Diagnostic Potential of Prostate-specific Antigen Expressing Epithelial Cells in Blood of Prostate Cancer Patients[1]

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ABSTRACT

Purpose: **Prostate-specific antigen (PSA) test has become a widely used screening test in prostate cancer (CaP). However, low specificity of serum PSA leads to many false-positive and false-negative results and clinical uncertainty. Development of CaP-specific diagnostic and prognostic markers is needed. Detection of circulating *PSA*-expressing cells (CPECs) in blood and bone marrow of CaP patients has potential in molecular diagnosis and prognosis. Our novel observations of the frequent presence of CPECs in CaP patients with organ-confined disease by reverse transcription (RT)-PCR-*PSA* assay in epithelial cells enriched from peripheral blood (ERT-PCR/*PSA*) have led us to test the hypothesis that CPECs have diagnostic potential for CaP.**

Experimental Design: **Epithelial cells from peripheral blood of radical prostatectomy patients or prostate biopsy patients were isolated using antiepithelial cell antibody, Ber-EP4-coated magnetic beads, and total RNA specimens from these cells were analyzed for *PSA* expression by RT-PCR.**

Results: **Peripheral blood specimens of 108 of 135 (80.0%) CaP patients were positive in ERT-PCR/*PSA* assay. Peripheral blood specimens from 45 control men were virtually negative (97.8%). In the blinded investigation, 84 patients who had biopsy for suspicion of CaP were evaluated by ERT-PCR/*PSA* assay. Eighteen of 22 (81.8%) patients with biopsy-proven CaP were positive, and 54 of 62 (87.1%) patients with biopsy negative for CaP were negative in this assay ($P < 0.001$).**

Conclusions: **Our study provides intriguing novel results showing that the majority of patients with clinically organ-confined CaP contain CPECs. Strong concordance between the biopsy results and ERT-PCR/*PSA* assay (sensitivity 81.8%; specificity 87.1%) suggests a potentially new diagnostic application of this type of assay in CaP diagnosis.**

INTRODUCTION

Detection of small number of cancer cells or tumor derived DNA in body fluids by sensitive PCR-based methods or the isolation of tumor cells from body fluids followed by immunohistochemical or fluorescent *in situ* hybridization analyses holds promise in molecular diagnosis and prognosis of cancer (1–3). Elevated serum levels of the prostate gland-specific protein, PSA,[3] is currently the most powerful and widely used CaP screening test (4). However, the utility of serum PSA test is limited because of high false-positive rates and up to 75% of patients with elevated serum PSA may not have cancer (4). Percentage-free PSA, complexed PSA, age-adjusted PSA, PSA velocity, and PSA density have added some accuracy, but sensitivity and specificity are still suboptimal (5). Additional CaP-specific molecular and cellular markers are clearly needed to enhance the diagnosis and prognosis of CaP (6). Exploiting the highly tissue-specific expression of *PSA* gene in normal and malignant prostatic epithelial cells, Moreno *et al.* (7) and Vessella *et al.* (8) reported the first studies of the detection of CPECs in blood of CaP patients by RT-PCR/*PSA* assay. Katz *et al.* (9–11) subsequently reported very promising results showing significant correlation of CPECs with nonorgan-confined disease in CaP patients who had RP. However, a number of reports could not demonstrate the clinical value of CPECs (12–19). Although the presence of CPECs in bone marrow has shown some correlation with early cancer recurrence after RP, additional studies are warranted (20, 21). A recent study (22) describes correlation between proliferation of CaP cells in bone marrow and recurrence in patients with localized CaP. *PSA*-expressing cells in lymph nodes have also been evaluated, and some studies showed a useful pretreatment prognostic value for patients undergoing RP (23–25).

Ideally, peripheral blood is the specimen of choice for a molecular marker-based test. However, diagnostic or prognostic value of CPECs as detected by RT-PCR-based assays remains uncertain and controversial (26, 27). To increase the sensitivity and specificity of detection of CPECs in CaP patients, we have now enriched the epithelial cells present in peripheral blood by

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[3] The abbreviations used are: PSA, prostate-specific antigen; CaP, prostate cancer; RT-PCR, reverse transcription-PCR; RP, radical prostatectomy; CPEC, circulating PSA-expressing cell.

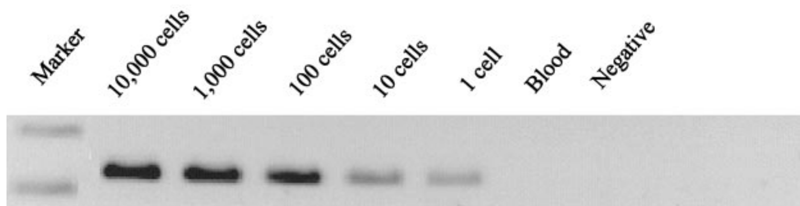

Fig. 1 Sensitivity of ERT-PCR/*PSA* assay: ERT-PCR/*PSA* sensitivity was determined by spiking of 1 ml of normal human female blood with known number of LNCaP cells. The detection limit was calibrated to detect as low as one cell in 1 ml of blood.

immunomagnetic bead capture followed by RT-PCR/*PSA* assay. Here, we provide data showing presence of CPECs in a very high proportion of CaP patients and additionally demonstrate the diagnostic potential of the ERT-PCR/*PSA* for CaP.

MATERIALS AND METHODS

Cells. LNCaP cancer cells, used as positive control for PSA expression or used for determining sensitivity of the ERT-PCR/*PSA* assays, were obtained from American Type Culture Collection (Manassas, VA) and were grown as recommended by supplier.

Blood Specimens. Peripheral blood from 135 CaP patients were obtained before incision in the operating room from patients undergoing RP at Walter Reed Army Medical Center. Peripheral blood of 84 biopsy patients with suspicion of CaP and recommended for biopsy were obtained before biopsy procedure. Biopsy was performed in a standardized manner in all patients with elevated serum PSA and/or abnormal digital rectal test; all of the patients had a minimum of 10 cores/biopsy (28). Blood specimens from 45 controls included men with normal serum PSA and normal digital rectal test or men with no evidence of cancer. None of the patients undergoing RP included in this study had received neoadjuvant hormonal therapy before blood extraction. All patients provided written informed consent and protocols were approved by the human use committee of the Department of Clinical Investigation, Walter Reed Army Medical Center.

Pathological Analysis of Surgical Specimen. The prostatectomy specimens were formalin-fixed for a minimum of 48 h before sectioning. The cut surfaces were inked, and the specimens cut at 2.25-mm intervals with a deli slicer. They were paraffin-embedded as whole mounts. Five-μm sections were stained with H&E. Each of the tumors was graded according to the WHO/Mostofi and Gleason grading systems and recorded separately. The margin status was recorded, and the tumors were staged according to the tumor-node-metastasis system (from 1997 on, both the 1992 and 1997 systems were applied to allow correlation of all of the stages). Also, the presence of benign glands in the surgical margin was reported.

Immunomagnetic Capture of Epithelial Cells from Blood and RNA Preparation. Five ml of blood were collected into a sodium citrate tube and transported to the laboratory on wet ice within 2–3 h after the blood was drawn. Eighty-μl suspensions of Dynabeads coated with Ber-EP4 monoclonal antibody, specific for enrichment of human epithelial cells (Dynal, Inc., Lake Success, NY), was used for isolation of epithelial cells from 5 ml of blood per supplier's procedures. RNA from rosetted cells on the beads was prepared using RNAzol B (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's recommendations. RNA was precipitated by isopropanol using 20 μg of glycogen as carrier and dissolved in 10 μl of diethyl pyrocarbonate water and stored at −80°C. Details of the procedure are available online.[4]

RT-PCR. Three μl of total RNA were reverse transcribed into cDNA, using random hexamer primer and murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA) in 20 μl volume, following the supplier's recommendations. PCR primers for *PSA* expression were same as described earlier (19) and information is also available on our web site.[4] The 25-μl volume of the PCR reaction mixture included 5 μl of cDNA, 1.0 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate, 25 ng of sense and antisense primers, and 0.5 units of AmpliTaq Gold Polymerase (Applied Biosystems). PCR amplification protocol included one cycle at 95°C for 10 min, 25 cycles at 94°C for 30 s, 66°C for 1 min, and 72°C for 1 min, followed by one cycle at 72°C for 5 min. Hemi-nested PCR reaction for *PSA* included 2 μl of the first PCR reaction, 25 ng each of nested sense primer, and same antisense primer used in the first PCR reaction. Other components and PCR cycles were the same as for the first PCR reaction.

Three μl of RNA aliquots from each patient sample were processed for two identical RT-PCR reactions and one control reaction without RT. PCR controls also included a no template PCR as control for reagents. Analysis of the RT-PCR-derived *PSA* fragment included (*a*) visual detection of the expected size DNA bands by SYBR Gold staining of Tris-borate EDTA-10% polyacrylamide gel from hemi-nested PCR reactions and (*b*) direct DNA sequencing of randomly selected PCR products confirming their identity as *PSA*. Quality control of Ampli TaqGold and reverse transcriptase enzymes was considered crucial to detect one *PSA*-expressing LNCaP cell/ml of blood. Stringent precautions were followed to avoid PCR contamination and carry over and were essentially the same as described earlier (21). Detailed protocols are available at our web site.[4]

Clinical Information. All patients with CaP in this study are enrolled in the Department of Defense, Center for Prostate Disease Research Triservice Multicenter Prostate Disease Longitudinal Research Database as described previously (29). Standardized data forms were used prospectively to collect demographic, clinical and pathologic stage, biopsy and RP grade, and laboratory information.

Statistical Analysis. Variation in ERT-PCR/*PSA* assay positivity by clinicopathological features and patient demo-

[4] Internet address: www.cpdr.org/Research/ertpcrpsa.html.

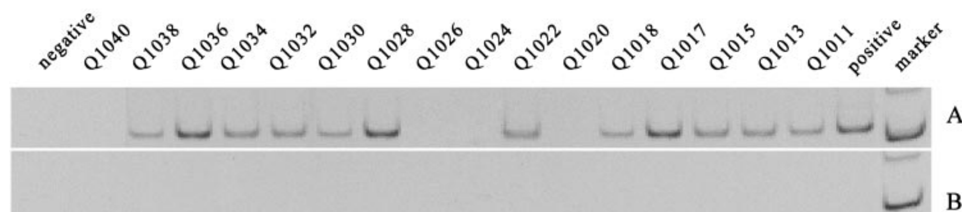

Fig. 2 ERT-PCR/*PSA* assay from peripheral blood of CaP patients: a representative ERT-PCR/*PSA* assay is shown here, *A* is with ERT-PCR/*PSA* assay with RT, and *B* is ERT-PCR/*PSA* with no-RT control. Patient samples Q1020, Q1024, Q1026, and Q1040 were negative, and the other samples were positive in this assay.

graphics was examined for statistical significance using Fisher's exact test for nominal variables and trend tests for ordinal and continuous variables. For 84 biopsy patients, sensitivity, specificity, and several other measures of association were calculated and used to measure the accuracy of the ERT-PCR/*PSA* assay in identifying patients with CaP. Variation in these measures by the clinical and demographic characteristics of the patients was examined in stratified analyses and with logistic regression.

RESULTS

High Frequency of Detection of CPECs in CaP Patients by ERT-PCR/PSA Assay. Sensitivity limit of the ERT-PCR/PSA assay used here was calibrated to detect one LNCaP cell/ml of whole blood (Fig. 1). Blood-derived epithelial cell RNA specimens from 135 CaP patients and 45 controls were analyzed in ERT-PCR/*PSA*. A total of 108 of 135 (~80.0%) CaP patients was ERT-PCR/*PSA* positive (Fig. 2, Table 1). Reproducibility of the ERT-PCR/*PSA* assay was 90% in two independent RT-PCR reactions. Only one of the control RNA samples from 45 men was positive in ERT-PCR/*PSA* assay (2.2%). In contrast to the high percentage (80%) of CaP patients showing CPECs in this study, we as well as others (12–19) have previously reported a much lower frequency (23–30%) of CPECs in CaP patients with organ-confined disease by RT-PCR/*PSA* assays that did not include epithelial cell enrichment. Except for patient age and pathologic grade of surgical specimen, no significant relationship of ERT-PCR/*PSA* positivity with ethnicity, pretreatment serum PSA, Gleason score of surgical specimen, and surgical margin status was observed in this patient cohort (Table 1). Because a very large proportion (80%) of patients harbored CPECs, we did not anticipate an association of positive ERT-PCR/*PSA* assay to specific clinicopathological features; however, qualitative or quantitative characterization of CPECs may provide additional prognostic use. Our preliminary results suggest that quantitative assessment of CPECs have significant association with PSA recurrence after RP (Gao *et al.*, unpublished observations). Age at surgery ranged from 39 to 76 years with a mean and median age of 60 years. Positivity was 75.0% for patients < 50 years of age, increased to 90.0% for 50–54-year-olds, then steadily declined to 61.5% for those 70 years and older (Table 1). When age at surgery was treated as a continuous independent variable in a third degree polynomial logistic regression model with ERT-PCR/*PSA* positivity as the dependent variable, the quadratic age term was statistically significant ($P = 0.038$). Positivity increased with increasing WHO grade, a nearly significant trend ($P = 0.054$).

Table 1 Relationship of ERT-PCR/*PSA* positivity to clinicopathological features of clinically localized stage CaP patients before radical prostatectomy

	Patient no.	ERT-PCR/*PSA* positive (%)
Overall	135	108 (80.0)
Age ($P = 0.150$ for linear trend test)[a]		
<50	16	12 (75.0)
50–54	20	18 (90.0)
55–59	34	29 (85.3)
60–64	26	22 (84.6)
65–69	26	19 (73.1)
70+	13	8 (61.5)
Ethnicity ($P = 1.000$ for Fisher's exact test)		
African American	33	27 (81.8)
White and others	102	81 (79.4)
Surgical margin status ($P = 1.000$ for Fisher's exact test)		
Negative	98	78 (79.6)
Positive	35	28 (80.0)
Data not available	2	
Surgical specimen Gleason score ($P = 0.268$ for linear trend test)		
4,5	6	3 (50.0)
6	57	45 (79.0)
7	51	45 (88.2)
8,9	16	12 (75.0)
Data not available	5	
Surgical specimen grade ($P = 0.054$ for linear trend test)		
Well	26	18 (69.2)
Moderate	56	45 (80.4)
Poor	49	43 (87.8)
Data not available	4	
Pretreatment serum PSA ng/ml ($P = 0.740$ for linear trend test)		
0–4	33	25 (75.8)
4.1–7	58	50 (86.2)
7.1–10	24	18 (75.0)
10+	16	12 (75.0)
Data not available	4	
Pathological T stage ($P = 0.708$ for linear trend test)		
T_{1b}, T_{1c}	76	60 (79.0)
T_{2a}	35	30 (85.7)
T_{2b}	13	11 (84.6)
T_{2c}, T_{3b}	9	7 (77.8)
Data not available	2	
Biochemical recurrence ($P = 0.0757$ for Fisher's Exact test)		
Recurrence positive	18	12 (66.6)
No recurrence	113	93 (82.3)
Data not available	4	

[a] Statistical significance increases ($P = 0.038$) when age is treated as a continuous independent variable using third degree polynomial logistic regression.

Diagnostic Potential of ERT-PCR/*PSA* Assay in Patients undergoing Biopsy for Suspicion of CaP. Because a majority of the CaP patients showed the presence of CPECs in blood, we hypothesized that the ERT-PCR/*PSA* may have potential in the

Table 2 Relationship of ERT-PCR/*PSA* and biopsy results in patients undergoing biopsy for suspicion of CaP[a]

ERT-PCR/*PSA* assay	Biopsy-positive number (%)	Biopsy-negative number (%)	Total no.
Positive	18 (81.8%)	8 (12.9%)	26
Negative	4 (18.2%)	54 (87.1%)	58

[a] ($P < 0.001$). Sensitivity: 81.8%; specificity: 87.1%; positive predictive value: 69.3%; negative predictive value: 93.2%; and accuracy: 85.7%.

Table 3 Relationship of sensitivity and specificity of ERT-PCR/*PSA* assay to age, ethnicity, pretreatment PSA, and biopsy history

	No.	Sensitivity (%)	Specificity (%)
Total	84	81.8	87.1
Age ($P = 0.918$)			
0–54	21	66.7	88.9
55–64	30	75.0	92.3
65+	33	86.7	77.8
Ethnicity ($P = 0.567$)			
Black	22	87.5	71.4
All other	62	78.6	91.7
Pretreatment serum PSA ng/ml ($P = 0.578$)			
<4.0	29	83.3	87.0
4.1–10.0	42	84.6	89.7
>10.0	13	66.7	80.0
Prior prostate biopsy ($P = 0.503$)			
No	65	82.4	89.6
Yes	19	80.0	78.6

The P shown is for the Breslow-Day test.

diagnosis of CaP. Blinded evaluation of the ERT-PCR/*PSA* was therefore performed in the peripheral blood from 84 patients who were recommended for prostate biopsy because of suspicion of CaP. Biopsies of 22 of 84 patients (26.2%) revealed CaP. Of these 22 patients, 18 (81.8%) were ERT-PCR/*PSA* positive. Sixty-two of 84 patients (73.8%) were negative for cancer on biopsy, and 54 of 62 (87.1%) were negative in ERT-PCR/*PSA*. Sensitivity and specificity of ERT-PCR/*PSA* in identifying patients with CaP were 81.8 and 87.1%, respectively. The positive predictive value was 69.2%, and negative predictive value was 93.1% (Table 2).

Eight patients were positive in ERT-PCR/*PSA* assay but negative on biopsy (false positive). The mean prebiopsy PSA was 8.06 ng/ml (range, 0.97–19.0 ng/ml), and the mean-free PSA was 3.7 ng/ml (range, 0.66–4.5 ng/ml). Four of 8 patients did not have cancer on a repeat biopsy, and the other 4 patients currently do not have repeat biopsies at the physicians' discretion. There was no strong relationship in these false-positive cases with age, race, PSA level, or prior biopsy history, but 5 of 8 (62.5%) did have evidence of histological prostatitis on biopsy histology compared with 19 of 69 (27.6%) evaluable patients in the overall study ($p = 0.032$). Notably, one of these cases had atypical glands on histopathogical examination.

In stratified univariate analyses, sensitivity and specificity did not vary significantly by age, race, pretreatment PSA, or prior biopsy status (Table 3). For the 19 patients who had a prior biopsy, the ERT-PCR/*PSA* assay accurately predicted the current biopsy result in 15 of 19 (78.9%). There were 3 false positives and 1 false negative and a remarkable sensitivity of 80.0% and specificity of 78.6% in this clinically challenging group (Table 4).

The simultaneous influence of age, race, pretreatment PSA, and prior biopsy status on the ability of ERT-PCR/*PSA* to predict CaP was evaluated by fitting a multivariable logistic regression model (age and serum PSA were modeled as continuous variables). The adjusted estimates of sensitivity and specificity were 77.3 and 87.1%, respectively. Estimates of the predictive accuracy of a model can be biased when they are obtained from the same data used to fit the model. Bias-reduced estimates of sensitivity and specificity were therefore computed (using an approximation to the jackknife procedure) for this multivariable logistic regression model: they were 68.2 and 85.5%, respectively.

DISCUSSION

The serum PSA screening test has revolutionized the early detection of CaP, but high false-positive rate of the PSA test leading to unnecessary biopsies has been of concern (4, 5). With the enormous increase in the rate of CaP incidence, review of current practices for screening, diagnosis, and staging have been highly investigated (30, 31). The majority of new CaP cases are clinically localized without obvious metastases. However, 30–40% patients show biochemical recurrence after treatment of localized disease (32). It is apparent that more accurate detection, staging, and prognostic tools are needed because many CaP are not cured by local therapies because of occult micrometastases.

Diverse technological approaches have been used to evaluate the presence of CPECs or tumor DNA in peripheral blood of CaP patients. Analysis of CPECs in the blood of CaP patients has been performed through cytokeratin-immunomagnetic isolation (33). Qualitative characterization of CPECs can be achieved through magnetic cell sorting and immunocytochemistry (34). Additional studies on CPECs by density gradient centrifugation and immunomagnetic beads selection of epithelial cells from peripheral blood were done by several groups (35, 36). Other techniques for CPEC isolation such as using ammonium chloride and distilled water erythrocyte lysis have been tested; however, isolation through density gradient separation and Ber-EP4 immunocapture are more sensitive and efficient (37). Analysis through flow cytometry on immunomagnetic-enriched epithelial cells was also investigated; however, sensitivity and specificity were limited because of the nature and technique of specimen collection (38). Studies on methylation-specific PCR targeting promoter hypermethylation of the glutathione *S*-transferase P1 gene for the possible molecular detection of CPECs have been performed in various human bodily fluids, which included plasma, serum, urine, and blood. Glutathione *S*-transferase P1 promoter hypermethylation was found in 90% of tumors, 72% of plasma or serum samples, and 76% of urine specimens (39). However, the nature and origin of the circulating DNA tested for by methylation-specific PCR has not been defined.

Ability to detect very small numbers of CPECs by sensitive

Table 4 Relationship of ERT-PCR/*PSA* and biopsy results in patients with prior history of biopsy[a]

ERT-PCR/*PSA* assay	Biopsy-positive number (%)	Biopsy-negative number (%)	Total no.
Positive	4 (80.0%)	3 (21.4%)	7
Negative	1 (20.0%)	11 (78.6%)	12

[a] Sensitivity: 80.0%; specificity: 78.6%; positive predictive value: 57.1%; negative predictive value: 91.7%; and accuracy: 78.9%.

RT-PCR-based methods, despite current limitations, represents the potential of molecular technology aiding in detection and staging of CaP (26, 27). In our efforts to enhance the sensitivity and reproducibility of CPECs detection, we combined ERT-PCR/*PSA* assay, which has revealed intriguing and novel data showing a very high rate of CPECs in CaP patients with clinically organ-confined disease. Similar results were obtained in a preliminary evaluation of CPECS by TaqMan Real-Time PCR assay (data not shown). We have observed stringent procedures to prevent/minimize contamination artifacts, and various controls have validated the data presented here. It is reasonable to suggest that enrichment of epithelial cells along with quality control of Taq polymerase and reverse transcriptase have increased the sensitivity of detection of CPECs in CaP patients.

Strikingly, high rate of CPECs in CaP patients has led to blinded evaluation of results from biopsy, and ERT-PCR/*PSA* assay in patients showed a strong correlation between an ERT-PCR/*PSA* positivity and biopsy positive for cancer and a negative ERT-PCR/*PSA* assay and biopsy negative for cancer. The unadjusted estimates of sensitivity and specificity of ERT-PCR/*PSA* in identifying patients with CaP were 81.8 and 87.1%, respectively. The jackknife estimates of sensitivity and specificity obtained from a logistic regression analysis that controlled for race, age, serum PSA, and prior biopsy status were 68.2 and 85.5%, respectively.

Additional studies and follow-up of false-positive and -negative patients are also required to prove the promise of ERT-PCR/*PSA* CaP diagnosis. Of note, this assay has future potential in reducing unnecessary repeated biopsies in patients with persistently high PSA and biopsies negative for cancer. One limitation of our data are the 20% false-negative rate (*i.e.,* patients with cancer are negative in ERT-PCR/*PSA* assay). Because the neoplastic process itself or treatment response is known to effect *PSA* expression in CaP cells, future improvement in the ERT-PCR/*PSA* assay may additionally increase the sensitivity and specificity. Multiplexing of ERT-PCR/*PSA* with additional CaP-specific genes [Prostate Specific Membrane Antigen (40), Differential Display 3 (41), Prostate Cancer Gene Expression Marker 1 (42) and Prostate Specific G-Protein Coupled Receptor (43)] that show elevated expression in CaP cells and refinements in methods of epithelial cell capture are being considered. However, it is possible that a subset of patients (<20%) do not present with CPECs either because of tumor biology or time of the tumor presentation. Although the true nature of CPECs is unclear, CPECs by RT-PCR methods have been almost exclusively detected in CaP patients and have been rarely detected in normal controls (26, 27). We suggest that detection of CPECs by ERT-PCR/*PSA* assay as well as future development of this assay in a quantitative multiplex format have potential in improving the specificity of CaP detection. Finally, isolation of CPECs from the blood of most CaP patients may additionally aid in genotyping these cells for prognostic evaluations in disease progression.

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