ABSTRACT

Purpose: There have been several studies on the presence of circulating tumor cells in the peripheral blood of patients with malignant tumors including prostate cancer (PCa) using reverse transcription-PCR (RT-PCR). One of the aims of these studies was to obtain high sensitivity that would enable early-stage diagnosis. However, they varied in their detection rates, and the methods were rather complicated. We have improved the RT-PCR assay combining three prostate-associated molecules, prostate specific antigen (PSA), prostate specific membrane antigen (PSMA), and prostate stem cell antigen (PSCA) to reveal patients with poor prognosis.

Experimental Design: Peripheral blood samples were obtained from 129 patients including 58 cases of PCa and 71 cases of nonmalignant disorders. Total RNA was extracted from 1 ml of whole blood using a commercially available kit.

Results: The sensitivity of PSA- and PSMA-RT-PCR was verified with positive signals of a single LNCaP cell in 1 ml of female blood sample. PSA-, PSMA-, and PSCA-mRNA were detected in 7 (12.1%), 12 (20.7%), and 8 (13.8%) PCa, and in 1, 2, and 0 samples in nonmalignant disorders, respectively. Among 58 PCa patients, each PCR indicated the prognostic value in the hierarchy of PSCA>PSA>PSMA-RT-PCR, and extraprostatic cases with positive PSCA PCR indicated lower disease-progression-free survival than those with negative PSCA PCR.

Conclusions: The present findings suggest that PSCA PCR would be most promising for the molecular staging of PCa. The present RT-PCR is a highly cost-effective and rapid procedure, enabling the molecular staging of PCa with RT-PCR as a diagnostic routine.

INTRODUCTION

PCa is the second leading cause of cancer-related death in men in the United States (1) and is a common cancer increasing in Japan (2). Current staging modalities in predicting locally-advanced and metastatic PCa include sPSA level, TRUS, and computed tomography scan (3) and these corresponded to prognosis to some extent. However, the frequency of detection of distant metastasis by routine clinical analysis is not concordant with the frequency of disease recurrence after definitive local therapy (4). Thus, new strategies are required for accurate staging of PCa to adapt appropriate treatment.

RT-PCR is a sensitive and useful method for detecting the presence of a specific cell type such as those cells that bear tumor-specific mRNA. There have been several studies on the presence of PSA-mRNA-bearing cells, most likely PCa cells, in the peripheral blood in patients without evidence of metastasis, and the higher frequency of PSA-mRNA-bearing cells in the peripheral blood was correlated with the extent of the disease (5). However, detection rates in previous studies in PCa patients varied from 0 to 88% (5–9), and the appropriate procedure still remains undetermined. Because one of the aims of these studies was to obtain high sensitivity enabling early-stage diagnosis, these RT-PCR methods demanded 5–15 ml of blood and great effort on the part of investigators. We have improved the RT-PCR assay, especially in RNA extraction from peripheral blood, and applied the method of combined nested RT-PCR for three prostate-associated molecules, PSA, PSMA, and PSCA, to detect circulating prostate cells with PCa and other nonmalignant disorders. PSA, which belongs to the tissue kallikrein family, is primarily produced by the prostatic epithelium (10). PSMA is a recently cloned Mr 100,000 integral transmembrane glycoprotein (11), for which a role as a potential prostatic marker protein has been suggested (12) and which has been reported as expressed by the neovascular endothelial cells of renal cell cancer.
PSCA is a 123-amino-acid glycoprotein, the mRNA of which was expressed predominantly in the basal cell epithelium of normal prostate and placenta and which is also detected in >80% of primary PCAs (14, 15).

In the present study, we further examined the relationship between clinicopathological findings including prognosis and nested RT-PCR findings for these three molecules in PCA patients’ peripheral blood, demonstrating the potential role of nested RT-PCR, which enables the molecular staging in PCAs as a diagnostic routine.

PATIENTS AND METHODS

Patients and Their Clinicopathological Characteristics. A total of 132 randomly selected patients complaining of dysuria, and/or with sPSA levels elevated more than 4.0 ng/ml, were enrolled for this study. Informed consent to perform the study was obtained from all of the patients. Systemic sextant prostate biopsy was performed using TRUS; 58 cases were histopathologically diagnosed as PCa and 71 cases as nonmalignant disorders including 69 BPH, 1 atypical adenomatous hyperplasia (AAH) and 1 prostatitis case. Three cases of prostatic intraepithelial neoplasm (PIN), considered to have malignant potential to some extent, were excluded from this study. Clinical staging of PCa cases was performed according to International Union Against Cancer (1997) guidelines (16) and was determined by TRUS, computed tomography scan, magnetic resonance image, isotoped bone scanning, and cystoscopy. Disease-specific survival and disease-progression-free survival were investigated in February, 2002, and the mean observation periods were 26.3 and 24.5 months, respectively. We defined disease progression if any of the following events took place: local enlargement of the tumor, appearance of new metastatic sites, or their enlargement. Also, we investigated prospectively the fraction of patients who had biochemical progressions (PSA failure) according to the definition by the American Society of Therapeutic Radiation Oncologists (ASTRO).

Blood Samples and Total RNA Extraction. Peripheral blood samples were obtained before prostate biopsy. RNA extractions from the previous studies required relatively large amounts of whole blood (5–15 ml; Refs. 5–9) and, thus, were complicated. In the present study, we extracted total RNA from 1 ml of whole blood anticoagulated with EDTA using a SV total RNA isolation system (Promega, Madison, WI). This method is concise and reduces time consumption, obtaining high-quality RNA within 1 h (17, 18). Moreover, it does not require any special equipment or apparatus except for a centrifuge and costs only two dollars per sample.

RT-PCR. Nested PCRs, using outer and inner primer sets for PSA, PSMA, and PSCA, were applied (Fig. 1). We previously reported the sequences used for the external and nested PSA 20-mer primers to identify circulating prostate tumor cells in blood (17). The sequences for PSMA and PSCA primers were determined on Primer express for Macintosh software (Perkin-Elmer Japan Applied Biosystems, Tokyo, Japan) based on the sequences obtained from the GenBank, and the specificity of the resulting primers’ sequences was examined by a BLAST search (19). The first-strand DNA was synthesized using a Single-strand cDNA synthesis kit (Boehringer Mannheim, Inc., Mannheim, Germany) according to the manufacturer’s instructions. To control for RNA integrity and to determine the significance of a negative PCR assay, we performed an RT-PCR assay involving the product of the housekeeping gene β-actin. The resulting cDNA was amplified by PCR using a set of outer primers. One μg of the RNA-equivalent cDNA was mixed in a 50-μl reaction volume with 10× PCR buffer [1.5 mM MgCl2, 0.2 mM each standard dNTP (Boehringer Mannheim), 0.1 μM forward and reverse primers, and 0.4 units AmpliTaq Gold polymerase (Applied Biosystems, Foster, CA)]. Amplification was performed in a thermal cycler 9700 (Perkin-Elmer, Norwalk, CT) for 40 cycles, each cycle consisting of denaturation at 95°C for 30 s; annealing at 57°C in PSA and PSMA, and 58°C in PSCA for 30 s; and extension at 72°C for 1 min, except for the last cycle for which the extension time was 10 min. Four μl of a 4:100 dilution of the resulting PCR product DNA was amplified in a nested PCR reaction for 40 cycles using a set of inner primers. The sensitivity of the PSA, PSMA, and PSCA RT-PCR assays was examined using total RNA isolated from diluted LNCaP human PCA cells in 1 ml of human female peripheral blood. The LNCaP cell suspension was diluted from 1000 to 1 cell/ml. In each sample, the presence of LNCaP cells was confirmed with all three PCR assays (Fig. 2). The PCR products were electrophoresed in agarose gel electrophoresis and stained with ethidium bromide to confirm the products were derived from specific mRNA, and the nucleotide sequences of some PCR products were sequenced. Each sequence was verified in both the sense and antisense directions (data not shown).

Statistical Analysis. Statistical comparisons were made using the Welch-corrected t test and χ² tests in StatView 5.0.
RT-PCR findings of nonmalignant disorders and those of organ-confined disease (stage II) were observed (PSA PCR and PSCA PCR, 0.01; PSCA PCR, 0.005; Table 1). Among the three RT-PCRs, patients with positive RT-PCR findings had higher sPSA levels and more cancer-involved biopsy cores than those with negative findings (P < 0.001; data not shown).

All of the 58 PCa patients were treated with combined androgen blockade after diagnostic procedures involving prostate biopsy. Six of these cases underwent radical prostatectomy after androgen blockade for 6 or 12 months, and two patients with local progression received radiation therapy. Three patients died of disease-specific causes within 10.2, 12.6, and 16.6 months. All of these patients had stage IV PCa and were found positive for all three of the marker PCRs. Disease progressions were observed in 3 (7.3%) of 41 patients with stage II, one (50%) of 2 with stage III, and 7 (46.7%) of 15 with stage IV PCa. The disease-progression-free survival rates of patients treated only hormonally were lower in groups with positive RT-PCR findings than in those with negative findings, and the differences were significant (Fig. 4, A–C). One case with positive PSMA PCR and another case with both positive PSA PCR and positive PSMA PCR with stage II PCa, showed no disease progression. However, one of two stage III cases, with positive PSCA as well as PSMA PCR, had disease progression in 25.1 months, whereas another stage III case with negative PCRs had no evidence of disease for 26.9 months. In addition, stage IV patients with positive PSCA PCR resulted in lower disease-progression-free survival than those with negative PSCA PCR (Fig. 4D). The probability of double or triple positive findings for each PCR did not appear to be more significant than for PSCA PCR as a single factor regarding disease-progression-free survival (data not shown).

DISCUSSION

Recent studies showed that tumor cells, circulating in peripheral blood, were observed in several cancers including renal cell cancer and breast cancer, and the consequence in disease staging has been demonstrated (21, 22). New strategies for accurate staging of PCa have been tried including flow-cytometric detection of PSA-positive cells in peripheral blood (21) and RT-PCR, which is the most sensitive method and is applicable to the molecular staging of PCa (24–27). One of the aims of these studies was to obtain high sensitivity enabling early-stage diagnoses and the detection of micrometastases that were not identifiable with images. However, it is still controversial whether circulating tumor cells cause micrometastasis. RT-PCR is an extremely sensitive method to detect specific mRNA-bearing cells in the blood stream. It can detect a single specific mRNA-bearing cell among 10⁷–10⁸ cells (28). Some studies reported that at least 10⁸ circulating tumor cells are required for metastasis (29). Moreover, only 0.01% of circulating cancer cells create a single metastasis (23, 30, 31). The methods in the present study provide the appropriate sensitivity and are highly cost-effective and rapid, which enables the molecular staging of PCa with RT-PCR to be a diagnostic routine. Thus, with more accurate molecular biological techniques, staging before surgery may be achieved. However, the described RT-PCR assays,
especially PSMA PCR, showed no differences between nonmalignant disorders and organ-confined PCa, although 12% of organ-confined PCa was reported to reach a biochemical failure within 10 years of treatment (32).

In the present study, PSA-, PSMA-, and PSCA-mRNA-bearing cells circulating in peripheral blood were identified at higher rates in PCa patients with extraprostatic disease. These findings suggest that detection of circulating prostate cells would have diagnostic value for patients with PCa. However, several cases with extraprostatic disease including one patient, with clinical stage T4, N0, M0 and 1121 ng/ml sPSA, who showed negative RT-PCR (Table 1). In addition, the current PCR assays furnished several false-positive cases. The only case with positive PSA PCR among nonmalignant cases was diagnosed as prostatitis by the prostate biopsy. Correspondingly, prostatitis was reported to induce hematogenous dissemination of prostate epithelial cells (33). Although PSMA PCR showed the highest sensitivity in the present study, it picked up 2 (2.8%) false-positive cases of 71 nonmalignant disorders. The present PSMA PCR is supposed to amplify its splicing variant PSM\textsuperscript{H11032}, which was identified by Grauer et al. (34, 35), and this PSM\textsuperscript{H11032} was presumably expressed in nonprostate tissues also. Thus, a correlation between prognosis and detection of cancer cells in blood are needed to clarify their clinical significance. Although the clinicopathological background and tumor biological behavior in patients in the present study varied, the RT-PCR-positive group demonstrated a tendency toward decreased disease-progression-free survival, which indicated that detection of circulating prostate cells might have a prognostic value. Interestingly, the above mentioned patient with clinical stage IV and 1121 ng/ml sPSA, who showed negative RT-PCR, has stayed alive without any evidence of disease or PSA failure for 26.9 months. Moreover, to our knowledge, the present study is the first to apply the PSCA PCR for detection of circulating prostate cells and to demonstrate that this can be used as a prognostic factor. Recent evidence showed that the expression levels of PSCA protein on the surface of PCa cells increased with tumor progression (36). A longer observation period is required to draw a definite conclusion, and another modality to improve the information, such as quantitative RT-PCR, appears to be necessary for estimating the correlation between the number of circulating prostate cells and the presence of metastases or further disease progression.

![Figure 3](image)

**Fig. 3**  A, the group with positive PSA PCR had a higher Gleason score (mean, 6.57) than that with negative RT-PCR (mean, 4.01). B, the group with positive PSCA PCR also had a higher Gleason score (mean, 5.71) than that with negative RT-PCR (mean, 4.14), but (C) PSMA PCR findings were not correlated with the Gleason score.

### Table 1  Summary of clinicopathological diagnoses, stages of 129 cases and RT-PCR findings

<table>
<thead>
<tr>
<th></th>
<th>PSA PCR (+)</th>
<th>PSMA PCR (+)</th>
<th>PSCA PCR (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonmalignant disorders (n = 71)</td>
<td>1/71 (1.4%)</td>
<td>2/71 (2.8%)</td>
<td>0/71 (0%)</td>
</tr>
<tr>
<td>PCa (n = 58)</td>
<td>7/58 (12.1%)</td>
<td>12/58 (20.7%)</td>
<td>8/58 (13.8%)</td>
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<tr>
<td>Organ-confined disease (n = 41)</td>
<td>1/41 (2.4%)</td>
<td>2/41 (4.9%)</td>
<td>0/41 (0%)</td>
</tr>
<tr>
<td>T\textsubscript{1} N\textsubscript{0} M\textsubscript{0} (n = 26)</td>
<td>0/26</td>
<td>1/26</td>
<td>0/26</td>
</tr>
<tr>
<td>T\textsubscript{2} N\textsubscript{0} M\textsubscript{0} (n = 15)</td>
<td>1/15</td>
<td>1/15</td>
<td>0/15</td>
</tr>
<tr>
<td>Extraprostatic disease (n = 17)</td>
<td>6/17 (35.3%)</td>
<td>10/17 (58.8%)</td>
<td>8/17 (47.1%)</td>
</tr>
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<td>T\textsubscript{1} N\textsubscript{0} M\textsubscript{0} (n = 2)</td>
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<td>1/2</td>
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<td>N\textsubscript{1} and/or M\textsubscript{1} (n = 14)</td>
<td>7/14</td>
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<tr>
<td>Total (n = 129)</td>
<td>8/129</td>
<td>14/129</td>
<td>8/129</td>
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It is concluded that nested RT-PCR targeting three different prostate-associated molecules, PSA, PSMA, and PSCA, could detect tumor cells in smaller volumes of peripheral blood with high specificity and appropriate sensitivity, enabling molecular staging of the tumor as a diagnostic routine. It is suggested that the detection of circulating prostate cells has prognostic value. In the present study, PSCA PCR demonstrated higher disease specificity and independent prognostic value, and PSMA PCR indicated higher sensitivity, although its specificity was lower and all of the PSCA PCR-positive cases were also PSMA PCR-double positive, which suggested that the PSCA PCR would be the most promising for molecular staging of PCa. A prospective study examining the clinical course is required to estimate the significance of detection of circulating prostate cells in the peripheral blood for metastasis and survival.

REFERENCES
Clinical Cancer Research

Reverse Transcription-Polymerase Chain Reaction Detection of Prostate-specific Antigen, Prostate-specific Membrane Antigen, and Prostate Stem Cell Antigen in One Milliliter of Peripheral Blood: Value for the Staging of Prostate Cancer

Noboru Hara, Takashi Kasahara, Takashi Kawasaki, et al.


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