolation of F-PSA by Immunosorption

The immunosorption methods used have recently been described in detail (19). The following protocol was used here: a suspension (2.5 ml) of streptavidin-coated magnetic beads (10.7 mg/ml) was placed in a 10-ml tube and washed with PBS. After the addition of 4 ml of biotinylated anti-F-PSA-M30-IgG (monoclonal antibody from mouse, used in the Roche Diagnostics ELECSYS test for F-PSA; 25 μg/ml in PBS containing 1% BSA and 0.1% Tween 20), the suspension was incubated for 30 min. The beads were collected, washed three times with PBS containing 20 mM N-octylglucoside and incubated with 8 ml of the PCa serum A (containing 180 ng/ml F-PSA) for 1 h to bind the F-PSA to the beads. The beads were collected and washed as described before, followed by a very short washing step with 200 μl of water to remove most of the detergent. The beads were then incubated under shaking with 500 μl of 1 M propionic acid for 1 h. After magnetic separation of the beads, the supernatant was removed, lyophilized in a vacuum concentrator, and stored at −20°C if further analysis was not performed immediately after lyophilization. In case of the other four PCa sera (F-PSA > 600 ng/ml) a volume of 100 μl of the sera was used for immunosorption, and the amounts of reagents were adjusted accordingly. For these sera, biotinylated IgG from the monoclonal antibody M36, which recognizes free as well as complexed PSA and is used in the Roche Diagnostics ELECSYS test for T-PSA was used for the immunosorption, because we wanted to isolate the PSA/ACT complex as well as the F-PSA by separation on SDS-PAGE (see Fig. 4).

SDS-PAGE and endo Lys-C Digestion

SDS-PAGE was performed under nonreducing conditions using the Mini-PROTEAN II gel electrophoresis system and precast 12% ready gels from Bio-Rad, using essentially the protocol as described by Laemmli (20). The gels were silver stained and the F-PSA band was digested with endo Lys-C as described by Shevchenko et al. (21).

MS

Determination of the Molecular Mass of F-PSA. The samples were analyzed in a Voyager Biospectrometry Workstation MALDI mass spectrometer equipped with delayed extraction, operating in the positive mode of detection. The spectrometer contains a nitrogen laser operating at 337 nm. TOF spectra were produced at 25 kV acceleration voltage by averaging 80 single spectra. A matrix consisting of a solution of ferulic acid (4-hydroxy-3-methoxy...
mixed with 0.75 mM acid. Spectra were acquired in positive ion mode.

desalted on a Poros R2,20 column packed in a GEloader tip (Eppendorf) and ionization Q-TOF MS, carried out on a SCIEX Qstar. The sample were averaged and externally calibrated using Angiotensin I.

The doubly charged ion of horse skeletal apomyoglobin ([M+2H]+, 66,431 Da) and the doubly charged ion of horse skeletal apomyoglobin ([M+2H]+, 16,953 Da) as references.

**Analysis of F-PSA Peptides.** The endo Lys-C in-gel digests of the purified F-PSA were analyzed by MALDI-MS on either a Bruker Reflex III or a PerSeptive Biosystems STR mass spectrometer both equipped with delayed extraction and reflectors. All of the spectra were acquired in positive ion mode using a reflector mode for higher resolution. Samples were prepared on top of a fast evaporating thin-layer preparation of 4-hydroxy-cyano-cinnamic acid; 10 mg/ml) in formic acid:water:acetonitrile (1:3:2, v/v/v) was used for all determinations. PSA from semen was used as a reference solution at a concentration of 2 pmol of protein/μl of distilled water. The eluates from the immunosorption procedures were dissolved in 10 μl of distilled water. An aliquot (0.5 μl) of this protein solution was mixed with 1 μl of the matrix solution on the target plate and allowed to dry at room temperature, prior to insertion into the mass spectrometer. All of the spectra were calibrated externally using the singly charged ion of BSA ([M+H]+, 66,431 Da) and the doubly charged ion of horse skeletal apomyoglobin ([M+2H]+, 16,953 Da) as references.

**RESULTS**

**Isolation of F-PSA from PCa Serum.** The F-PSA from 8 ml of PCa serum A (containing 1890 ng/ml of T-PSA and 180 ng/ml of F-PSA) was purified by immunosorption on streptavidin-coated magnetic beads according to published methods (19) using a biotinylated antibody that was specific for F-PSA only (monoclonal antibody M30, which is being used in the Roche Diagnostics ELECSYS test for F-PSA). After the immunosorption, the amount of F-PSA in the PCa-serum was measured with an immunoassay for F-PSA and showed no detectable level, which indicated a quantitative isolation of the F-PSA. After elution of the bound F-PSA with 1 M propionic acid, the molecular mass of the F-PSA was determined by MALDI-TOF MS, and we observed an ion with an average value of 29,180 Da for the intact PSA molecule (Fig. 1B). This molecular mass is about 600 Da above the value for F-PSA from seminal fluid (28,580 Da). This reference spectrum is shown in Fig. 1A.

Recently, we reported that PSA can be liberated from the PSA/ACT complex by a nucleophilic cleavage reaction (22). Thus, for PCa serum A, the PSA liberated from the complex was found to have a molecular mass of 28,560 Da. This mass is similar to that of the F-PSA from seminal fluid. The molecular mass difference of about 600 Da indicates that the F-PSA of this PCa serum is somewhat larger than PSA isolated from seminal fluid or from the PSA/ACT complex. This finding led us to speculate that pro-PSA forms were present in the PCa serum.

**Analysis of F-PSA Peptides.** F-PSA (180 ng) isolated from PCa serum A separated by SDS-PAGE. The silver-stained band of F-PSA was cut out, alkylated under reducing conditions, and digested by endo Lys-C as described under Methods. Fig. 2 shows the peptide patterns obtained by MALDI-TOF MS from this band (A) and a PSA band from seminal fluid (B) which had been treated similarly. Table 1 contains a list of molecular masses for all of the PSA peptides observed in these two preparations and the expected theoretical values.

It is obvious that the peptides containing the NH2-terminal part of PSA are different in these two samples. The expected regular NH2-terminal peptide of PSA (amino acids 1-9) was only present in the PSA from seminal fluid and could not be found in the F-PSA from this PCa serum. From the latter sample, several peptides could be assigned to forms carrying an elongation at the NH2 terminus. The data obtained are in accordance with the theoretical values expected from the pro-PSA forms with 7, 5, 3, and 2 additional amino acids at the regular NH2 terminus (see Table 1). All of the other peptides originating from PSA in the two samples were identical. Moreover, the peptide patterns that were obtained from PSA released from the PSA/ACT complex (22) and PSA from seminal fluid were identical to each other and showed only the NH2-terminal peptide amino acids 1-9. In these cases, pro-PSA forms were absent.

The sequence coverage obtained by endo Lys-C digestion was calculated to be 83%. One minor peptide (amino acids 168–170; Mr, 1346.2) and the peptide carrying the predicted N-glycosylation site were missing (amino acids 10–46). We assume that the N-glycopeptide was not observed because of signal suppression. Because all of the mass spectrometric experiments were carried out in reflector mode and the glycan was not derivatized, significant metastable losses of the glycan may have occurred, and these altered fragments are normally not detected in reflector mode MALDI-MS.

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Sequencing of the Pro-PSA Peptide (Amino Acids −7 to +9) by Electrospray Ionization Q-TOF MS. To unequivocally identify the pro-PSA forms, the longest pro-PSA peptide (amino acids 7 to +9; Mr, 1827.97) was sequenced by Q-TOF MS. The resulting spectrum is displayed in Fig. 3, and the observed masses were assigned to the resulting sequences as shown in Table 2.

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* The direct immunosorption method was used in this case because it gives higher yield of a reasonably pure F-PSA compared with the indirect method using digoxigenin-Lys.HCL for elution, which provides higher purity but lower yield (see Ref. 19).
Table 2: m/z values of the signals observed in the Q-TOF MS sequencing analysis of the (−7) pro-PSA peptide and the amino acid sequences corresponding to these values

<table>
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<tr>
<th>Ion signal (m/z)</th>
<th>Ion (charge)</th>
<th>Measured mass</th>
<th>Calculated mass</th>
<th>Amino acid sequence*</th>
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<td>a₂</td>
<td>141.11</td>
<td>141.10</td>
<td>P</td>
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<tr>
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<td>169.10</td>
<td>169.10</td>
<td>EK</td>
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<td>y₃</td>
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<td>276.16</td>
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<td>y₃</td>
<td>436.19</td>
<td>436.19</td>
<td>CEK</td>
</tr>
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<td>565.24</td>
<td>ECEK</td>
</tr>
<tr>
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<td>y₅</td>
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<td>565.24</td>
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<td>1320.66</td>
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<td>1433.74</td>
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<td>773.912</td>
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<td>1546.82</td>
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<td>830.454</td>
<td>y₁₄ (2⁻)</td>
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<td>1827.99</td>
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<td>1828.01</td>
<td>1828.00</td>
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* X, leucine/isoleucine.

It can be seen that each of the observed peaks is in perfect agreement with the Y-ion sequence resulting from the seven amino acids of the precursor part and the nine amino acids of the regular NH₂ terminus of PSA.

Analysis of Additional PCa Sera for the Presence of Pro-PSA Forms. Four other sera (S1, S5, S6, and S9) of PCa patients with a high content of T-PSA (>6000 ng/ml) were further examined for the presence of pro-PSA peptides. The analysis followed the same protocol as described above for the analysis of F-PSA peptides, and Fig. 4 shows a representative silver-stained SDS-PAGE gel of the PSA fractions isolated from these four sera. The biotinylated monoclonal antibody M36, which recognizes T-PSA, and the immunosorption method with 1 M propionic acid elution, as described above, were used in these studies. The bands representing the PSA/ACT complex and F-PSA could clearly be identified on the gel, although some other contaminating protein bands were still present. The F-PSA bands were cut out and treated as described above, digested with endo Lys-C and the resulting peptides analyzed by MALDI-TOF MS. The result of the analysis is shown in Table 3, where the relative signal heights of the NH₂-terminal PSA peptides obtained from the F-PSA of these four sera are compared with the peptide signals obtained from F-PSA of PCa serum A (in each case calculated as percentage of the total amount of F-PSA present in that sample).

It was found that the (−4), (−5) and (−7) pro-PSA forms were present in all of the four sera investigated. In contrast to PCa serum A and serum S9, the regular NH₂ terminus as well as the (−1) pro-PSA form were detected in the other three sera. The values listed in Table 3 should be regarded with caution, considering that they were obtained in different experiments and that quantification of MALDI-TOF MS is generally problematic. However, it can be assumed that the F-PSA form with a regular NH₂ terminus represents a major part of the F-PSA of the sera S1 and S 5. The (−3) and (−6) pro-PSA forms could not be detected in any of the samples investigated thus far.

**DISCUSSION**

The structural properties of F-PSA present in human blood have not yet been unequivocally determined. It seems obvious that F-PSA should exist in a form that does not display protease activity, because otherwise it would be complexed by the protease inhibitors present in the sera.

**Table 3** Relative signal intensities of the precursor (NH₂-terminal) peptides of F-PSA obtained from the sera of five individual PCa patients, observed by MALDI-TOF MS

<table>
<thead>
<tr>
<th>Peptide calculated mass (Da)</th>
<th>S1</th>
<th>S5</th>
<th>S6</th>
<th>S9</th>
<th>Serum A</th>
</tr>
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<tr>
<td>1 to 9 (1077.50)</td>
<td>34</td>
<td>30</td>
<td>16</td>
<td>0</td>
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<tr>
<td>−1 to 9 (1233.61)</td>
<td>11</td>
<td>10</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>−2 to 9 (1320.49)</td>
<td>32</td>
<td>34</td>
<td>20</td>
<td>0</td>
<td>16</td>
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<tr>
<td>−4 to 9 (1546.81)</td>
<td>5</td>
<td>7</td>
<td>14</td>
<td>28</td>
<td>29</td>
</tr>
<tr>
<td>−5 to 9 (1659.88)</td>
<td>12</td>
<td>12</td>
<td>30</td>
<td>44</td>
<td>35</td>
</tr>
<tr>
<td>−7 to 9 (1827.97)</td>
<td>6</td>
<td>7</td>
<td>13</td>
<td>28</td>
<td>20</td>
</tr>
</tbody>
</table>
abundant amounts in the blood (mainly by α1-ACT and α2-macroglobulin). Whether this inactivity is related to internal nicking of the PSA or the presence of pro-PSA forms has largely been a matter of speculation (12, 13). Some evidence for the presence of a nicked form came from data by Noldus et al. (14), but this was not corroborated by Mikolajczik et al. (15), who did not find substantial amounts of a nicked form. Instead their HIC high-performance liquid chromatography data provided evidence that about 25% of the F-PSA seemed to be present as pro-PSA forms when compared with the elution of a pro-PSA reference obtained from cell cultures. A structural analysis of that peak was not reported. The contradictory results may be related to the use of pooled PCa sera. Moreover only low amounts of partially purified F-PSA were available, which prevented a thorough structural analysis (14). In a recent paper by Hilz et al. (23), it was reported that pro-PSA forms could not be detected in the F-PSA preparation that was isolated by immunoabsorption from blood aspirated during transvesical prostatectomy of BPH patients. This was concluded from the sequencing of the F-PSA by Edman degradation on polyvinylidene difluoride membranes. Furthermore, it was stated in that paper that no pro-PSA forms could be detected in PCa and BPH sera when probing with an antibody that had been raised against the propeptide moiety of thezymogen.

The development of efficient immunopurification procedures using streptavidin-coated magnetic beads (19) enabled us to isolate sufficient amounts of F-PSA from individual PCa sera for an analysis using state-of-the-art MS. Thus, we could unequivocally demonstrate the presence of pro-PSA forms in the serum of a PCa patient and identify their structures. Moreover, by looking at other PCa sera, we found that F-PSA with a regular NH2 terminus can also be present. This could explain the results obtained by Noldus et al. (14) as well as those of Mikolajczik et al. (15), because F-PSA, with a regular NH2 terminus, as well as pro-PSA forms can be present in PCa sera and were, therefore, very likely present in the pooled PCa sera used by those authors. In contrast to Mikolajczik et al. (15), the presence of pro-PSA forms with seven and five amino acids in the pro-PSA part could be detected in all of the PCa sera investigated by us. However, the form with the regular NH2 terminus was only present in some of the sera. Thus far, we have not investigated the lack of a complex produced by prostate inhibitors of this F-PSA form carrying the major NH2 terminus. It is tempting to speculate about the occurrence of a nicked form, but this needs to be examined in additional experiments, using an approach different from the one described above (e.g., using SDS–PAGE separation under reducing conditions in combination with suitable specific detection methods for the analysis of F-PSA forms, which has not been done by us thus far).

The results presented above demonstrate that F-PSA in sera from PCa patients occurs as a combination of different pro-PSA forms, and that a form with the regular NH2 terminus can be present as well in some sera. Among the individual sera, the presence of, and the relative amounts of, the different PSA forms seem to vary. The origins of these differences are not known. The five sera used in this study contained very high amounts of PSA. In PCa and BPH sera, in which there are low amounts of PSA (e.g., in the diagnostically interesting range of 2–15 ng/ml), the relative amounts of the various F-PSA forms could differ significantly from samples with a high PSA content or might not be present at all. For this reason comparisons to the results obtained by Mikolajczik et al. (15) and Hilz et al. (23), who were using sera with a much lower PSA concentration, have to be made with much caution because they may or may not be relevant. However, to be able to analyze sera with low PSA content for the presence of pro-PSA forms, we have prepared a monoclonal antibody that binds to the three pro-PSA forms (–7, –6, –5), and used it to develop an immunoassay that specifically recognizes these precursor forms. A preliminary analysis of panels of PCa and BPH sera of high and low PSA content showed that the pro-PSA forms could be detected almost in all of the sera analyzed.6 Whether this test can be used to better differentiate between BPH and PCa in the diagnostically gray area of 2–15 ng/ml T-PSA compared with the state-of-the-art methods (e.g., by using the ratio of F-PSA:T-PSA) will be evaluated.

In this context, a very recent report from Mikolajczik et al. (24), dealing with the identification of pro-PSA forms in PCa and benign transition zone prostate tissue, is also shedding light on the issue of F-PSA isoforms. As reported earlier for sera (15), they identified the (–4) and (–2) forms of pro-PSA in tissue extracts after immunopurification by NH2-terminal sequencing. In contrast to our report, they again did not find pro-PSA forms with the longer precursor sequences (e.g., –7 or –5), and the reason for this is not clear to us at this time. Interestingly enough, the pro-PSA forms were present more in PCa tissues than in BPH. Whether this reflects a similar situation in the sera has to be elucidated in the future using a panel of specific antibodies for the different pro-PSA forms.

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