Quantitation of Serum Prostate-specific Membrane Antigen by a Novel Protein Biochip Immunoassay Discriminates Benign from Malignant Prostate Disease

Zhen Xiao, Bao-Ling Adam, Lisa H. Cazaures, Mary Ann Clements, John W. Davis, Paul F. Schellhammer, Enrique A. Dalmasso, and George L. Wright, Jr.


Abstract

The lack of a sensitive immunoassay for quantitating serum prostate-specific membrane antigen (PSMA) hinders its clinical utility as a diagnostic/prognostic biomarker. An innovative protein biochip immunoassay was used to quantify and compare serum PSMA levels in healthy men and patients with either benign or malignant prostate disease. PSMA was captured from serum by anti-PSMA antibody bound to ProteinChip arrays, the captured PSMA detected by surface-enhanced laser desorption/ionization mass spectrometry, and quantitated by comparing the mass signal integrals to a standard curve established using purified recombinant PSMA. The average serum PSMA value for prostate cancer (625.1 ng/ml) was significantly different (P < 0.0001) from that for benign prostate hyperplasia (117.1 ng/ml) and the normal groups (age <50, 272.9 ng/ml; age >50, 359.4 ng/ml). These initial results suggest that serum PSMA may be a more effective biomarker than prostate-specific antigen for differentiating benign from malignant prostate disease and warrants additional evaluation of the surface-enhanced laser desorption/ionization PSMA immunoassay to determine its diagnostic utility.

Introduction

PSMA is a 100,000 transmembrane protein initially identified from the human PCA cell line LNCaP (1). Since its discovery, studies using a variety of approaches such as tissue immunohistochemistry, reverse transcription-PCR, in situ hybridization, and Western blot analysis have demonstrated that PSMA is expressed predominantly in prostate tissue and that PSMA is down-regulated in BPH and overexpressed in primary and metastatic PCA (2–6). Additional studies confirmed that the highest expression of PSMA was found in bone and lymph node metastatic prostate carcinomas and patients with recurrences after androgen deprivation therapy (7, 8). Although these studies have suggested the potential of PSMA as a diagnostic or prognostic biomarker, its clinical utility has been hampered primarily by the unsuccessful attempts to develop a sensitive immunoassay for quantitation of this glycoprotein in body fluids. Recently Sokoloff et al. (9) reported the development of a fluorescence-based dual-monoclonal sandwich assay to measure the levels of PSMA in tissues, seminal fluid, and urine. Their study did not include the analysis of serum; therefore, it remains uncertain if their assay is capable of detection and quantitation of serum PSMA. Murphy et al. (11–13) and our laboratory reported the successful detection of PSMA in serum by Western blot analysis (2, 10–14). However, Western blotting is far from a precise technique for quantitating proteins and, therefore, does not provide a useful format for development of a clinical assay. In a previous report we described the feasibility of developing a quantitative immunoassay for detecting PSMA in body fluids using a novel ProteinChip mass spectrometry platform (15, 16). We describe in this report the initial success of this SELDI immunoassay for quantitating PSMA in serum and show the clinical potential of this assay for the differential diagnosis of PCA from benign prostate diseases.

Materials and Methods

Materials. The procedure for the generation and purification of a recombinant PSMA protein was described in detail in a previous report (15). The purified rPSMA protein was quantitated by the BCA protein assay (Pierce Chemical, Rockford, IL) and stored at −20°C until used. Anti-PSMA monoclonal antibody 7E11-C5 (IgG1) was purified from the cell culture medium of American Type Culture Collection hybridoma line HB-10494 using Affi-Gel Protein A MAPS II kit (Bio-Rad, Hercules, CA).

Patients and Serum Samples. The serum samples used in this study were obtained from the serum bank of the Virginia Prostate Center at Eastern Virginia Medical School and were the same group of samples that had been used previously to analyze the serum levels of PSMA by Western blotting (14). All of the sera were collected from consented patients following the study protocol approved by the Institutional Review Board. The demographics of the healthy donors and patients are summarized in Table 1. The normal male population was defined by a negative DRE and a PSA <4.0 ng/ml The age range for normal males <50 years of age was 27–39 (mean, 33.9), and 54–71 (mean, 60.3) for normal males >50 years of age. Pretreatment serum samples from patients with benign or malignant prostate disease were used in this study. BPH patients were those who had bladder outlet obstructive symptoms, increased PSA levels (>4 ng/ml), and confirmed to be BPH on transrectal ultrasound biopsy. The prostate-specific antigen population consisted of patients with chronic symptom complex attributed to prostatic source and absent of infection by urine analyses and culture at the time of sample collection. PCA patients included clinical stages (T1-T3).

SELDI Immunoassay for PSMA. The SELDI ProteinChip immunoassay for the detection and quantitation of PSMA was performed essentially as described previously with slight modifications (15, 16). After coating the preactivated ProteinChip array (Ciphergen Biosystems, Inc., Fremont, CA) with 1 µg of G protein (Sigma Chemical Co., St. Louis, MO), residual active sites were blocked by incubating the entire array in 1 M ethanolamine (pH 8.0) for 30 min with agitation. The array was subsequently washed 2 × 5 min with 0.5% Triton X-100 (Sigma Chemical Co.) in PBS and 2 × 5 min with PBS. Monoclonal antibody 7E11 (1.5 µg) was added to the arrays and incubated at room temperature for 3 h. The unbounded antibodies were washed off by incubating the array 2 × 5 min in 0.1% Triton X-100 in PBS and 2 × 5 min in PBS. A 96-well bioprocessor was assembled over the arrays, creating multiple sample wells in the format of a 96-well plate. According to the rPSMA standard curve established in the previous report (15), 15–90 ng of rPSMA was used in this study as the working range and applied to the arrays in 30 µl of PBS.
containing 0.1% Triton X-100. To detect PSMA in serum, samples were diluted to within the working range of the standard curve, and 400 μl of serum (1/2–1/10 diluted in PBS with 0.5% Triton X-100) was applied to the arrays. The samples were incubated overnight at 4°C with vigorous agitation. The array was subsequently washed 2 × 5 min with 0.1% Triton X-100 in PBS, 2 × 5 min with PBS, and briefly with high-performance liquid chromatography H2O. Sinapinic acid (Ciphergen Biosystems) saturated in 50% (v/v) acetoniitile, 0.5% (v/v) trifluoroacetic acid, and 0.05% Triton X-100 was used as the matrix solution along with β-galactoglobulin (Mr 18,363.3) included as an IS for normalization. The matrix solution (2 × 0.5 μl) was applied to the array, and the array was air dried at room temperature. The detection of serum PSMA was performed using the PBS-I mass reader (Ciphergen Biosystems) with a laser intensity of 60. The data in each spectrum was averaged from 120 laser shots. The peak area of each spectrum obtained from a PSMA SELDI immunoassay was normalized against the peak area of IS. The PSMA:IS ratios were calculated, and the levels of serum PSMA were determined by comparing the serum PSMA:IS peak area ratios with rPSMA:IS area ratios of the standard curve. To assess the assay performance, a total of three averaged spectra were collected from each sample. The averages and SDs were calculated to determine the intra-assay variability. A number of serum samples were repeated on different days to assess the inter-assay variability.

### Biostatistical Analysis

The differences of PSMA levels among the normal and patient populations were analyzed using one-way ANOVA. First, Levene’s test was performed to examine the homogeneity of variances among the PSMA values of normal and patient populations. If the equality of variances was assumed (P > 0.05), then Student’s t test was performed to compare the differences of the PSMA levels. If the equality of variances could not be

![Image](image_url)

**Fig. 1.** Measurement of serum PSMA levels by SELDI ProteinChip immunoassay. A, Western blot analysis showing amount of rPSMA added to chip array (Before) and amount of rPSMA remaining in supernatant after incubation (After). Analysis shows that up to 90 ng of rPSMA could be bound to the array. B, representative example of the mass spectrum obtained from a PSMA SELDI immunoassay. Mr 101,000 PSMA peak was captured by 7E11 and detected in spectra of normal, BPH, PCA, and prostatitis serum samples. When mouse IgG was substituted for 7E11, no PSMA was detected in spectra of normal, BPH, and PCA, and prostatitis serum samples. When mouse IgG was substituted for 7E11, no PSMA was detected in spectra of normal, BPH, PCA, and prostatitis serum samples. When mouse IgG was substituted for 7E11, no PSMA was detected in spectra of normal, BPH, PCA, and prostatitis serum samples. When mouse IgG was substituted for 7E11, no PSMA was detected in spectra of normal, BPH, PCA, and prostatitis serum samples.
The overall significance level was set at 5%. Differences of the PSMA levels. In both Student’s t test and Tamhane’s test, the equality of variances was not assumed. Therefore Tamhane’s post hoc test was adopted to compare the differences of PSMA values among different populations. The average serum PSMA level in older normal males (age >50) was slightly higher than that in the younger normal males (age <50), and the difference was not significant (P = 0.83). On the other hand, significant differences were found when comparing the average PSMA level of the normal population versus PCA (normal <50 versus PCA, P < 0.001 and normal >50 versus PCA, P < 0.01), normal versus BPH (normal <50 versus BPH, P = 0.04 and normal >50 versus BPH, P < 0.01), and BPH versus PCA (P < 0.001). In addition, the mean level of serum PSMA in prostatitis patients differed from that of the normal and PCA groups (normal <50 versus prostatitis, P = 0.04; normal >50 versus prostatitis, P = 0.001; and PCA versus prostatitis, P < 0.001) but not from the BPH group (P = 1.00).

PSMA serum levels in individual patients with BPH and PCA are shown in Tables 2 and 3. These two populations were age matched (BPH, 68.6 ± 7.5 years and PCA, 68.6 ± 8.7 years). Within the BPH group (Table 2), the average PSA level was 4.9 ng/ml with six patients with a serum PSA of >4 ng/ml and four patients with a PSA of <4 ng/ml. No apparent correlation was observed between the levels of PSA and the levels of PSMA. Within the PCA group (Table 3), the average PSMA level in the low-stage cancer patients (T1a-T2a; n = 11; 668.8 ng/ml) was higher than that in the high-stage cancers (T2b-T3; n = 6; 539.5 ng/ml); however, this difference was not statistically significant (P = 0.16, Student’s t test). Collectively these results demonstrate that PSMA levels in PCA were significantly higher than in BPH.

### Table 2 Demographics and serum PSA and PSMA levels for BPH patients

<table>
<thead>
<tr>
<th>BPH sample</th>
<th>Age yr</th>
<th>PSA ng/ml</th>
<th>PSMA ng/ml</th>
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<tbody>
<tr>
<td>1</td>
<td>68</td>
<td>9.6</td>
<td>138.25</td>
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<td>2</td>
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<td>6</td>
<td>54</td>
<td>4.4</td>
<td>35.6</td>
</tr>
<tr>
<td>7</td>
<td>81</td>
<td>0.9</td>
<td>136.3</td>
</tr>
<tr>
<td>8</td>
<td>71</td>
<td>0.2</td>
<td>148.3</td>
</tr>
<tr>
<td>9</td>
<td>74</td>
<td>0.6</td>
<td>146.1</td>
</tr>
<tr>
<td>10</td>
<td>64</td>
<td>13.7</td>
<td>92.4</td>
</tr>
</tbody>
</table>

**Average** 68.6 4.9 117.1

**Range** 54–81 0.2–13.7 35.6–193.8

**SD** 7.5 4.2 48.7

A rPSMA linear standard curve was developed with the R^2 (the square of the correlation coefficient or the strength of the association) ranging from 0.92 to 0.99 (mean, 0.95) and an average intra-assay variability of 8.9%. Pre- and postassay rPSMA samples were analyzed using 7E11 Western blotting, which demonstrated that the SELDI ProteinChip immunoassay was capable of capturing up to 90 ng of rPSMA on the chip array (Fig. 1A). The SELDI immunoassay was used to detect and quantitate PSMA in serum ranging from 1.2% to 20.1% with an average of 10.1%, whereas the inter-assay variability ranged from 2.9% to 18.7% with an average of 11.8%. Fig. 1B shows the representative mass spectra of the M_101,000 PSMA peak detected in serum from a normal age-matched donor and in sera from patients with BPH, PCA, or prostatitis.

### Table 3 Demographics and serum PSA and PSMA levels for PCA patients

<table>
<thead>
<tr>
<th>PCA sample</th>
<th>Age yr</th>
<th>Stage</th>
<th>Grade^a</th>
<th>Gleason score</th>
<th>PSA ng/ml</th>
<th>PSMA ng/ml</th>
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<tr>
<td>1</td>
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<td>T1c</td>
<td>WD</td>
<td>2 + 2</td>
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<tr>
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<td>63</td>
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<td>2 + 2</td>
<td>3.0</td>
<td>455.8</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>T1c</td>
<td>PD</td>
<td>4 + 4</td>
<td>7.7</td>
<td>632.6</td>
</tr>
<tr>
<td>4</td>
<td>66</td>
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<td>WD</td>
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<td>6.4</td>
<td>687.6</td>
</tr>
<tr>
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<td>64</td>
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<td>MD</td>
<td>3 + 4</td>
<td>2.6</td>
<td>471.0</td>
</tr>
<tr>
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<td>55</td>
<td>T2a</td>
<td>MD</td>
<td>2 + 3</td>
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<td>946.6</td>
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<td>423.8</td>
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<td>74</td>
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<td>NR^b</td>
<td>5.5</td>
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<td>67</td>
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<td>765.4</td>
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<td>3 + 4</td>
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<td>716.3</td>
</tr>
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<td>T2b</td>
<td>MD</td>
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<td>624.2</td>
</tr>
<tr>
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<td>T2b</td>
<td>PD</td>
<td>NR</td>
<td>9.3</td>
<td>730.9</td>
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<tr>
<td>14</td>
<td>67</td>
<td>T2b</td>
<td>MD</td>
<td>3 + 3</td>
<td>5.5</td>
<td>349.5</td>
</tr>
<tr>
<td>15</td>
<td>61</td>
<td>T2b</td>
<td>WD</td>
<td>2 + 2</td>
<td>8.2</td>
<td>645.0</td>
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<tr>
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<td>PD</td>
<td>4 + 4</td>
<td>1230.0</td>
<td>419.2</td>
</tr>
<tr>
<td>17</td>
<td>93</td>
<td>T3</td>
<td>MD</td>
<td>3 + 4</td>
<td>455.7</td>
<td>467.9</td>
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<tr>
<td><strong>Average</strong></td>
<td><strong>68.6</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>107.2</strong></td>
<td><strong>623.1</strong></td>
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<tr>
<td><strong>Range</strong></td>
<td><strong>55–93</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>2.4–1230</strong></td>
<td><strong>349.5–946.6</strong></td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td><strong>8.7</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>309.1</strong></td>
<td><strong>177.7</strong></td>
</tr>
</tbody>
</table>

**Low stage (T1a/T2a), 11 patients**

- Average 66.4 5.7 668.8
- Range 55–74 2.4–7.9 423.8–946.6
- SD 5.1 2.2 181.6

**High stage (T2b/T3), 6 patients**

- Average 72.7 293.2 539.5
- Range 61–93 5.5–1230 349.5–730.9
- SD 12.5 491.5 148.8

^a WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.
^b NR, not recorded. Score cannot be given because of limited specimen.
^c P = 0.16 when compared to the average PSMA level in the low stage cancers.
higher than those observed for the BPH and the two normal male populations.

**Discussion**

Several lines of evidence suggest that PSMA may be a suitable target for gene and immunotherapy strategies and as a biomarker of progressive malignant prostate disease (17, 18). Observations that PSMA appeared to be differentially expressed in BPH and PCA (6–8, 14) also suggested that PSMA might be useful as a marker, either alone or in concert with PSA, to differentiate PCA from BPH in the PSA 4–10 ng/ml gray zone, thus reducing the number of unnecessary biopsies. However, to critically evaluate PSMA as a biomarker, it would first be necessary to develop an assay format to quantitatively measure PSMA in body fluids. Although Western blot analysis has been used to detect PSMA in body fluids, our laboratory and others have found it unreliable for quantitation and unsuitable as a clinical assay (2, 10–14). Attempts to develop an immunoassay for quantitation of PSMA have been unsuccessful, and, to the best of our knowledge, no test has been developed that accurately measures PSMA in sera.

This report describes a novel immunoassay using a ProteinChip platform to capture the PSMA followed by mass spectrometry to detect and quantitate the antigen. This SELDI immunoassay format was successful in measuring PSMA in serum from normal healthy men and men diagnosed with either benign or malignant prostate disease. The highest PSMA levels were found in sera from patients with PCA, and these values differed significantly from the normal and benign groups. The results confirm a similar trend of difference among the normal, BPH, and PCA samples observed by Western blot analysis in our previous study (14) and therefore validate the sensitivity, specificity, and feasibility of SELDI ProteinChip technology as an immunoassay format for measuring serum PSMA.

Most noteworthy was the observation that PSMA levels significantly discriminated BPH from the PCA samples that had PSA values between 4–10 ng/ml. When a PSA cutoff of 4 ng/ml is used as the parameter for diagnosis, a substantial overlap will be present between BPH and PCA. It is in this context that we believe the ability of serum PSMA to differentiate BPH from PCA will be of most interest for clinical application. PSA has been considered the standard tumor biomarker for PCA diagnosis, but its clinical utility is hampered by the sensitivity and specificity issues that have been raised by many investigators (19, 20). For instance, ~25% of patients who are diagnosed with PCA have a PSA value <4 ng/ml (21), whereas an equal percentage of BPH patients have PSA >4 ng/ml. Therefore, the sensitivity and specificity of a cutoff at 4 ng/ml is problematic. If the PSA cutoff for recommending a prostate biopsy is lowered to improve sensitivity, it will trigger a significant number of additional and unnecessary biopsies (22). Free to total PSA ratio or percentage of free PSA has been used to improve the operating characteristics; however, 5–10% of PCA still remained undetected (19, 20). Using the SELDI/PSMA immunoassay described in this report, all of the samples with a PSA between 4 and 10 ng/ml were correctly “diagnosed” as either BPH or PCA. This observation is encouraging and suggests that PSMA may be a clinically valuable biomarker, either used alone or in combination with PSA, for the diagnosis of PCA.

This is the first report describing not only the quantitation of PSMA in serum but the use of an unconventional immunoassay format. Whereas the conventional assays usually detect antigen indirectly based on the colorimetric or fluorometric changes in solution, SELDI quantitates antigen directly according to the mass signal. This enables SELDI to bypass the problem of cross-reactivity of antibody with other molecules similar to the specific antigen, which can be a problem that affects the performance of conventional assays and leads to the overestimation of antigen. Because SELDI is a new quantitative immunoassay method, other PSA antibodies that recognize either the intracellular or extracellular domains of the PSMA glycoprotein are being evaluated to assess if these antibodies can enhance assay sensitivity.

Another advantage of the SELDI ProteinChip immunoassay system is that multiple biomarkers can be measured simultaneously in a multiplex format. In a preliminary study, we described the successful measurement of both PSMA and PSA (both the free- and antichymotrypsin-complexed PSA) in seminal plasma and sera (16). The multiplex format would have a major benefit, because it could simultaneously measure a combination of PSMA with the various PSA forms that have proven to have an advantage over PSA or PSA alone in discriminating BPH from PCA.

Whereas the serum PSMA levels could effectively differentiate benign from malignant prostate diseases, they were not able to distinguish low-stage from high-stage cancers or Gleason grades <6 from 7–10. However, this observation is still inconclusive because of the small sample size, requiring additional studies with larger numbers of samples to evaluate correlations with grade and stage and the prognostic utility of serum PSMA.

In summary, a SELDI ProteinChip immunoassay was developed for quantitating PSMA in sera. The levels of serum PSMA from PCA patients were significantly different from the levels in sera from patients with BPH, making it possible to discriminate BPH from PCA, especially in the PSA 4–10 ng/ml gray zone. These results suggest that PSMA may be a clinically useful diagnostic biomarker for improving the specificity in differentiating PCA from BPH and warrants additional studies to evaluate the clinical utility of the SELDI/PSMA immunoassay.

**Acknowledgments**

We thank biostatisticians Dr. Michael Doviak, Seemit Sheth, and Thomas Abbott of the Office of Research at Eastern Virginia Medical School for the assistance with the biostatistical analysis.

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