Combined Nested Reverse Transcription-PCR Assay for Prostate-specific Antigen and Prostate-specific Membrane Antigen in Detecting Circulating Prostatic Cells

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ABSTRACT
Accurate staging is an important issue in managing patients with prostate cancer. Current staging modalities are poor predictors for locally advanced disease. In the present study, we investigated the role of a peripheral blood-based, nested reverse transcription-PCR (RT-PCR) for prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSM) in prostate cancer staging. Our nested RT-PCR could detect both PSA and PSM mRNA in one LNCaP cell diluted in 10⁶ mononuclear cells. None of the controls, including patients with benign prostate hyperplasia, normal male subjects, and female subjects, were positive for either marker, confirming the assay's specificity for prostate cancer. In patients with bony metastases, 100% were positive by combined PSA/PSM assays (64% by PSA and 91% by PSM). In patients with clinically localized prostate cancer, 29% were positive by combined PSA/PSM assays (13% by PSA and 23% by PSM). The combined PSA/PSM assay is more sensitive than the PSA assay alone in detecting circulating prostatic cells (P = 0.0071). PSM is a more sensitive marker than PSA (P = 0.042). We also correlated preoperative nested RT-PCR results with pathological findings in prostatectomy patients. Nested RT-PCR for PSA/PSM has an odds ratio of 20 in predicting tumor extracapsular penetration (P = 0.017). These results indicate that a nested RT-PCR result may provide the staging information unavailable from other modalities, including the clinical stage, initial serum PSA, and Gleason score. Additional investigation is needed to determine the ultimate role of this assay in the management of patients with prostate cancer.

INTRODUCTION
Prostate cancer is the second leading cause of cancer-related deaths in men in the United States. Current staging modalities in predicting locally advanced and metastatic prostate cancer include serum PSA level, transrectal ultrasound, pelvic computed tomography scan, and radionucleotide bone scan. However, it is reported that up to 40-50% of patients with initial diagnosis of localized disease were found to be understaged following radical surgery (1). To identify patients with true organ-confined disease, a more sensitive method of detecting locally advanced and micrometastatic prostate cancer is needed.

RT-PCR is a sensitive and powerful technique in detecting the presence of a specific cell type based on its ability to identify tissue- and/or tumor-specific mRNA. Recently, several laboratories used RT-PCR for PSA to detect circulating prostatic cells in prostate cancer patients (2-4). On the basis of these findings, the role of hematogenous prostatic cells as potential occult prostate cancer micrometastases has been suggested. Different techniques have also been explored to increase PCR sensitivity. Some of the commonly used techniques are Southern analysis (5), inclusion of digoxigenin-labeled dUTP in PCR reactions (6), and nested PCR (5, 7-9).

Whereas most of the RT-PCR assays detect PSA mRNA in the circulation, efforts are being made to seek new prostate-specific markers. PSM is a recently cloned (10) M₁ 100,000 integral transmembrane glycoprotein. Its role as a potential prostatic marker protein has been suggested (11). It has been shown that, in contrast to PSA, PSM is highly expressed by hormone-deprived cells (11), suggesting that PSM could be more informative than PSA in patients under hormonal therapy. It has also been demonstrated that PSM-based nested RT-PCR has improved sensitivity over the PSA-based assay (8, 9).

The concept of “molecular staging” of prostate cancer using PSA RT-PCR appeared 3 years ago (6) in response to the insufficient correlation of imaging modalities in predicting extracapsular disease. Katz et al. (6) demonstrated that in patients with clinically localized disease, preoperative PSA RT-PCR data could predict final pathological stage. Their results also showed that RT-PCR was superior to other staging methods.
such as serum PSA level, DRE, computed tomography scan, and endorectal coil magnetic resonance imaging. Their follow-up study (12) demonstrated that preoperative PSA RT-PCR results could predict surgical failure.

In the present study, we examined the relationship between clinical findings and nested RT-PCR for PSA and PSM in prostate cancer patients’ peripheral blood. We compared sensitivities between the PSA and PSM assays. We also compared the results of the PSA assay alone with the combined PSA/PSM assay results. We investigated the role of hematogenous dissemination in patients with clinically localized prostate cancer by comparing their pre-radical prostatectomy nested RT-PCR results with final pathological findings. The potential of using combined PSA/PSM nested RT-PCR assays to molecularly stage prostate cancer is addressed.

MATERIALS AND METHODS

Patient Selection. Seventy-five prostate cancer patients were entered into the study. Among them, 48 patients had newly diagnosed clinically localized disease (T1–T2), 16 were surgical failure subjects (rising serum PSA after prostatectomy), and 11 were stage D1 patients with positive bone scans. Controls included nine BPH patients; six healthy, age-matched male subjects with normal serum PSA and DRE; and five female subjects. Patients’ participation was approved by the Institutional Review Board.

Cell Culture. The human prostate cancer cell line LNCaP, which synthesizes both PSA and PSM, was used as a positive control. LNCaP was obtained from American Type Culture Collection and propagated in RPMI containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml fungizone (Life Technologies, Inc.).

Isolation of Mononuclear Cells by Ficoll Gradient. Six ml of venous blood were collected in EDTA-treated tubes, placed on ice, and processed within 4 h of collection. Whole blood was mixed with an equal volume of PBS (pH 7.4) layered with 8 ml of Ficoll (Pharmacia) in a 15-ml polystyrene tube. The samples were centrifuged at 400 X g for 20 min at 4°C. The mononuclear cell layer was collected, washed with PBS (pH 7.4), and pelleted.

Total RNA Extraction. Total RNA was isolated using Trizol reagent (Life Technologies) following the manufacturer’s instructions. Briefly, Trizol reagent was added to the mononuclear cell pellet and incubated for 5 min at room temperature. After chloroform extraction, RNA in the aqueous phase was precipitated with isopropanol, washed with 75% ethanol, dried, and resuspended. To ensure the lack of genomic DNA contamination, RNA preparation was treated with DNase I (Life Technologies) for 15 min at room temperature. The final RNA preparation was resuspended in diethyl pyrocarbonate-treated water and quantitated by absorbance analysis at 260 nm.

cDNA Synthesis. cDNA was synthesized using the Superscript Preamplification System (Life Technologies) according to the instruction manual. In brief, a 12-μl template-primer mixture containing 1 μg of total RNA and 0.2 μg of oligodeoxynthymidylate was denatured at 70°C for 10 min and chilled on ice. Seven μl of reverse transcription master mix were added, and RNA template-primer annealing was carried out at 42°C for 5 min. The reverse transcription reaction was completed by incubating with 200 units Superscript II reverse transcriptase for 50 min at 42°C. The reaction was terminated at 70°C for 15 min. Excess RNA was degraded by RNase H treatment.

Nested PCR. The primers and reaction parameters for the PSA nested PCR were chosen according to Israeli et al (8). Fifty μl of PCR reaction mixture contained 2 μl of cDNA, 2.5 units of Taq DNA polymerase (Perkin-Elmer Corp.), 200 μM of each deoxynucleotide triphosphate, 1 μM of each primer, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 1.5 mM MgCl₂. The PCR reaction was carried out in a Perkin-Elmer thermal cycler (model 9600). The parameters were as follows: 25 cycles of 94°C for 15 s, 60°C for 15 s, and 72°C for 45 s. Two μl of the reaction product served as the template for another 25 cycles of PCR using the inner primers. The nested PCR product is a 355-bp fragment.

The primers and reaction parameters for the PSM nested PCR were chosen according to Loric et al. (9). The PCR parameters were: 94°C for 2 min followed by 25 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 1 min, and finally one cycle of 72°C for 10 min. The nested PCR product is a fragment of 186 bp. Control PCR for human GAPDH, which generated a 360-bp fragment, was carried out on each sample according to Wainstein et al. (13). A tube with no addition of cDNA to the PCR mixture was included in each PCR assay as a negative control. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Specimens were numbered and processed in the sequence of their arrival, regardless of the patients’ clinical status.

Statistical Analysis. χ² analysis for proportion comparison was used to analyze the nested RT-PCR results. The Fisher’s exact test was used if the expected frequency in any cell was less than 5. A conclusion of significant difference was drawn when the P value was less than 0.05. The following parameters were calculated from 2 × 2 tables: sensitivity = a/(a + c), specificity = d/(b + d), PPV = a/(a + b), NPV = d/(c + d), and OR = ad/bc, where a = number of true positive, b = false positive, c = false negative, and d = true negative results.

RESULTS

Sensitivity of PSA/PSM Nested RT-PCR. We determined the sensitivity of nested RT-PCR by using the LNCaP cell line. Serially diluted LNCaP cells were mixed with 2.5 × 10⁶ mononuclear cells isolated from peripheral blood samples in the following ratios: 1:10², 1:10³, 1:10⁴, 1:10⁵, and 1:10⁶. Total RNA was extracted using Trizol reagent (Life Technologies). cDNA was synthesized using oligodeoxynthymidylate primer and the Superscript II reverse transcriptase (Life Technologies; see “Materials and Methods”). Nested PCRs for PSA and PSM were performed using specific primers according to Israeli et al. (8) and Loric et al. (9), respectively. Fig. 1 shows the result after nested PSA/PSM RT-PCR. Mononuclear cells alone were negative for both PSA and PSM mRNA. Control RT-PCR for GAPDH confirmed the integrity of RNA in all samples. The mRNAs for both PSA and PSM from an average of 1 LNCaP cell diluted in 10⁶ mononuclear cells could be detected after nested RT-PCR. Thus, our assay detection limits for PSA and PSM were similar. The results demonstrate that nested RT-PCR
Nested PSA/PSM RT-PCR in Control Subjects. We performed nested RT-PCR on six male subjects with normal DRE and serum PSA, five female subjects, and nine biopsy-proven BPH patients. Fig. 2 shows that GAPDH mRNA was present in all female (Fig. 2, Lanes 1–5) and normal male (Fig. 2, Lanes 6–11) peripheral blood samples. Neither normal male subjects nor female subjects were positive for either PSA or PSM mRNA. No circulating prostatic cells could be detected by nested PCR for either marker in nine BPH patients (data not shown). Therefore, the PSA and PSM nested RT-PCR assays were specific for prostate cancer.

Table 1: Nested RT-PCR for PSA and PSM in prostate cancer patients

<table>
<thead>
<tr>
<th>PCA category</th>
<th>(No. of patients)</th>
<th>PSA</th>
<th>PSM</th>
<th>PSA and/or PSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinically localized (48)</td>
<td>6 (13%)</td>
<td>11 (23%)</td>
<td>14 (29%)</td>
<td></td>
</tr>
<tr>
<td>T1, T2 (25)</td>
<td>2 (8%)</td>
<td>4 (16%)</td>
<td>5 (20%)</td>
<td></td>
</tr>
<tr>
<td>T3 (23)</td>
<td>4 (17%)</td>
<td>7 (30%)</td>
<td>9 (39%)</td>
<td></td>
</tr>
<tr>
<td>Surgical failure (16)</td>
<td>1 (6%)</td>
<td>5 (31%)</td>
<td>5 (31%)</td>
<td></td>
</tr>
<tr>
<td>Metastatic (11)</td>
<td>7 (64%)</td>
<td>10 (91%)</td>
<td>11 (100%)</td>
<td></td>
</tr>
<tr>
<td>Total (75)</td>
<td>14 (19%)</td>
<td>26 (35%)</td>
<td>30 (40%)</td>
<td></td>
</tr>
</tbody>
</table>

is a sensitive technique in detecting PSA/PSM-synthesizing cells diluted with mononuclear cells.

**Nested PSA/PSM RT-PCR in Patients with Metastatic Prostate Cancer.** We performed nested PCR on peripheral blood samples from 11 stage D2 patients with positive bone scans. A representative experiment on four patients shown in Fig. 2 (Lanes 12–15) demonstrates that nested RT-PCRs for both PSA and PSM were able to detect circulating prostatic cells in patients with prostate cancer bony metastasis. Results from all 11 stage D2 patients studied (Table 1) showed that 6 patients were positive for both PSA and PSM, 1 patient was PSA positive only, and 4 were PSM positive only. Therefore, the assay sensitivity of combined RT-PCR for PSA and PSM was 100%. Nested RT-PCR sensitivity was 91 and 64% for PSA and PSM, respectively.
Nested PSA/PSM RT-PCR in Prostate Cancer

Detection of Circulating Prostatic Cells in Patients with Clinically Localized Prostate Cancer. Blood samples from 48 patients with clinically localized prostate cancer were analyzed by nested RT-PCR. Among this group of patients, 25 had clinical stage T1–T2 disease, and 23 had clinical stage T3 disease. The number of positive PCRs increased with the clinical stage (39% by combined assays in stage T1 versus 20% in stage T1–T2; Table 1). Overall, 29% of these 48 patients were positive by combined screening for PSA and PSM (Table 1). Fig. 3 shows a representative experiment on 13 patients. GAPDH signal was positive in all samples. The data show that nested RT-PCR assay is able to detect circulating prostatic cells in patients with clinically localized disease, and that the result correlates with clinical stage. The data demonstrate that, in addition to PSA, PSM also is a sensitive marker in identifying circulating prostatic cells in this group of patients.

Comparison of PSA and PSM Nested RT-PCR. A total of 75 prostate cancer patients was analyzed by nested RT-PCR. There were 48 patients with clinically localized disease, 16 surgical failure patients who had rising serum PSA (>0.2 ng/mL) after prostatectomy, and 11 stage D2 patients. Twenty-three of the patients with clinically localized disease were PSM positive, whereas only 13% were PSA positive. In surgical failure patients, PSA and PSM RT-PCR positivity was 31% and 6%, respectively. Table 1 summarizes the nested RT-PCR results on these 75 patients. Overall, 40% of patients were positive for the combined PSA/PSM assay. Thirty-five percent of patients were positive for PSM, whereas 19% were positive for PSA. Chi-squared analysis showed that the difference in results between PSA and PSM RT-PCR assays is statistically significant (P = 0.042). The combined nested RT-PCR for PSA/PSM is more sensitive than for PSA alone (P = 0.0071). Therefore, the data demonstrate that PSM is a more sensitive marker than PSA and that the combined screening approach for both PSA and PSM is the most sensitive in detecting circulating prostatic cells.

Correlation of RT-PCR Results with Pathological Stage. We studied the role of nested RT-PCR in predicting final pathological stage in patients with clinically localized disease. We compared preoperative RT-PCR results with pathological findings in 20 patients undergoing radical prostatectomy, including 11 stage T1 patients, 4 stage T2 patients, and 5 stage T3 patients. Among the 20 patients, 9 had extracapsular tumor penetrating the prostatic capsule, 14 had perineural invasion, 3 had tumors invading the seminal vesicles, and 1 had lymph node metastasis. Only one patient had a positive surgical margin. This patient had clinical stage T1 disease, with a Gleason score of 8. His surgical pathology report showed that his tumor had extracapsular penetration, perineural invasion, and seminal vesicle invasion. He also had lymph node metastasis. This patient was negative for PSM but positive for PSA nested RT-PCR. Our nested PSM RT-PCR identified more tumors involving extracapsular penetration and perineural invasion than PSA nested RT-PCR did. We correlated the pathological findings with the combined PSA/PSM nested RT-PCR assay. The PCR positivity rate is 67% in patients with extracapsular extension and 9% in patients with pathologically organ-confined disease. The difference is statistically significant (P = 0.0166). The results (Table 2) show that nested RT-PCR for PSA/PSM has a 67% sensitivity in predicting extracapsular penetration, with a specificity of 91%. The PPV and NPV of this combined assay for extracapsular penetration are 86% and 77%, respectively. The results also suggest that a patient with a positive preoperative RT-PCR result is 20 times more likely to have tumor extracapsular penetration than one with a negative preoperative RT-PCR result (P = 0.017). The 95% confidence interval for this OR is 1.68–238.7.

Comparison of Nested RT-PCR with Other Staging Parameters. In the total 75 patients examined in this study, there is a significant relationship between PCR results and serum PSA (>10 ng/mL; P = 0.0072). However, in the 20 prostatectomy patients, no statistically significant relationship was found between preoperative RT-PCR results and initial serum PSA levels (P = 0.651), Gleason score (P = 0.651), and clinical stage (P = 0.29).

We compared the sensitivity and specificity of standard staging parameters, including clinical stage, initial serum PSA value, and Gleason score in predicting tumor extracapsular penetration, with nested RT-PCR. The cutoffs for analysis were T1–T2 for clinical stage, >10 ng/mL for serum PSA, and ≥7 for Gleason score. The result (Table 3) demonstrates that nested RT-PCR has the highest sensitivity, specificity, PPV, NPV, and OR among the parameters analyzed. Clinical stage and Gleason score ranked second and third, with ORs of 8 and 5.6, respectively, in predicting extracapsular penetration. Among these 20 prostatectomy patients, their initial serum PSA level did not have any predictive value for tumor extracapsular penetration. Our data suggest that nested PSA/PSM RT-PCR is a better predictor for tumor extracapsular penetration than the current staging parameters analyzed in the study.

DISCUSSION

In the present study, we performed peripheral blood-based nested RT-PCR assays for PSA and PSM in prostate cancer patients, using specific primers that were designed to span intron-exon junctions (8, 9). Our nested RT-PCR could detect one PSA/PSM-producing cell diluted in 1,000,000 mononuclear cells. The nested PSA/PSM RT-PCR assays were specific for prostate cancer, given that none of our controls (BPH patients, healthy male subjects, and female subjects) were positive for either marker. In agreement with previous studies (8, 9), our results showed that PSM was a more sensitive marker than PSA.
Table 2 Role of preoperative nested PSA/PSM RT-PCR in predicting prostate cancer pathological stage

<table>
<thead>
<tr>
<th>Pathological stage</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracapsular penetration</td>
<td>67</td>
<td>91</td>
<td>86</td>
<td>77</td>
<td>20</td>
</tr>
<tr>
<td>Perineural invasion</td>
<td>43</td>
<td>83</td>
<td>86</td>
<td>38</td>
<td>3.75</td>
</tr>
<tr>
<td>Seminal vesicle invasion</td>
<td>67</td>
<td>69</td>
<td>29</td>
<td>92</td>
<td>4.4</td>
</tr>
</tbody>
</table>

*Values are calculated from 2 x 2 tables.

Table 3 Comparison of RT-PCR with standard staging modalities in their abilities to predict tumor extracapsular penetration

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR</td>
<td>67</td>
<td>91</td>
<td>86</td>
<td>77</td>
<td>20</td>
</tr>
<tr>
<td>Clinical stage a</td>
<td>44</td>
<td>91</td>
<td>80</td>
<td>67</td>
<td>8</td>
</tr>
<tr>
<td>Serum PSA b</td>
<td>33</td>
<td>64</td>
<td>43</td>
<td>54</td>
<td>0.875</td>
</tr>
<tr>
<td>Gleason score c</td>
<td>56</td>
<td>82</td>
<td>71</td>
<td>69</td>
<td>5.6</td>
</tr>
</tbody>
</table>

a Cutoff, ≥T1c.  
b Cutoff, ≥10 ng/ml.  
c Cutoff, ≥7.

Statistical analysis also demonstrated that the combined PSA/PSM screening approach was more sensitive than the PSA assay alone in detecting circulating prostatic cells. Although the biological significance of circulating prostatic cells expressing either PSA or PSM (or both) in prostate cancer spread is not fully understood, the combined assays enabled us to detect more circulating cells expressing prostate-specific markers. This observation suggests that both markers should be examined to reduce the false negative rate of the RT-PCR assay.

In contrast to the previous studies (8, 14), which identified more PSM-positive patients in lower clinical stages than in higher clinical stages, our study showed that the RT-PCR positivity increased in patients with clinical stage T1 disease versus lower stages by either PSA assay alone or the combined PSA/PSM assays. One potential reason for this is the different PSM primers used. We used PSM primers described by Loric et al. (9), which were designed and confirmed (15) to span an intron-exon junction. Because the PSM genomic sequence is not known completely at the present time, the two earlier studies (8, 14) could have used primers that did not cross intron-exon junctions, leading to false positive result if genomic DNA contamination was present. Sokoloff et al. (16) obtained lower sensitivity rates from PCR for PSM than for PSA. The discrepancy may be explained partially by the different PCR parameters used in their study, including primers and detection methods. It is possible that different PSM isoforms are detected in the different series. It has been shown (17) that an alternatively spliced variant of PSM is present, and that the ratios between the two isoforms are different among normal prostate, BPH, and cancerous tissues. The effect of different primers on the sensitivity of PSM RT-PCR in prostate cancer patients should be investigated further.

Although we have analyzed only a small number of surgical patients, the presence of circulating PSA/PSM-expressing cells in preprostatectomy patients correlated with the final pathological stage. The nested PSA/PSM RT-PCR assay has an OR of 20 in predicting extracapsular tumor penetration. This is higher than any other staging parameters analyzed in the study, including standard modalities such as the clinical stage, serum PSA, and Gleason score. Our data are consistent with larger studies (6) suggesting that the nested PSA/PSM RT-PCR assay may have the potential to increase accuracy in staging of prostate cancer. These observations also suggest that a positive result of PSA/PSM RT-PCR in a patient with apparently localized disease (as determined by standard staging methods) should make the urological surgeon suspicious of extracapsular disease and may dictate a surgical technique designed to obtain the widest possible margin.

In summary, we demonstrate that a combined nested PSA/PSM RT-PCR assay has superior sensitivity and specificity in predicting the presence of circulating prostatic cells compared to PSA alone. The ultimate impact of this technique in the management of patients with prostate cancer will require continued investigation.

REFERENCES

Nested PSA/PSM RT-PCR in Prostate Cancer

Clinical Cancer Research

Combined nested reverse transcription-PCR assay for prostate-specific antigen and prostate-specific membrane antigen in detecting circulating prostatic cells.

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