Published in final edited form as:

Prostate. 2009 August 1; 69(11): 1188–1194. doi:10.1002/pros.20963.

Analysis of a Serum Test for Prostate Cancer That Detects a Second Epitope of EPCA-2

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Abstract

BACKGROUND—We have previously shown that EPCA-2 can serve as a highly specific and sensitive serum marker for prostate cancer. As a component of our validation of this marker, we have performed an initial evaluation of an assay that detects a distinct epitope of the same protein: EPCA-2.19. The goals of this study are to characterize the sensitivity and specificity of the EPCA-2.19 assay, in a non-screening population, and to demonstrate that such test based has similar characteristics as the initial assay produced.

METHODS—Three hundred twenty-eight serum samples from men with PSA values < and >2.5 ng/ml who had negative biopsies, men with BPH, men with organ-confined and non-organ-confined prostate cancer, as well as control populations were evaluated using the EPCA-2.19 assay.

RESULTS—At a cut-off of 0.5 ng/ml and above, EPCA-2.19 has a specificity of 94% and a sensitivity of 91% in separating normal men with PSA < and >2.5 ng/ml, and men with BPH from those with prostate cancer. Receiver Operator Curve analyses of the EPCA-2.19 assay demonstrate an area under the curve of 0.982 (95% CI 0.952–0.996, $P < 0.0001$).

CONCLUSIONS—This study confirms our earlier findings that the assay that detects against a second epitope of EPCA-2 yields almost identical results to those obtained for the first published assay (EPCA-2.22). While this provides some validation of our earlier studies, larger multi-institutional studies still need to be performed.

Keywords

prostate cancer; proteomics; biomarkers; nuclear matrix; immunoassay

INTRODUCTION

Our laboratory has previously identified novel prostate cancer biomarkers utilizing focused proteomic analysis. These efforts have concentrated on determining the molecular underpinnings of what pathologists observe under the microscope. Alterations in nuclear structure and architecture are one of the characteristics of cancer. During neoplastic transformations in concordance with changes in nuclear structures are modifications in the composition of nuclear structural proteins. Assessment of the protein components that comprise nuclear structure/architecture have resulted in the discovery of cancer-associated biomarkers that are specific to cancer. These types of biomarkers have been identified in

breast, prostate, colon, bladder, lung, ovarian, renal as well as in squamous cell carcinoma of the head and neck (reviewed in Ref. [1]). As it relates to prostate cancer, differences in nuclear structural proteins have been demonstrated in the cancer and normal rat prostate [2], a transgenic mouse model for prostate cancer [3], as well as in benign prostatic hyperplasia (BPH) and prostate cancer [4–6].

Utilizing the above described approach, early prostate cancer antigen-2 (EPCA-2) was identified as a nuclear structural protein associated with prostate cancer [7]. Based upon an assay that we constructed to detect the protein, EPCA-2 has been shown to be elevated in human prostate cancer but not in normal prostate. EPCA-2 has been sequenced and antibodies have been raised against three epitopes of this protein: EPCA-2.19, 2.22, and 2.4. The antibodies against each of the three epitopes of the EPCA-2 protein have been utilized to develop ELISA assays, and these assays were used to screen serum samples from control population as well as men with prostate cancer and BPH.

We recently demonstrated that an assay which detects one of the EPCA-2 epitopes, EPCA-2.22 in the serum is a highly sensitive and specific test for prostate cancer [7]. This epitope of EPCA-2 is also able to differentiate men with organ-confined from those with non-organ-confined prostate cancer. The main objective of this study is to determine if the assay which detects a unique epitope of EPCA-2 (EPCA-2.19) in the serum can be utilized for prostate cancer detection. Using an indirect ELISA approach, our studies demonstrate that an ELISA assay for EPCA-2.19 is indeed highly sensitive and specific for detecting men with prostate cancer and differentiating them from normal controls, those with BPH, as well as other benign conditions and cancer types.

MATERIALS AND METHODS

EPCA-2 Sequencing and Antibody Production

The EPCA-2 protein (MW 40 kDa, pI 5.91) was isolated from two-dimensional gels and the spots were then thawed, pooled, stained with 0.25% Coomassie blue and concentrated on an acrylamide/agarose gel. The concentrated protein band was then excised and sent for peptide sequencing (Department of Biochemistry, Michigan State University, East Lansing, MI). Three epitopes were generated from the EPCA-2 sequencing, and they were designated as EPCA-2.4, EPCA-2.19, and EPCA-2.22, respectively. A standard protocol was followed in the production of antibodies raised against these epitopes. Utilizing the peptide sequence derived from the EPCA-2 epitopes, peptides and antibodies against EPCA-2.19 were produced by Cocalico Biologicals, Inc. (Reamstown, PA) under an Institutional Animal Care and Use Committee approved protocol.

Serum Sample Populations

Serum samples were collected from consented patients under an IRB approved protocol (supported by the NCI Early Detection Research Network). Five distinct populations of individuals served as the source of serum samples obtained for this study. The first group consists of serum samples from 43 normal men with no clinical evidence of prostate cancer and PSA levels <2.5 ng/ml. These men were part of a prostate cancer screening program. The second group consists of 30 normal men with PSA >2.5 ng/ml that have had repeated negative biopsies for prostate cancer. The last three groups consist of 43 men with organ confined prostate cancer, 45 men with non-organ-confined prostate cancer, and 33 men who were diagnosed with highly symptomatic BPH (AUA symptom score >20). Men with organ confined prostate cancer are classified as those at the time of surgery without any extracapsular extension, perineural invasion or seminal vesicle invasion, whereas men with non-organ-confined prostate cancer are those with either extracapsular extension, invasion

of the lymph nodes or seminal vesicle. Twenty-five of the 43 men with organ confined prostate cancer and 23 of the 45 men with non-organ-confined prostate cancer were previously used for the analysis of the first EPCA-2 epitope (EPCA-2.22). A total of 194 serum samples were screened for EPCA-2. Profiles of the patients screened for EPCA-2.19 are outlined in Table I.

Additional control groups consisted of serum samples from normal females, women with benign breast disease and breast cancer, benign and colorectal cancer, benign and liver cancer, benign and pancreatic cancer, benign and renal cancer, bladder cancer, as well as benign and lung cancer patients. The cancer patients were diagnosed with non-metastatic disease and their sera were collected prior to surgery. Profiles of the patients from the control group are listed in Table I Supplement. The serum samples were then stored at -80°C before the EPCA-2.19 ELISA assays were performed. A total of 134 control serum samples were provided as blinded samples that were decoded upon completion of the analysis.

EPCA-2.19 Pilot Study and Indirect ELISA

To evaluate the assay, initial training sets were established for EPCA-2.19 using sera from normal men with PSA <2.5 ng/ml ($n = 10$), as well as men with organ confined ($n = 10$) and non-organ-confined prostate cancer ($n = 10$). These training sets were used to develop the optimal conditions for the EPCA-2.19 assays as well as the cut-off point, which would then be applied prospectively. Once the conditions and cut-off points were reproducible from assay to assay, these studies were expanded to larger groups of men and control populations (as outlined above). Fifty microliters of serum was diluted in 50 μl of coating buffer (KPL, Gaithersburg, MD) and plated in duplicate and in random order using 96-well Nunc Immunoplate MaxiSorb plates. The serum samples and controls (IgG as positive control, blank wells, peptide controls at various concentrations, and pre-immune serum as negative control) were plated overnight at room temperature with shaking. Following overnight incubation, the samples were aspirated using a plate washer (Beckman Coulter, Fullerton, CA) and blocked in Super Block blocking buffer (Pierce, Rockland, IL) at 300 μl /well for 45 min with shaking at 37°C . The blocking buffer was removed and the plates were then washed for three times with 1X TBS with 0.05% Tween-20 (TBS-T). Antibodies for EPCA-2.19 were diluted in Super Block blocking buffer (Pierce) at 1:10 dilution. One hundred microliters of the diluted EPCA-2.19 antibodies were added to the wells with the exception of the blank and IgG wells. The plates were incubated for 2 hr at 37°C with shaking. After this incubation, the plates were rinsed three times with TBS-T. Secondary goat anti-rabbit peroxidase antibody (KPL) was diluted at 1:2,000 in Super Block blocking buffer, added at 100 μl to each well, and incubated at 37°C for 2 hr with shaking. The plates were then washed three times with TBS-T, followed by incubation with 100 μl /well of room temperature TMB substrate (KPL) for 3 min. The plates were then analyzed at 630 nm using Pherastar plate reader (BMG Lab Tech, Durham, NC). Inter-plate and intra-assay variability for each assay was verified by plating various concentrations of the EPCA-2.19 peptides (0–1 mg/ml) and rabbit IgG in duplicates in each plate. To estimate the concentration of each sample, various concentrations (0.05, 0.1, 1, 10, 100, and 1,000 ng/ml) of peptide controls for EPCA-2.19 were fitted on a sigmoidal dose–response curve using Graph Pad Prism 5 software, and the concentration was calculated from the equation generated by the curve.

Statistical Analysis

The receiver operating characteristic (ROC) curves were constructed and the area under the curve (AUC) was used to evaluate the performance of EPCA-2.19 in both the study and control populations. All statistical analyses (two-tailed, $P < 0.05$ was considered significant), as well as upper and lower bounds for confidence intervals for sensitivity and specificity

were calculated by exact methods based on the binomial distribution using MedCalc (Version 9.210, Mariakerke, Belgium) and SPSS (Version 15.0, SPSS, Chicago, IL).

RESULTS

The performance of the EPCA-2.19 immunoassays was examined in a set of serum samples consisting of normal healthy men ($n = 10$), men with organ-confined prostate cancer ($n = 10$) and men with non-organ-confined prostate cancer ($n = 10$). The pilot assays for EPCA-2.19 demonstrated that both organ-confined and non-organ-confined prostate cancer populations were distinct from the normal healthy individuals (Fig. 1). The cut-off point for EPCA-2.19 at 0.5 ng/ml was determined based upon the pilot data and applied prospectively. At the time that these studies were performed, the recombinant protein for EPCA-2.19 was not available. Thus, the concentrations that correspond to the absorbance levels were estimated by the equation that resulted from fitting various peptide concentrations to a sigmoidal curve. Upon completion of the pilot studies, inter-assay variability was measured by running repeated samples on the assays. The studies demonstrated values that are within 10% of one another. Using the established cut-point, the EPCA-2.19 assays were tested on the larger sample populations ($n = 328$) that did not include samples from the pilot study. This population of samples was distinct from that previously evaluated for the other epitope of EPCA-2 although there was a 70% overlap of the samples being used for both sets of analyses.

The mean levels of EPCA-2.19 are presented in Table I (normal healthy men, men with BPH and prostate cancer) and in Table II (for individuals with other benign diseases and other types of cancer). The individual values for EPCA-2.19 are represented in dot-plot graphs (Fig. 2). The sensitivity and specificity of EPCA-2.19 assay are summarized in Table III. The EPCA-2.19 assay at a cut-off of 0.5 ng/ml demonstrated a sensitivity of 91% (95% CI 83–96%) in detecting individuals with prostate cancer (both organ-confined and non-organ-confined). At the same cut-off, the EPCA-2.19 assay has a specificity of 94% (95% CI 88–98%) in separating normal men (both PSA <2.5 ng/ml and PSA >2.5 ng/ml) and men with BPH from those with prostate cancer (Table III). When individuals with other benign diseases and other types of cancer are taken into account, the EPCA-2.19 assay has a specificity of 98% (95% CI 95–99%).

The sensitivity and specificity values of the EPCA-2.19 assay were further analyzed by performing Receiver Operating Characteristics (ROC). As shown in Figure 3, the area under the curve (AUC) for EPCA-2.19 is 0.98 (95% CI 0.95–0.99; $P < 0.0001$) in separating normal men (with PSA < and >2.5 ng/ml) and men with BPH from those with prostate cancer (both organ-confined and non-organ-confined).

In our previous study, we showed that the first epitope of this protein, EPCA-2.22 was able to differentiate men with organ-confined prostate cancer from those with non-organ-confined disease. ROC analyses of the EPCA-2.19 immunoassays, however, did not differentiate the organ-confined from the non-organ-confined prostate cancer (AUC 0.55, 95% CI 0.44–0.66; $P = 0.275$). Twenty-five of the 43 men with organ-confined prostate cancer (58%) and 23 of the 45 men (51%) with non-organ-confined prostate cancer were used in the analyses of both EPCA-2.19 and EPCA-2.22 epitopes. ROC analyses of these men (Table IV) demonstrated that EPCA-2.19 did not differentiate men with organ confined from men with non-organ-confined prostate cancer (AUC 0.52, 95% CI 0.35–0.69, $P = 0.81$), whereas the first epitope EPCA-2.22 showed a distinct separation (AUC 0.84, 95% CI 0.72–0.96, $P < 0.0001$). In addition, the EPCA-2.19 immunoassays did not show any separation between men with high grade prostate cancer (Gleason Score of 7 or higher) from those with low grade prostate cancer (Gleason Score of 6 or less) (results not shown),

although our study population contained only a limited number of men with high Gleason scores.

DISCUSSION

Utilizing a proteomics approach that focused on nuclear structure, we have previously identified prostate cancer associated markers [2]. We have recently shown that an assay that detects the first epitope of EPCA-2 (EPCA-2.22) serves as a highly sensitive and specific marker for prostate cancer detection [7]. The purpose of our present study is to examine whether an assay which detects a second epitope of EPCA-2 (EPCA-2.19), can also be utilized to detect prostate cancer. In addition, this study may further validate EPCA-2 as a marker of prostate cancer by demonstrating that a test based upon an antibody raised to this distinct region has similar characteristics as the initial assay produced.

Utilizing a cut-off of 0.5 ng/ml and above, EPCA-2.19 has a specificity of 94% and a sensitivity of 91% in separating the normal men in the study, as well as men with BPH from those with prostate cancer. Unlike the previous assay for EPCA-2.22, the assay for EPCA-2.19 epitope is not able to differentiate men with localized and extracapsular disease. There are a couple of potential reasons to explain this difference. First, the populations utilized in this study are somewhat distinct from those in our earlier work, which may contribute to this difference. Another potential explanation is the differences in the assays. The two distinct epitopes of EPCA-2, EPCA-2.22 and EPCA-2.19 reflect different regions of the same protein. We have recently characterized this protein as an alternatively spliced member of the hnRNP family. The epitope for EPCA-2.22 is relatively unique for EPCA-2 whereas the epitope encoding EPCA-2.19 appears to contain more overlap with other members of the hnRNP family. This similarity in sequence may result in some lack of specificity in the differentiation between the organ confined and non-organ-confined prostate cancers. Since this lack of specificity is not observed in the “control” samples analyzed in these studies, we believe that it may be due to the reactivity with another hnRNP(s) associated with prostate cancer. In addition, absorbance values for the EPCA-2.19 assays were obtained after 3 min of incubation with the TMB solution. This was slightly different than the EPCA-2.22 assays, where the absorbance values were obtained 30 sec after incubation with TMB solution. Future studies on biological properties and functions of EPCA-2 epitopes and the implications they have on EPCA-2 as a prostate cancer biomarker are clearly warranted.

Overall, the findings presented in this study demonstrate that EPCA-2.19, a second epitope of prostate cancer associated nuclear proteins EPCA-2, can be utilized as a potential serum-based marker for prostate cancer detection. This study is important since it further validates EPCA-2 as a marker of prostate cancer by demonstrating that a test based upon an antibody raised to this distinct region has similar characteristics as the initial assay produced. These findings can also serve as groundwork for the development of a more standardized assay for EPCA-2 as prostate cancer serum marker, where both epitopes can be utilized in a sandwich assay format. As with the first EPCA-2 epitope, the EPCA-2.19 immunoassay results represent characterization of an assay and it is not designed to determine the performance of this assay in screening patients for prostate cancer. Future studies will be focused on multi-institutional analyses of the performance of these two assays.

Abbreviations

AUC	area under the curve
BPH	benign prostatic hyperplasia

ELISA	enzyme linked immunosorbent assay
EPCA	early prostate cancer antigen
PCa-OC	organ confined prostate cancer
PCa-NOC	non-organ-confined prostate cancer
PSA	prostate specific antigen
ROC	receiver operating characteristic

Acknowledgments

Grant sponsor: Patana Fund of the Brady Urological Institute; Grant sponsor: Prostate Cancer Foundation; Grant sponsor: NCI EDNRN; Grant number: U02CA86323.

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Fig. 1.

Pilot studies collected from 30 men show that indirect EPCA-2.19 ELISA assays using 0.5 ng/ml cut-off results in 90% sensitivity and 100% specificity in separating men with prostate cancer from those without the disease. PCa-OC: organ-confined prostate cancer, PCa-NOC: non-organ-confined prostate cancer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 2.

Using serum samples collected from 328 individuals, indirect ELISA assays demonstrate that EPCA-2.19 separates normal men as well as those with BPH from prostate cancer populations. A cut-off of 0.5 ng/ml was applied prospectively. BPH: benign prostatic hyperplasia, PCa-OC: organ-confined prostate cancer, PCa-NOC: non-organ-confined prostate cancer.

Fig. 3.

Receiver Operating Characteristic (ROC) curves for EPCA-2.19 in separating normal men and men with BPH from men with prostate cancer. ROC analyses of EPCA-2.19 results in an area under the curve (AUC) of 0.982 (95% CI 0.952 – 0.996, $P < 0.0001$).

TABLE I

Clinicopathologic Characteristics of Healthy, BPH and PCa Patients

Characteristic	Cohorts with PSA available				
	Healthy (PSA <2.5 ng/ml)	Healthy (PSA >2.5 ng/ml)	BPH	PCa (OC)	PCa (NOC)
Patients (n)	43	30	33	43	45
Age					
Median (range)	59 (43–80)	63 (45–71)	58 (40–84)	58 (45–67)	60 (43–72)
Mean $\pm$ SD	60.3 $\pm$ 8.6	60.7 $\pm$ 7.0	59.3 $\pm$ 10.9	58.3 $\pm$ 5.1	59.0 $\pm$ 6.4
Race (%)					
Caucasian	38 (88)	26 (87), 18 (55)	40 (93)	43 (96)	
African-American	4 (9)	3 (10)	4 (12)	2 (5)	0 (0)
Other	1 (2)	1 (3)	11 (33)	1 (2)	2 (4)
PSA (ng/ml)					
Median (range)	1.0 (0.1–2.2)	8.8 (2.5–39.9)	1.2 (0.1–4.1)	5.0 (1.6–11.8)	5.9 (0.8–24.8)
Mean $\pm$ SD	1.1 $\pm$ 0.6	8.8 $\pm$ 6.7	1.4 $\pm$ 0.9	5.2 $\pm$ 2.2	7.3 $\pm$ 4.6
EPCA-2.19 (ng/ml)					
Median (range)	0.11 (0.04–0.36)	0.15 (0.07–0.43)	0.23 (0.10–2.23)	1.71 (0.27–22.72)	2.11 (0.31–10.30)
Mean $\pm$ SD	0.13 $\pm$ 0.07	0.18 $\pm$ 0.09	0.35 $\pm$ 0.37	2.94 $\pm$ 3.87	2.62 $\pm$ 2.27
Clinical stage (%)					
cT1	N/A	N/A	N/A	36/41 (88)	32/45 (71)
cT2	N/A	N/A	N/A	5/41 (12)	13/45 (29)
cT3	N/A	N/A	N/A	0/41 (0)	0/45 (0)
Biopsy Gleason sum (%)					
2–6	N/A	N/A	N/A	33 (77)	27 (60)
7	N/A	N/A	N/A	9 (21)	16 (36)
8–10	N/A	N/A	N/A	1 (2)	2 (4)
Prostatectomy Gleason sum (%)					
2–6	N/A	N/A	N/A	32 (74)	19 (42)
7	N/A	N/A	N/A	10 (23)	25 (56)
8–10	N/A	N/A	N/A	1 (2)	1 (2)
Extraprostatic extension	N/A	N/A	N/A	0 (0)	45 (100)

Cohorts with PSA available					
Characteristic	Healthy (PSA <2.5 ng/ml)	Healthy (PSA >2.5 ng/ml)	BPH	PCa (OC)	PCa (NOC)
Seminal vesicle invasion	N/A	N/A	N/A	0 (0)	4 (9)
Lymph node invasion	N/A	N/A	N/A	0 (0)	1 (2)
Positive surgical margin	N/A	N/A	N/A	2 (5)	9 (21)

Clinicopathologic Characteristics of Healthy Individuals and Patients With Diseases Other Than BPH and PCa

TABLE II

Patient cohort	n	Males (%)	Mean age (years) ± SD	Median age (years), RANGE	Mean EPCA-2.19 (ng/ml ± SD)	Median EPCA-2.19 (range)
Healthy females	10	0 (0)	39.5 ± 11.0	43 (23–55)	0.26 ± 0.12	0.23 (0.12–0.42)
Benign breast	9	0 (0)	53.4 ± 11.3	50 (36–68)	0.22 ± 0.14	0.18 (0.05–0.46)
Breast cancer	10	0 (0)	55.4 ± 15.1	51 (37–82)	0.15 ± 0.08	0.12 (0.06–0.34)
Benign colon	9	4 (44)	59.2 ± 10.4	56 (42–75)	0.21 ± 0.11	0.20 (0.06–0.39)
Colorectal cancer	10	8 (80)	63.4 ± 13.0	63 (47–87)	0.25 ± 0.14	0.23 (0.05–0.46)
Benign liver	10	3 (30)	51.6 ± 13.9	50 (27–74)	0.23 ± 0.10	0.20 (0.09–0.41)
Liver cancer	10	9 (90)	64.6 ± 9.4	66 (51–76)	0.23 ± 0.11	0.23 (0.07–0.41)
Benign pancreas	10	6 (60)	62.7 ± 18.9	64 (17–85)	0.21 ± 0.13	0.18 (0.06–0.47)
Pancreatic cancer	10	7 (70)	71.6 ± 6.7	71 (60–80)	0.22 ± 0.13	0.18 (0.05–0.42)
Benign renal	10	4 (40)	44.1 ± 15.1	45 (18–69)	0.12 ± 0.06	0.10 (0.07–0.24)
Renal cell cancer	9	8 (89)	58.3 ± 12.7	62 (37–71)	0.18 ± 0.13	0.16 (0.05–0.44)
Bladder cancer	9	8 (89)	67.7 ± 8.5	69 (58–80)	0.20 ± 0.11	0.22 (0.06–0.37)
Benign lung	9	9 (100)	53.4 ± 12.3	56 (35–71)	0.21 ± 0.08	0.22 (0.11–0.34)
Lung cancer	9	9 (100)	65.2 ± 10.8	69 (49–78)	0.19 ± 0.11	0.16 (0.08–0.40)

TABLE III

Specificity and Sensitivity of PSA and EPCA-2.19

Population	Specificity $\pm$ SE (95% CI)		
	PSA (n) (2.5 ng/ml cut-off)	PSA (4.0 ng/ml cut-off)	EPCA-2.19 (0.5 ng/ml cut-off)
Normal males and BPH (106)	67 $\pm$ 4.6 (57–76)	73 $\pm$ 4.3 (63–81)	94 $\pm$ 2.2 (88–98)
Normal males, BPH and other diseases (240)	N/A	N/A	98 $\pm$ 1.0 (95–99)
Population (n)	Sensitivity $\pm$ SE (95% CI)		
	PSA (2.5 ng/ml cut-off)	PSA (4.0 ng/ml cut-off)	EPCA (0.5 ng/ml cut-off)
Prostate cancer, OC and NOC (88)	95 $\pm$ 2.2 (89–99)	78 $\pm$ 4.4 (68–86)	91 $\pm$ 3.1 (83–96)
Prostate cancer, OC (43)	93 $\pm$ 3.8 (81–99)	67 $\pm$ 7.2 (51–81)	88 $\pm$ 4.9 (75–96)
Prostate cancer NOC (45)	98 $\pm$ 2.2 (88–99)	89 $\pm$ 4.7 (76–96)	93 $\pm$ 3.7 (82–99)

TABLE IV

ROC Analyses of EPCA-2.19 and EPCA-2.22 on Men With Organ Confined (n = 25) and Non-Organ-Confined Prostate Cancer (n = 23) Cohort Groups

	EPCA-2.19	EPCA-2.22
Area under the curve (AUC)	0.52	0.84
95% CI	0.35–0.69	0.72–0.96
<i>P</i> -value	0.81	<0.0001