

Prognostic Value of Prostate Secretory Protein of 94 Amino Acids and its Binding Protein after Radical Prostatectomy

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Abstract Purpose: To establish the prognostic value of total and free prostate secretory protein of 94 amino acids (PSP94) and the PSP94-binding protein (PSPBP) following radical prostatectomy. **Experimental Design:** One hundred and eighty-five serum samples were obtained from patients with localized prostate cancer prior to treatment with radical prostatectomy at Virginia Urology (Richmond, VA). Patients were followed up for a median of 48 months (range, 1-66 months) and biochemical relapse was indicated as total prostate-specific antigen (tPSA) levels increasing to >0.1 ng/mL. The available clinical variables included initial tPSA, Gleason score, surgical margin status, and clinical stage. Total PSP94, free PSP94, and the PSPBP were quantified in the pretreatment serum using new ELISA tests (Medicorp, Inc. and Ambrilia Biopharma, Inc., Montreal, Quebec, Canada). Univariate and multivariate Cox proportional hazards models were used to assess the ability of PSP94 and PSPBP to predict time to recurrence. **Results:** Thirty-one patients had biochemical recurrence. Gleason score, margin status, clinical stage, and initial tPSA significantly predicted recurrence risk (all $P < 0.001$). In addition, PSPBP was negatively associated with recurrence risk ($P = 0.005$), and, consistent with previous studies, the bound/free PSP94 ratio was positively associated with recurrence risk ($P = 0.008$). Multivariate analysis showed that PSPBP, as well as the bound/free PSP94 ratio, were independent predictors of biochemical relapse risk adjusting for tPSA, Gleason score, and margin status. **Conclusions:** Bound/free PSP94 and PSPBP are novel and independent prognostic markers following radical prostatectomy for prostate cancer.

The variable nature of prostate cancer yields two main groups of patients: the minority with rapidly progressing disease causing significant health issues and a high probability of death resulting from the condition, and the majority with relatively indolent prostate cancer that can be controlled for the remainder of the patient's life with modest or no intervention. Unfortunately, there is no absolute way to assess disease aggressivity at diagnosis; many patients may be overtreated to benefit those who need it. Pretreatment nomograms have been developed to determine outcome probabilities from measurements including initial prostate-specific antigen (PSA) levels

and biopsy-derived pathologic grading (1). These are valuable tools for assisting with clinical management decisions, but they go only some way to providing an accurate forecast of the disease course, particularly in their mid range in which the predictive value diminishes.

Studies involving patients with localized prostate cancer, treated conservatively with 15 years of follow-up, have shown that 70% to 82% of Gleason 6 and 30% to 58% of Gleason 7 patients (depending on age) had a nonlethal form of the disease (2). With the widespread use of PSA screening and associated stage shift in the diagnosed population (3), the proportion of patients with indolent disease may be increasing. Furthermore, as the population in North America ages (4), the number of patients having a limited life expectancy from other causes at the time of prostate cancer diagnosis will increase. These factors contribute to the increasing need to identify those patients who have aggressive disease requiring immediate intervention from those with indolent disease who can be managed conservatively to avoid or postpone the morbidity and cost associated with unnecessary treatments. Consequently, there is a requirement for additional pretreatment prognostic factors to develop more precise nomograms that predict the outcome probabilities for each of the possible clinical management options.

Prostate secretory protein of 94 amino acids (PSP94) is one of the most abundant proteins in semen (5). The 10.7 kDa, nonglycosylated, and cysteine-rich protein is also known as β -microseminoprotein or prostate inhibin peptide. Along with roles in fertility, PSP94 has postulated systemic functions including growth regulation and induction of apoptosis in

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Table 1. Serum analyte concentrations in categories based on demographic, clinical, and pathologic variables

	N (%)	Total PSP94, ng/mL median (range)	Free PSP94, ng/mL median (range)	PSPBP, ng/mL median (range)	Initial PSA, ng/mL median (range)	Bound/free PSP94 median (range)
All patients	185 (100)	4.3 (0.5-22.8)	2.2 (0.20-17.1)	655 (330-1,424)	5.6 (1.0-34)	1.10 (0.00-35)
Age						
<60	59 (32)	3.9 (0.5-19.6)	1.9 (0.2-14.5)	661 (343-1,424)	5.2 (2.2-26)	1.29 (0.0-9.5)
60-65	61 (33)	4.6 (0.5-22.8)	2.4 (0.2-17.1)	651 (459-989)	5.8 (1.0-34)	1.10 (0.0-7.5)
>65	65 (35)	4.6 (0.5-16.8)	2.4 (0.2-9.9)	658 (332-932)	5.8 (1.4-25)	1.07 (0.23-35)
<i>P</i> values		NS	NS	NS	NS	NS
Race						
Black	26 (14)	2.4 (0.5-22.8)	0.8 (0.2-17)	597 (376-1,424)	6.3 (1.1-21)	1.83 (0.33-9.5)
Other	159 (86)	4.5 (0.5-19.6)	2.4 (0.2-15)	663 (330-1,006)	5.6 (1.0-34)	1.07 (0.0-35)
<i>P</i> values		<i>P</i> = 0.0103	<i>P</i> = 0.0061	<i>P</i> = 0.0185	NS	<i>P</i> = 0.0015
Family history						
None	134 (74)	4.8 (0.5-19.6)	2.5 (0.2-14.5)	655 (332-1,424)	5.8 (1-27.5)	1.08 (0.0-35)
First or second degree	46 (26)	3.9 (0.5-22.8)	1.8 (0.2-17.1)	656 (376-1,006)	5.5 (2-25.9)	1.23 (0.23-9.5)
<i>P</i> values		NS	NS	NS	NS	NS
Stage						
T ₁ and T ₂	150 (83)	4.4 (0.5-22.8)	2.2 (0.2-17.1)	654 (332-1,424)	5.5 (1.0-26)	1.11 (0.0-9.5)
T ₃	31 (17)	4.3 (0.5-16.8)	2.2 (0.2-9.9)	626 (343-933)	6.6 (2.3-34)	1.25 (0.0-35)
<i>P</i> values		NS	NS	NS	<i>P</i> = 0.028	NS
Gleason						
<6	22 (12)	4.3 (1.5-22.8)	2.55 (0.2-7.6)	648 (493-817)	4.9 (1.1-11)	1.40 (0.33-9.5)
6	109 (60)	4.4 (0.5-22.8)	2.40 (0.2-17.1)	660 (343-1,424)	5.5 (1-24)	1.08 (0.0-9.5)
7	39 (21)	4.6 (0.5-17)	2.10 (0.2-11.4)	658 (296-947)	7.1 (3.7-26)	1.21 (0.25-7.5)
>7	15 (8)	3.6 (0.5-15.9)	1.40 (0.2-9.1)	566 (434-889)	6.9 (2.0-33.7)	1.25 (0.71-35)
<i>P</i> values		NS	NS	NS	<i>P</i> = 0.01*	NS
Margins						
Clear	139 (79)	4.6 (0.5-22.8)	2.4 (0.2-17.1)	660 (330-1,424)	5.3 (1.0-23)	1.09 (0.0-9.5)
Not clear	37 (21)	4.1 (0.5-15.9)	2.1 (0.2-9.1)	593 (343-889)	6.8 (3.7-34)	1.08 (0.0-35)
<i>P</i> values		NS	NS	<i>P</i> = 0.007	<i>P</i> < 0.001	NS

Abbreviation: NS, not significant.

*Pairwise tests indicate that total PSA is significantly lower in Gleason <6 group compared with the Gleason 7 and >7 groups.

prostate cancer cells *in vitro* and *in vivo* (6), as well as regulation of calcium levels during hypercalcemia of malignancy (7). As with other prostate-secreted proteins, PSP94 can leak into the blood upon benign or malignant prostate epithelial disruption and can be measured within serum. PSP94 was previously studied as a prostate cancer blood biomarker in the early PSA era (8–11). More recently, PSP94 was found to circulate in low

and high molecular weight forms, suggesting the presence of a high-molecular weight blood-binding factor (9, 12). In the pretreatment serum of patients treated with radiation, it was shown that tests differentiating between the free and bound forms of PSP94 had significant and independent prognostic value adjusting for initial PSA and Gleason score (13). More recently, a serum protein with high binding affinity for PSP94

Table 2. Univariate Cox proportional hazards analysis

Variable	<i>P</i>	Range	Hazard ratio (95% confidence interval)
Gleason score	<i>P</i> < 0.001	3-9	2.683 (1.837-3.920)
Stage*	<i>P</i> < 0.001	T ₂ or T ₃	6.170 (2.986-12.752)
Resection margins	<i>P</i> < 0.001	Involved or not	5.461 (2.629-11.34)
lnPSA*	<i>P</i> < 0.001	0-3.52 (1-33.8 ng/mL)	3.264 (1.864-5.716)
Free PSP94	NS	—	—
Total PSP94	NS	—	—
Bound/free PSP94	<i>P</i> = 0.008	0-34.5	1.113 (1.029-1.205)
PSPBP	<i>P</i> = 0.005	330-1,424 ng/mL	0.996 (0.993-0.999)
Age	NS	—	—
Family history	NS	—	—
Race	NS	—	—

NOTE: Hazard ratio and confidence limits for significant predictors of instantaneous recurrence risk are shown.

Abbreviation: NS, not significant.

*To maximize the strength of the association with recurrence risk, stage was expressed as a binary variable (T₂ or T₃) and PSA was log-transformed. Age was assessed as a continuous variable.

was identified (14), facilitating the development of monoclonal antibodies and immunoassays that specifically recognize the free and total forms of PSP94.

This retrospective study is a follow-up of a previous prognostic study (13) and uses new immunoassays to investigate whether pretreatment serum measurements of free PSP94, total PSP94, their ratios, or the PSP94-binding protein (PSPBP) have the potential to be useful prostate cancer prognostic factors using time to biochemical relapse as a surrogate end point following radical prostatectomy.

Materials and Methods

Patients. Blood was drawn and serum banked at -80°C from consenting patients attending Virginia Urology (Richmond, VA) prior to clinical evaluation for prostate cancer. Out of those patients diagnosed with localized prostate cancer and treated by radical prostatectomy between January 1999 and May 2000, 185 subjects were selected randomly for this retrospective study. Patient information relating to age, race, family history of prostate cancer, and clinical and pathologic data relating to Gleason score, initial PSA level, surgical margin status, and clinical stage were obtained. Patients were followed up initially at 3-month intervals and at 6-month intervals thereafter. At follow-up, serum was assessed for total PSA content, and the time from surgery to biochemical recurrence was noted (indicated by a sustained increase in serum PSA to >0.1 ng/mL). All patients provided informed consent and the study was conducted under appropriate ethical board approval.

Immunoassays. ELISA assays based on the 96-well plate format, specific for total PSP94, free PSP94, and PSPBP were developed and validated (Medicorp, Inc. and Ambrilia Biopharma, Inc., Montreal, Quebec, Canada) according to industry guidelines for bioanalytic method validations (15). The free PSP94 assay detects only uncomplexed PSP94 and uses a rabbit polyclonal antibody recognizing total PSP94 as a capture reagent, and a peroxidase conjugated monoclonal antibody specific for the free form of PSP94 as detection reagent. The total PSP94 assay detects both the uncomplexed and complexed forms of PSP94 (bound to PSPBP) and uses a rabbit polyclonal antibody recognizing total PSP94 as the capture reagent and a peroxidase conjugated monoclonal antibody specific for total PSP94 as the detection reagent. The PSPBP assay detects both free and complexed binding protein and uses a monoclonal antibody as capture reagent and a monoclonal antibody recognizing a different PSPBP epitope as detection reagent.

During assay validation, the lower limit of detection for each assay was established, and interassay and intraassay variabilities were determined by repeated measurements of three quality controls with analyte concentrations within the lower, mid, and upper range of the standard curve. For the free PSP94 assay, the total PSP94 assay, and the PSPBP assay the lower limit of detection was 0.37, 1.1, and 27 ng/mL, respectively, and the intraassay and interassay coefficients of variation were within 5%, 5%, and 9%, respectively.

Interference studies with molecules known to be relevant to serum samples from prostate cancer patients were done by spiking three lots of human serum with: PSA (10 $\mu\text{g/mL}$), α -fetoprotein (10 $\mu\text{g/mL}$), carcinoembryonic antigen (10 $\mu\text{g/mL}$), human chorionic gonadotrophin (10 $\mu\text{g/mL}$), prostatic acid phosphatase (1 $\mu\text{g/mL}$), lactalbumin (10 mg/mL), hemoglobin (5 mg/mL), bilirubin (200 $\mu\text{g/mL}$), triglycerides (10 mg/mL), cyclophosphamide (500 $\mu\text{g/mL}$), methotrexate (50 $\mu\text{g/mL}$), doxorubicin-HCl (20 $\mu\text{g/mL}$), diethylstilbestrol (2 $\mu\text{g/mL}$), and flutamide (10 $\mu\text{g/mL}$). Spiked and unspiked sera were analyzed for free PSP94, total PSP94, and PSPBP and the analyte recovery in the spiked serum samples was within 85% to 115% of the concentration measured within the unspiked serum. Patient serum sample analysis was done in a blinded fashion to outcome variables and covariates.

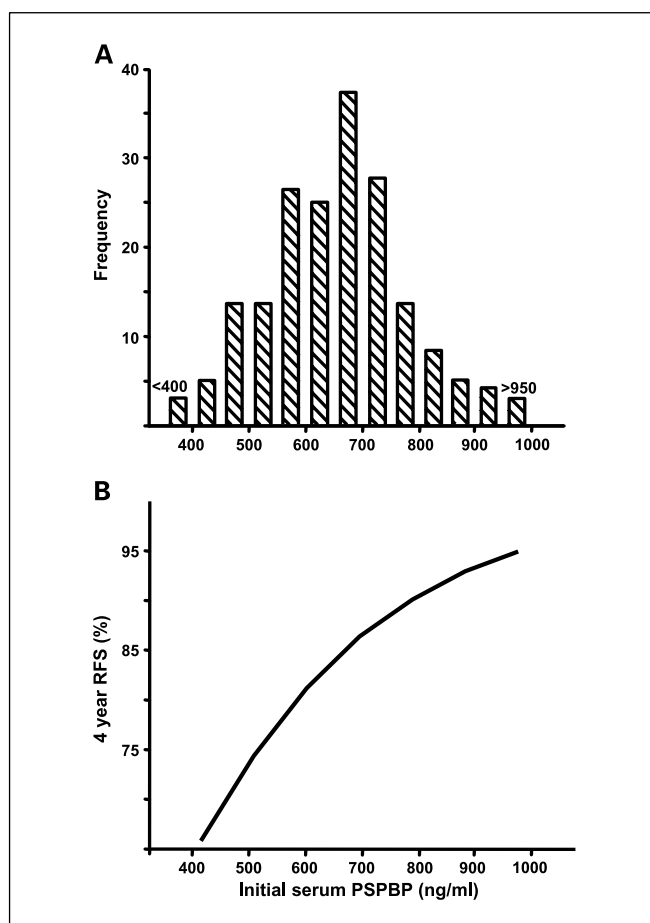


Fig. 1. A, pretreatment serum PSPBP frequency distribution in 185 patients with localized prostate cancer. B, biochemical relapse-free survival (RFS) probability at 4 years in each PSPBP level, based on the model generated by univariate Cox proportional hazards regression.

Statistics. The duration of follow-up was calculated from the date of radical prostatectomy to the first indication of PSA recurrence or date of last follow-up for censored patients. Serum analyte levels within categorical variable groupings were compared using nonparametric tests (Mann-Whitney rank sum test for two independent variables, or ANOVA on ranks with *post hoc* tests for variables with more than two independent groups). Univariate and multivariate survival analyses were carried out using Cox proportional hazards models. Statistical tests were two-sided at the 5% level of significance.

Results

Relationship of serum measurements to clinical and pathologic variables. Nine of the 185 patients had total PSP94 levels of <1 ng/mL (the sensitivity of the assay), and for the purpose of the analysis, a value of 0.5 ng/mL was assigned to these patients. Thirty-one patients had free PSP94 levels of <0.5 ng/mL, and for the purpose of the analysis, these patients were assigned a free PSP94 level of 0.2 ng/mL. All patients had a quantifiable level of the PSPBP. The makeup of the data set in terms of clinical and pathologic criteria is presented in Table 1, which also shows the analysis of the pretreatment blood marker variables (PSP94 variables and PSA) in each of the clinical and pathologic category groups. None of the biochemical markers were associated with age grouping or family history of prostate

Table 3. Multivariate Cox proportional hazards analysis

(1) Adding PSPBP to PSA	P	Hazard ratio (95% confidence interval)	-2 log likelihood
lnPSA	<0.001	3.264 (1.864-5.716)	287.2
lnPSA and PSPBP	<0.001 0.010	3.129 (1.772-5.525) 0.996 (0.993-0.999)	280.3
(2) Adding PSPBP to Gleason score			
Gleason	<0.001	2.683 (1.837-3.920)	280.4
Gleason and PSPBP	<0.001 0.018	2.658 (1.778-3.975) 0.997 (0.994-0.999)	274.4
(3) Adding PSPBP to PSA and Gleason score			
Gleason	<0.001	2.428 (1.628-3.620)	271.2
lnPSA	0.001	2.491 (1.425-4.353)	
Gleason lnPSA and PSPBP	<0.001 0.002 0.025	2.390 (1.567-3.644) 2.404 (1.371-4.216) 0.997 (0.994-1.000)	265.9

cancer. Free PSP94, total PSP94, and PSPBP were significantly lower in the Black population compared with the non-Black population. In a separate study of cancer patients and normal controls, PSPBP was also significantly lower in Black individuals compared with non-Blacks (data not shown). PSA was no different in the two race categories. A high pretreatment serum PSA level, but none of the PSP94 variables, was significantly associated with advanced clinical stage, and high Gleason score. PSPBP was significantly lower in patients with a positive surgical excision margin, and in these patients, PSA levels were significantly higher.

Univariate survival analysis. Thirty-one patients had evidence of biochemical recurrence during the follow-up period (median, 48 months). Univariate survival analysis (Table 2) showed that clinical stage, Gleason score, pretreatment PSA, and surgical excision margin status were significant predictors of instantaneous recurrence risk. The bound/free PSP94 ratio was positively associated with recurrence risk, and PSPBP was negatively associated with recurrence risk. Bound/free PSP94 was correlated with PSPBP, but PSPBP was the stronger marker in multivariate assessment. Free PSP94, total PSP94, age, race, and family history for prostate cancer were not significant predictors of recurrence. Based on the results generated by the univariate Cox proportional hazards model with PSPBP as a covariate, we see that patients with an initial PSPBP level of >800 ng/mL have a biochemical relapse-free survival of $\geq 90\%$ at 4 years (Fig. 1B), whereas those patients with <500 ng/mL of PSPBP have a $\geq 25\%$ risk of suffering a PSA recurrence in 4 years.

Multivariate survival analysis. Multivariate Cox regression models (Table 3) showed that PSPBP was an independently significant predictor of instantaneous recurrence risk when incorporated into models including established indicators of prognosis. PSPBP was also independently significant when combined with excision margin status or clinical stage (not shown in Table 3). Bound/free PSP94 was also a significant independent predictor recurrence risk, but PSPBP was the stronger marker.

Discussion

This study follows on from previous work in which a multistep biochemical test was used to assess the relative levels of bound and free PSP94 in the initial serum of localized

prostate cancer patients subsequently treated with radiation (13). In that study, the ratio of bound PSP94 to free PSP94 was an independent predictor of time to biochemical recurrence. Those patients with a short relapse-free interval tended to have a high bound/free PSP94 ratio. In the current study, we applied recently developed and validated ELISAs, specific for free PSP94, total PSP94, and PSPBP to the initial serum of localized prostate cancer patients treated with radical prostatectomy. Consistent with the previous study, the bound/free PSP94 ratio was positively associated with recurrence risk. In addition, the pretreatment serum concentration of PSPBP was negatively associated with recurrence risk. In multivariate analysis, PSPBP was a stronger marker than the ratio, and it also has the advantage of being a single marker rather than a derivation from two separate assays. These findings suggest that there may be a functional link between the levels of PSPBP and bound/free PSP94, but the mechanisms behind the relationship are yet to be elucidated.

PSPBP is a recently identified 50 kDa glycosylated secreted protein with significant amino acid sequence similarity to members of the CRISP family of proteins (14). Of particular note is the high sequence similarity to glioma pathogenesis-related protein (RTVP-1), a p53-regulated tumor suppressor down-regulated in prostate carcinoma (16). The function of PSPBP, other than binding to PSP94, is unknown, but the members of the CRISP family of proteins are structurally related and bind to cellular ion channels resulting in the regulation of ion transport across the plasma membrane (17). Assuming that PSPBP also binds to cellular ion channels, PSP94 binding may alter ion transport in some way. mRNA for PSPBP is found in human prostate tissue, and localization studies suggest that the protein is predominantly expressed in the stromal cells (14). PSPBP has the potential to be linked to the plasma membrane through glycosyl phosphatidyl inositol anchorage, and may therefore serve as a cell surface receptor as well as a soluble binding protein for PSP94. Release of PSP94 through the prostate epithelial basement membrane during benign or malignant prostate disorders may result in binding to cell surface stromal PSPBP with downstream signaling and physiologic effects. Whether the serum levels of PSPBP are in some way influenced by this process (through differential cleavage of the glycosyl phosphatidyl inositol-anchored PSPBP by

endogenous phosphatidyl inositol-specific phospholipases for example) or are simply a risk factor for the development of aggressive prostate cancer is unknown.

Additional appropriately powered studies on a larger groups of patients will more clearly define the prognostic value of bound/free PSP94 and PSPBP. As the end point of this study was increasing PSA, and this may be the result of local recurrence or metastatic progression, the preferred end point of subsequent studies should be clinical evidence of metastatic progression. To establish the extent of applicability, this should be done in observational and in the range of interventional settings. To assess the clinical value, the gain in predictive accuracy by the inclusion of PSPBP or bound/free PSP94 in preoperative validated nomograms should be established. This

is of particular relevance to the groups of patients who are unable to decide their most appropriate treatment from the information available to them.

There are a number of potential prostate cancer prognostic markers currently being researched or developed (18, 19). Many of these involve the assessment of the prostate tissue itself, making them less applicable in a pretreatment setting. The PSPBP assay is a serum-based test of a simple, transferable, and minimally invasive nature which lends itself well to the regulatory approval process. With that said, each of the candidate prognostic factors has potential value, and there is a requirement for an integrated approach to assess how combinations of these factors perform in terms of practicality and effect on predictive accuracy.

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