

Detection of Prostate Cancer with a Blood-Based Assay for Early Prostate Cancer Antigen

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Abstract

Prostate-specific antigen lacks specificity for prostate cancer, so the identification and characterization of a unique blood-based marker for the disease would provide for a more accurate diagnosis, reducing both unnecessary biopsies and patient uncertainty. We previously identified a novel biomarker for prostate cancer, early prostate cancer antigen (EPCA). EPCA antibodies positively stained the negative biopsies of men who, as much as 5 years later, were diagnosed with prostate cancer. The goal of this study was to determine whether EPCA antibodies could be used in a clinically applicable plasma-based immunoassay to specifically detect prostate cancer. Using an EPCA-based ELISA, the protein was measured in the plasma of 46 individuals, including prostate cancer patients, healthy individuals, other cancer patients, spinal cord injury victims, and patients with prostatitis. With a predetermined cutoff value of 1.7 absorbance at 450 nm, only the prostate cancer population, as a whole, expressed plasma-EPCA levels above the cutoff. Statistical analysis showed a significant difference in EPCA levels between the prostate cancer population and each of the other groups, specifically the healthy donors ($P < 0.0001$), bladder cancer patients ($P = 0.03$), and spinal cord injury patients ($P = 0.001$). Sensitivity of the EPCA assay for prostate cancer patients was 92% whereas the overall specificity was 94%. Specificity for the healthy donors was 100%. Although larger trials are required, this initial study shows the potential of EPCA to serve as a highly specific blood-based marker for prostate cancer. EPCA, when coupled with prostate-specific antigen, may help reduce the number of both unnecessary biopsies and undetected prostate tumors. (Cancer Res 2005; 65(10): 4097-100)

Introduction

The identification of specific cancer biomarkers can have a significant effect on the prognosis, diagnosis, and treatment options for patients. Prostate cancer is the most commonly diagnosed cancer in men living in the United States, and it is the second leading cause of cancer death in that same population (1). Prostate-specific antigen (PSA) and digital rectal exams remain the hallmark assessments for screening individuals for prostate cancer. The combined use of these diagnostic techniques has changed the clinical course of the disease by allowing for an earlier detection of tumors (2). However, PSA has limited specificity in that it is not a tumor marker, but actually a protease that is normally expressed in the organ. Abnormal PSA values, specifically those in the 4 to

10 ng/mL range, can occur in men with benign prostate conditions such as benign prostate hyperplasia and prostatitis. Indeed, only 25% of individuals that undergo biopsies are actually positive for prostate cancer (2). Conversely, low PSA findings are not always a true indicator of a healthy prostate. A recent study showed that ~15% of individuals with PSA levels below 4 ng/mL have prostate cancer as detected by biopsy (3). Therefore, more specific prostate cancer biomarkers need to be identified.

Traditional diagnosis of cancer by a pathologist relies on architectural alterations in the cells and/or tissues (4). Many of these changes occur in the nucleus, including an increase in nuclear size, a deformed nuclear shape, variations in patterns of lamin expression, and the alterations in the composition of nuclear matrix proteins (NMP). The nuclear matrix is responsible for maintaining nuclear shape, function, and organization of its components. Whereas some NMPs are common to all cell types and physiologic states, others are tissue specific or altered with the state of the cell (5). There have been several NMPs isolated and characterized that are specific for individual cancers. For example, Partin and colleagues have identified both PC-1 and YL-1 as NMPs unique to prostate cancer (6, 7). Additionally, we have developed a urine assay for the detection of bladder cancer based on BLCA-4, which is one of six unique bladder cancer NMPs identified in our laboratory (8).

Early prostate cancer antigen (EPCA) is a novel prostate cancer biomarker recently discovered in our laboratory. Antibodies directed against EPCA positively stained the negative biopsies of men who, as much as 5 years later, were diagnosed with prostate cancer (9). A significant difference existed in EPCA staining intensity between tumor tissue from the prostate cancer population and tissue from the donor controls. At the same time, normal adjacent prostate tissue from cancer patients also had significantly higher EPCA staining when compared with the donor controls, indicating the presence of a field effect (9). The purpose of these studies was to determine whether EPCA can function as a highly specific and sensitive serum-based biomarker for prostate cancer. Using an indirect ELISA approach, plasma from prostate cancer patients was compared with samples from healthy donors, other cancer patient populations, and patients with benign urological conditions.

Materials and Methods

Patients. Plasma samples were obtained from consenting patients under an Institutional Review Board–approved protocol. Samples from 12 patients that had undergone radical prostatectomies and 16 nondiseased individuals were examined. In addition, samples from bladder (6 samples), colon (2 samples), and renal (1 sample) cancer patients, as well as specimens from spinal cord injury victims (7 samples) and those presenting with a diagnosis of prostatitis with no other urological symptoms (2 samples) were analyzed. See Table 1A and B for a summary of the patient characteristics.

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Table 1. Characteristics of patients studied

(A) Prostate cancer patient profile							
Patient	Age range	PSA	Gleason score	Pathology stage	Extracapsule extension	Perineural invasion	Seminal vesicle invasion
GB73	50-59	4.33	6	pT _{2c} N ₀ M _x	No	Yes	No
GB79	50-59	4.7	7	pT _{2c} N ₀ M _x	No	Yes	No
GB83	50-59	8.3	7	pT _{2c} N ₀ M _x	No	Yes	No
GB84	60-69	7.1	6	pT _{2c} N ₀ M _x	No	No	No
GB100	60-69	8.5	7	pT _{3a} N ₀ M _x	Yes	Yes	No
GB101	60-69	12.1	7	pT _{2a} N ₀ M _x	No	Yes	No
GB102	60-69	4.9	7	pT _{3a} N ₀ M _x	Yes	Yes	No
GB122	60-69	6.2	6	pT _{2c} N ₀ M _x	No	No	No
GB136	50-59	5.1	7	pT _{2c} N ₀ M _x	No	No	No
GB145	50-59	na	6	pT _{2c} N ₀ M _x	No	Yes	No
GB148	60-69	5.5	6	pT _{2a} N ₀ M _x	No	No	No
PR163	70-79	7	9	pT _{3b} N ₁ M _x	Yes	Yes	Yes
(B) All other patient profiles							
Donor	Age range	Bladder cancer	Age range				
E242	20-29	#1	60-69				
FB499	30-39	#2	80-89				
FB519	20-29	#3	70-79				
E460	10-19	#4	80-89				
B1091	10-19	#5	70-79				
GB01-1	10-19	#6	50-59				
GB02-05	50-59	Colon cancer	Age range				
GB02-2	10-19	RS01-002	na				
F437	20-29	RS001-078	na				
GB02-52	60-69	Renal cancer	Age range				
E806	40-49	#1	50-59				
EB671	20-29	Spinal cord injury	Age range				
GB02-21	60-69	#1531	50-59				
PR32	10-19	#1532	40-49				
PR33	40-49	#1534	40-49				
G009	30-39	#1535	40-49				
Prostatitis	Age range	#1536	40-49				
#38	na	#1537	40-49				
#39	50-59	#1538	30-39				

Indirect ELISA. Using Nunc Immunoplate Maxisorb plates, 100 μ L of plasma per well, in triplicate, were allowed to incubate at room temperature overnight. As a positive control, 100 μ L of unlabeled rabbit immunoglobulin G (IgG), diluted 1:1,000 with 1 \times TBS, were plated overnight as well. The following day, all wells were washed 3 \times with 400 μ L of sterile water. All wells, with the exception of the blank wells, were blocked with 200 μ L of 1% bovine serum albumin (BSA) blocking solution (1 \times TBS, 1% BSA, 1% dry milk, and 0.05% Tween 20) for 30 minutes. After blocking the wells, rinsing again took place before the addition of the primary antibody. The primary antibody for the sample wells consisted of 100 μ L of diluted polyclonal anti-EPCA (previously described in ref. 9) in 2.5% BSA blocking solution. The negative control wells contained rabbit preimmune serum. Following a 2-hour incubation period at room temperature, the plate was emptied, washed with sterile water (400 μ L, 3 \times), and then secondary antibody was added to all the wells for another 2 hours. The secondary antibody applied was 1 mg/mL goat anti-rabbit IgG-horseradish peroxidase (KPL, Baltimore, MD), diluted 1:5,000 with 2.5% BSA blocking solution. After washing wells with sterile water (3 \times 400 μ L), 100 μ L of 3,3',5,5'-tetramethylbenzidine (KPL), which had been diluted 1:1 with sterile water, were added to each well and allowed to react for 4 minutes. The reaction was stopped with 100 μ L of stop solution (KPL) and absorbance was read at 450 nm on a Bio-Rad (Hercules, CA) microplate reader.

Statistical analysis. Differences across all groups as a whole were tested using the Kruskal-Wallis test. Because an overall significant difference existed ($P = 0.0001$), pairwise differences were tested using the Wilcoxon rank-sum test. Nonparametric tests were favored because of the small sample sizes and distributions that may not be normal. No adjustments were made for multiple comparisons because the study was exploratory in nature.

Results

Using anti-EPCA antibodies previously described (9), we developed an indirect ELISA to measure the level of EPCA in the plasma of various patient populations. Plasma samples were obtained from patients diagnosed with prostate cancer, bladder cancer, spinal cord injuries, colon cancer, prostatitis, and renal cell carcinoma, as well as from healthy donors. A predetermined cutoff of 1.7 absorbance was chosen based on the measurements of plasma-EPCA in a prostate cancer and healthy donor training set. Figure 1 clearly shows that only the prostate cancer population, as a whole, expressed plasma-EPCA levels above the set cutoff. Indeed, a Wilcoxon rank-sum analysis showed a highly significant

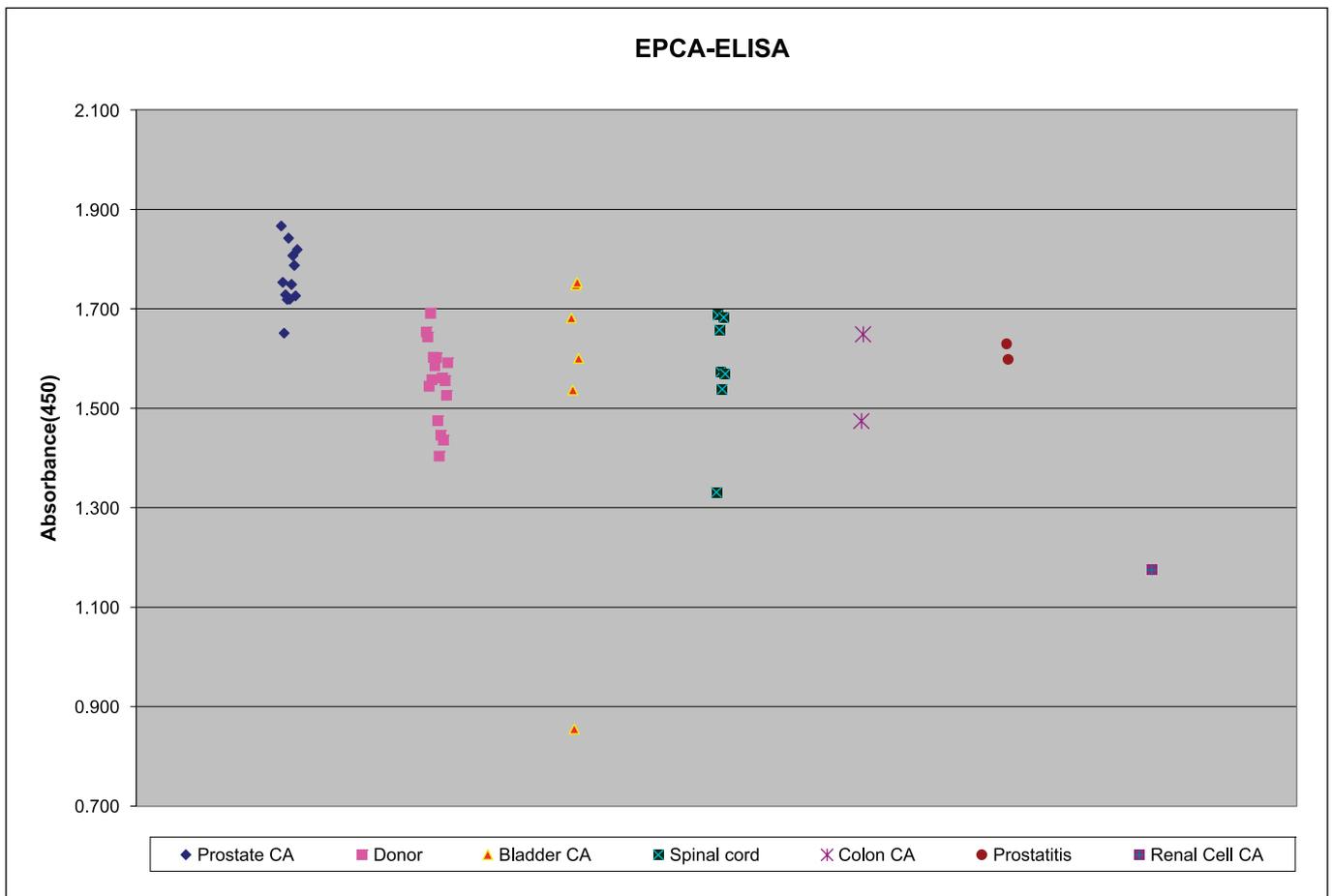


Figure 1. Plasma-EPCA levels. Plasma-EPCA levels were measured in various patient populations using the EPCA ELISA. Each sample was done in triplicate and reported as the mean. Representative of two independent experiments.

difference in plasma-EPCA levels between the prostate cancer population and each of the other groups, specifically populations comprised of healthy donors ($P < 0.0001$), bladder cancer patients ($P = 0.03$), and spinal cord injury patients ($P = 0.001$; Table 2). No significant difference was seen between these nonprostate cancer populations (Table 2).

Cancer diagnostic assays must be both sensitive and specific. Our EPCA blood-based assay detected plasma-EPCA levels above 1.7 absorbance in 11 of 12 prostate cancer patients, demonstrating

a sensitivity of 92%. None of the healthy donors had plasma-EPCA levels above 1.7 absorbance, thus, specificity for this population was 100% (Table 3). Furthermore, when considering the entire study population, only two bladder cancer patients presented with plasma-EPCA levels above 1.7 absorbance, resulting in an overall specificity of 94% (Table 3).

PSA levels for each of the men studied were compared with their EPCA levels. Although an inverse correlation was identified, there is no apparent significance because most of the PSA levels fell within the clinically indistinguishable range of 4–10 ng/mL (data not shown). In all but one case, the Gleason grade was either 6 or 7 (Table 1). Not surprisingly, no statistically significant correlation existed between the Gleason grade and the EPCA level (data not shown).

Population pairs	Significance (P)
Prostate cancer vs donor	<0.0001
Prostate cancer vs bladder cancer	0.03
Prostate cancer vs spinal cord injury	0.001
Donor vs bladder cancer	0.29
Donor vs spinal cord injury	0.45
Bladder cancer vs spinal cord injury	0.73

NOTE: Wilcoxon rank-sum analysis comparing differences in plasma-EPCA levels in individual pairs of populations.

Discussion

Early treatment of cancer can result in a significant decrease in disease-related morbidity and mortality; therefore, it is imperative to develop assays capable of detecting cancer in its preliminary state. Elevated serum PSA and digital rectal exams remain the primary diagnostic tools for the early detection of prostate cancer. However, several problems are associated with the use of PSA. Patients with benign prostate conditions often present with elevated serum PSA levels, which leads to unnecessary biopsies. At the same time, prostate tumors do not always produce increased levels of PSA. To address this problem, we developed a serum-based

Table 3. Specificity of blood EPCA assay

	No. samples <1.7 absorbance / total no. samples	Specificity (%)
Donors	16/16	100
All populations	32/34	94

immunoassay able to detect the prostate cancer-specific biomarker, EPCA. The assay employed antibodies previously shown to stain the negative biopsies of men who were eventually diagnosed with prostate cancer (9).

This study shows that the EPCA ELISA is both sensitive and specific for prostate cancer. Despite a small sample size, there was a statistically significant increase in serum EPCA levels in samples obtained from prostate cancer patients as compared with samples from healthy donors, bladder cancer patients, and spinal cord injury victims. Furthermore, the vast majority of prostate cancer patients (11 of 12) had plasma-EPCA levels above the preestablished cutoff. Obviously, a larger study is required for definitive conclusions and we are now in the process of initiating such a study. The EPCA ELISA also showed a high specificity for prostate cancer although a complex series of samples representing "real world" patient scenarios was used. Indeed, the overall specificity was 94%. Plasma obtained from two bladder cancer patients were the only samples falsely positive for EPCA. Unlike this study, men suffering from other malignancies normally represent only a small fraction of the total number of men screened for prostate cancer. Thus, if the sample sizes used for this study more accurately reflected the typical population screened for prostate cancer, then it could be argued that the specificity would be closer to 100%. Importantly, the two patients diagnosed with prostatitis, a disorder often associated with elevated PSA, had plasma-EPCA levels below the cutoff. If this trend remains consistent in a larger study, then this assay would help differentiate prostate cancer from more benign prostate conditions. Although these studies examined plasma EPCA levels, serum levels of the marker seem similar (data not shown). Using immunohistochemical staining, it seems that prostatic intraepithelial neoplasia (PIN) and proliferative inflammatory atrophy (PIA) do indeed express EPCA in individuals with prostate cancer. The studies described here were not designed to examine whether individuals with PIN or PIA alone have elevated EPCA serum levels but from our control individuals that do have PIN lesions but not prostate cancer, it would seem

that they do not. Therefore, from this work, it would appear that PIN or PIA alone is not sufficient to result in detectable serum levels of EPCA.

The reason that this assay does not result in 100% sensitivity is not easily apparent from our studies. Several reasons are possible for this result. Among them is the fact that even at the immunohistochemical level, not all prostate cancers express EPCA. A small percentage seem to not express the marker. Secondly, the tumor burden in these individuals may be so small that the assay is not able to detect the marker. This is being addressed by increasing the sensitivity of the assay with the utilization of different detection reagents. Even if the sensitivity is not increased, this should not limit the utility of the assay in that it can be used in conjunction with PSA, which has a high sensitivity, and will still detect almost all men with prostate cancer. There is no reason to believe that these results will be less promising in an even larger sample set. Previous immunohistochemical studies do not reveal a correlation between EPCA staining and tumor stage. This is further supported by a manuscript from an independent group from Japan (10). It seems that EPCA expression occurs early during the development of cancer and that this expression stays on in the tumor. A slightly different question is whether blood levels of EPCA correlate with the amount of cancer. Whereas this study does not directly examine individuals with large tumor burdens, our preliminary studies have revealed an increased expression of EPCA in the serum of men with metastatic disease.

The age range of the donor population was considerably less than that of the prostate cancer population (Tables 1A and B), which could contribute to the difference in EPCA levels observed between the two groups. However, other patient populations consisted of individuals more closely matched in age to the prostate cancer patients (Table 1B) and the differences in EPCA levels remained between these groups, indicating that age probably does not influence EPCA levels.

This is the first study demonstrating the ability of EPCA antibodies to specifically identify prostate cancer patients in a clinically applicable test. Whereas larger trials need to be done, the sensitivity and specificity of the EPCA ELISA, coupled with the ability of EPCA antibodies to identify individuals with prostate cancer before a positive biopsy, make EPCA potentially a very powerful complement to PSA for the early diagnosis of prostate cancer.

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