

Prostate-specific Membrane Antigen-derived Primers in a Nested Reverse Transcription Polymerase Chain Reaction for Detecting Prostatic Cancer Cells

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The detection of prostate-specific membrane antigen (PSM) mRNA in the peripheral blood of prostate cancer patients by a nested reverse transcriptase-polymerase chain reaction (RT-PCR) assay is a useful and sensitive method for the identification of small foci of metastatic lesions. In this study, a nested RT-PCR assay was performed using the two different PSM-derived oligonucleotide primer sets reported by Israeli *et al.* and Loric *et al.* (termed PSM primers-1 and primers-2, respectively, in this report), and the differences in the specificity and sensitivity of these primer sets for detecting prostate cancer cells in the blood are discussed. The PCR assay using PSM primers-1 showed DNA bands for 4 of 7 cases of metastatic prostate cancer and amplified the untreated genomic DNA, while that using PSM primers-2 showed 6 bands without the amplification of the genomic DNA. In conclusion, PSM primers-2 is superior to PSM primers-1 for the detection of PSM mRNA in the peripheral blood of prostate cancer patients.

Key words: Prostate-specific membrane antigen — Prostate cancer — Nested RT-PCR — Diagnosis

Prostate-specific membrane antigen (PSM) is a tissue-specific antigen that was first identified in the human prostate cancer cell line LNCaP.^{1–3} PSM is glycosylated, with an apparent molecular weight of 110 kDa. It is a type II transmembrane protein defined by monoclonal antibody 7E11.C5,^{1–4} and was shown to have the biological activities of folate hydrolase and N-acetylated α -linked acidic dipeptidase (NAALADase).^{5–7} The effect of such activities on the growth of prostate cancer cells has not yet been elucidated. PSM has been shown to be abundantly expressed by not only normal or benign prostate hyperplasia (BPH) tissues but also by malignant prostate tissue.^{8,9} The detection of PSM mRNA in the blood is a useful method for diagnosing micro-metastases and malignancies of prostate cancer patients. Israeli *et al.* showed that the nested reverse transcriptase-polymerase chain reaction (RT-PCR) method for PSM can be used to demonstrate the presence of circulating metastatic prostate cancer cells in the peripheral blood of patients with prostate cancer.¹⁰ Various oligonucleotide primer sets have been used for detecting PSM mRNA in the blood by the nested RT-PCR method,^{10–13} but appreciable percentages of false-positive or false-negative cases have been reported.

In this study, a nested RT-PCR analysis was performed using two different sets of oligonucleotide primers^{10,11} in order to compare the specificity and sensitivity of these primer sets for detecting prostate cancer cells in the peripheral blood.

MATERIALS AND METHODS

Cell culture The human metastatic prostate adenocarcinoma cell line LNCaP was obtained from American Type Culture Collection (Rockville, MD). The cells were grown in RPMI-1640 supplemented with 10% fetal calf serum and antibiotics in a 5% CO₂ incubator at 37°C.

Genomic DNA and total RNA extraction from LNCaP cells Genomic DNA was extracted from LNCaP cells in fresh medium, using a SepaGene extraction system (San-kyo Junyaku, Tokyo). Total RNA was extracted from the cells with a modification of the method described by Israeli *et al.*¹⁰ Cells were briefly washed twice with phosphate-buffered saline (PBS) and lysed with ISOGEN-LS solution (Nippon Gene, Tokyo). The cell lysate was collected with a cell-scraper and transferred to a polypropylene tube. After chloroform extraction and centrifugation, the aqueous phase was extracted twice with phenol/chloroform, and total RNA was precipitated with isopropyl alcohol. The RNA pellet was washed with 75% ethanol and dissolved in RNase-free distilled water. The concentration and purity of DNA and RNA were determined by ultraviolet spectroscopy at 260 nm and 280 nm.

Total RNA extraction from blood samples Whole peripheral blood (10 ml) was mixed with an equal volume of ice-cold PBS and transferred to a Lymphoprep tube (Nycomed Pharma AS, Div. Diagnostica, Oslo, Norway). The tube was centrifuged at 800g for 20 min at room temperature. The buffy coat layer that might contain cancer cells was carefully collected with a Pasteur pipette and rediluted with 50 ml of ice-cold PBS in a new 50-ml tube. This tube was centrifuged at 900g for 20 min at room

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temperature, and the supernatant was carefully decanted. The pellet was suspended in 250 μ l of PBS and transferred to a 1.5-ml polypropylene tube before the addition of 750 μ l of ISOGEN-LS solution. The mixture was kept at room temperature for 5 min before addition of 100 μ l of chloroform. It was then centrifuged at 12,000g for 15 min at 4°C after having been vigorously vortexed. The aqueous phase was transferred to a new tube, and Tris-EDTA (TE)-saturated phenol/chloroform was added. The solution was vigorously mixed and centrifuged at 12,000g for 10 min at 4°C. Phenol/chloroform extraction was performed twice more. Finally, the aqueous phase was transferred to a new tube and an equal volume of isopropyl alcohol was added, followed by centrifugation at 12,000g for 15 min at 4°C. The RNA pellet was washed with 75% ethanol and dissolved in RNase-free distilled water. The RNA concentration and purity were determined by ultraviolet spectroscopy at 260 nm and 280 nm.

Complementary DNA synthesis and RT-PCR Two micrograms of total RNA was denatured for 5 min at 65°C, followed by chilling on ice for 1 min. The denatured RNA was incubated for 1 h at 37°C in 50 μ l of a reaction mixture containing 5 \times RT buffer, 10 mM dithiothreitol, 0.5 mM each dNTPs, 500 ng of oligo dT primers (Boehringer-Mannheim, GmbH, Mannheim, Germany), 0.5 μ l of RNase inhibitor (Nippon Gene), and 2 μ l of MMLV-RT (GIBCO-BRL, Gaithersburg, MD). The mixture was heated for 5 min at 99°C to inactivate RT after the incubation.

A nested RT-PCR for PSM was then performed with the methods described by Israeli *et al.* and Loric *et al.*^{10, 11}) PSM cDNA-specific oligonucleotide primers designed by Israeli *et al.* and Loric *et al.* were used (Table I). The position of each outer and inner primer and the length of each PCR product are shown in Fig. 1. Five microliters of cDNA was amplified in 50 μ l of PCR reaction mixture,

Table I. Primers for PSM RT-PCR and GAPDH RT-PCR

PSM primers	
PSM primers-1	
Outer primers	5'-CAG ATA TGT CAT TCT GGG AGG TC-3' 5'-AAC ACC ATC CCT CCT CGA ACC-3'
Inner primers	5'-CCT AAC AAA AGA GCT GAA AAG CCC-3' 5'-ACT GTG ATA CAG TGG ATA GCC GCT-3'
PSM primers-2	
Outer primers	5'-GAA TGC CAG AGG GCG ATC TA-3' 5'-TTC TAG GAG CTT CTG TGC ATC ATA GTA TCC-3'
Inner primers	5'-AGG GGC CAA AGG AGT CAT TCT CTA CTC CGA-3' 5'-CTC TGC AAT TCC ACG CCT AT-3'
GAPDH primers	
	5'-ATG GGG AAG GTG AAG GTC GG-3' 5'-GCC AGT GGA CTC CAC GAC GT-3'

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

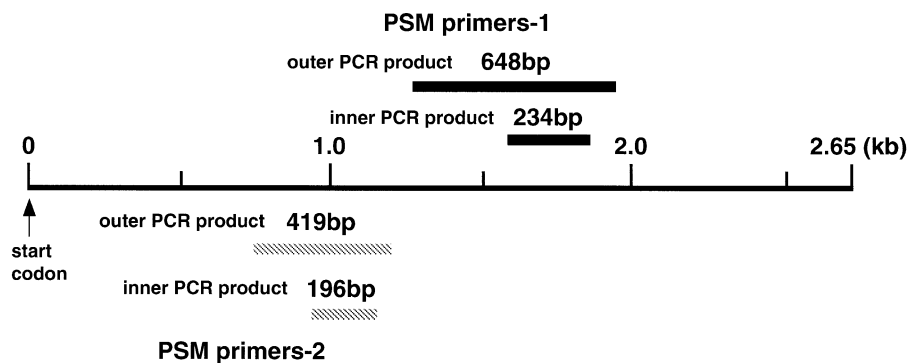


Fig. 1. Physical location of amplified outer and inner PCR products from PSM cDNA with PSM primers-1 (Israeli *et al.*) and PSM primers-2 (Loric *et al.*).

which contained cDNA, 1× PCR buffer, 10 μ M of sense and antisense oligonucleotide primers, 10 mM each dNTP mix and 1 unit of *Taq* DNA polymerase (Boehringer-Mannheim). The PCR was performed in a Thermal Cycler (GeneAmp 9600, Perkin Elmer, Norwalk, CT) under the following conditions: 94°C for 15 s, 60°C for 15 s and 72°C for 45 s for 25 cycles for PSM primers-1; 94°C for 2 min for 1 cycle, 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min for 25 cycles, followed by 72°C for 10 min for PSM primers-2. Five microliters of the reaction mixture was used as the template for another 25 cycles of PCR with a new reaction mixture containing inner PSM primers. As a control reaction, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) PCR was performed under

conditions of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 25 cycles. Ten microliters of each PCR product was electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining.

RESULTS

A control RT-PCR was performed with RNA extracted from LNCaP cells using either PSM primers-1 or primers-2. Each PCR mixture presented a single band on electrophoresis. The sizes of the PCR products obtained with PSM primers-1 were 648 base pairs (bp) for the 1st outer PCR and 234 bp for the 2nd inner PCR, while those of the PCR products obtained with PSM primers-2 were 419

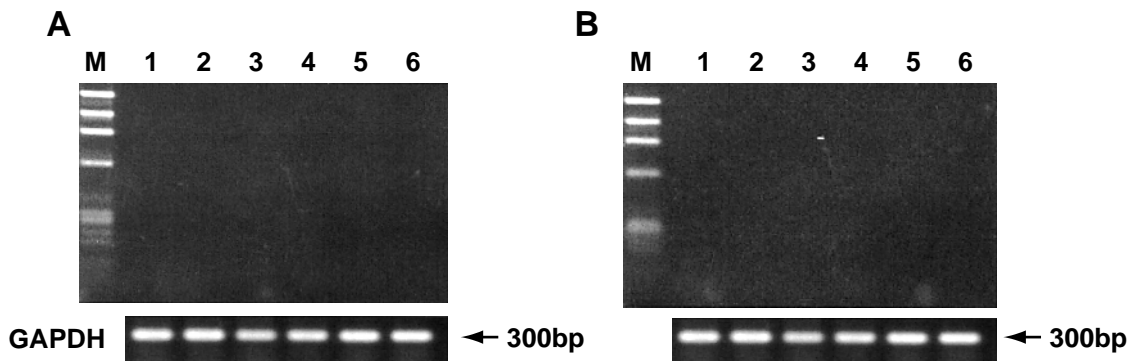


Fig. 2. Agarose gel electrophoresis of RT-PCR products obtained with PSM primers from 3 healthy males (lanes 1–3) and 3 healthy females (lanes 4–6). A, amplified with PSM primers-1; B, amplified with PSM primers-2. M, molecular weight marker ϕ X174/*Hae*III. The corresponding control reaction performed with GAPDH primers is shown below.

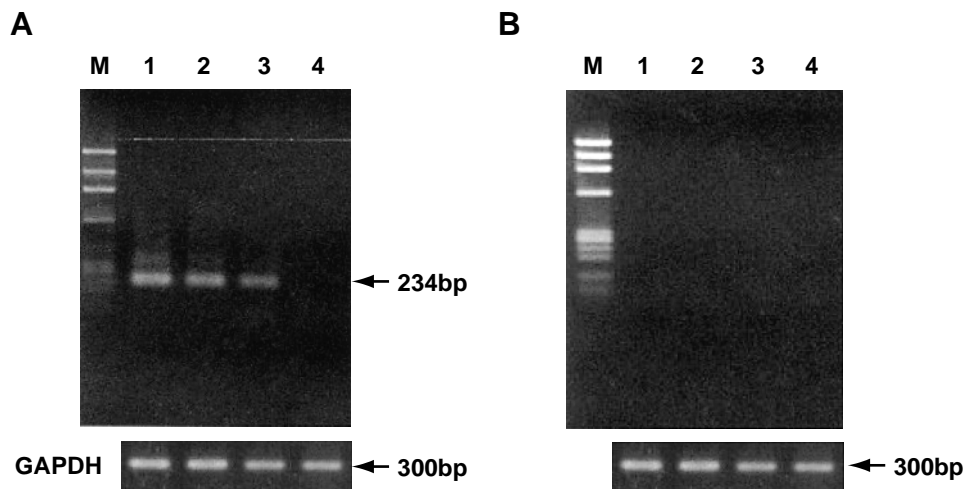


Fig. 3. Agarose gel electrophoresis of RT-PCR products obtained with PSM primers for BPH patients. A, inner PCR products amplified with PSM primers-1; B, amplified with PSM primers-2. M, molecular weight marker ϕ X174/*Hae*III. Lower panels show RT-PCR products with GAPDH primers to assure RNA integrity.

bp for the 1st outer PCR and 196 bp for the 2nd inner PCR (data not shown). The positive control amplification for GAPDH afforded a single band of 300 bp.

No amplified PCR product was visualized by RT-PCR with PSM primers-1 or primers-2 for any of 3 peripheral blood samples from normal healthy males and 3 samples from healthy females (Fig. 2). RT-PCR assays were performed with primers-1 or primers-2 to detect PSM mRNA in the peripheral blood of 4 pathologically confirmed BPH patients. The PCR using PSM primers-1 gave a positive band for 3 of the 4 patients, while the PCR using PSM primers-2 showed no positive PCR product (Fig. 3).

RT-PCR assays were also performed for peripheral blood samples from 5 metastatic and 2 locally invasive prostate cancer patients, in order to compare the sensitivity of PSM mRNA detection with primers-1 and primers-2. Table II shows the clinical characteristics and treatment background of each patient. The PCR with PSM primers-1 showed a positive PCR product in 4 of these 7 patients, while PSM primers-2 showed 6 positive bands (Fig. 4). This result suggested that the detection sensitivity of PSM

mRNA in peripheral blood might differ with the design of PSM primers.

It is possible that the appearance of a false-positive band for BPH patients in PCR using PSM primers-1 was due to genomic DNA contamination in the total RNA samples. To determine whether such a PCR product was derived from genomic DNA or total RNA, genomic DNA was treated with RNase A or DNase I before being examined by PCR. Genomic DNA was treated with RNase A or DNase I for 1 h at 37°C in each PCR reaction mixture, extracted with phenol/chloroform and precipitated with ethanol before amplification with the PSM-derived primer sets. The amplification with PSM primers-1 showed a positive PCR result for untreated genomic DNA, and the size of the PCR product was equal to that of the product derived from the total RNA template. In contrast, PSM primers-2 developed no PCR product (Fig. 5A). The PCR product was detected from RNase A-treated genomic DNA samples, whereas no product was detected from genomic DNA treated with DNase I when PSM primers-1 was used (Fig. 5, B and C; lane 1). No PCR product was

Table II. Clinical Characteristics and Treatment Background of Patients with Prostate Cancer

Patient	Age	Stage	Differentiation	Serum PSA value (ng/ml)	Invasion/metastasis	Treatment
1	78	D2	Mod.	1305	Bone	LH-RH, Flut
2	79	D2	Mod.	0.5	LN	LH-RH, Flut
3	60	D2	Well	16.5	Bone	LH-RH, Flut, UFT
4	64	D2	Poor	0.6	Bone, LN	LH-RH
5	77	D2	Mod.	1.9	Bone, NUV	LH-RH, Flut
6	75	C	Mod.	1.1	SV	Estracyte
7	76	C	Well	0.7	SV	LH-RH

LN, lymph node; SV, seminal vesicle; NUV, neurovascular. LH-RH, agonist; Flut, flutamide.

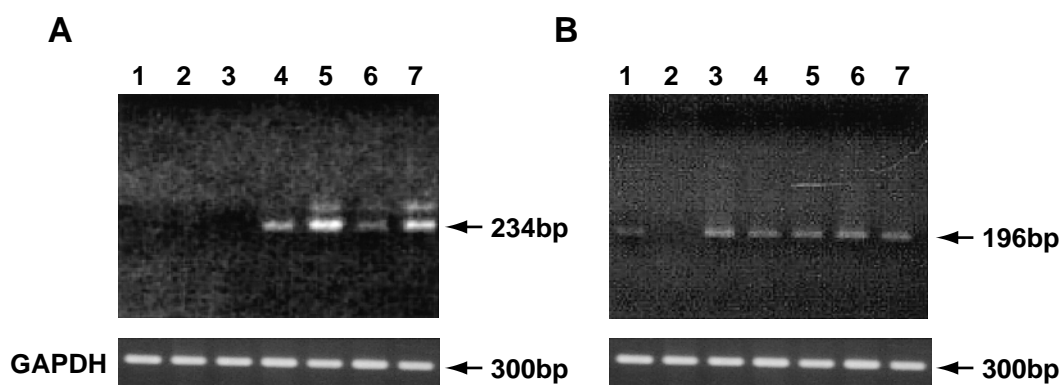


Fig. 4. Agarose gel electrophoresis of RT-PCR products obtained with PSM primers for prostate cancer patients. A, inner PCR products amplified with PSM primers-1; B, amplified with PSM primers-2. Lanes 1–5 are from Stage D2 patients; lanes 6 and 7 are from Stage C patients. The corresponding control reactions performed with GAPDH primers are shown below.

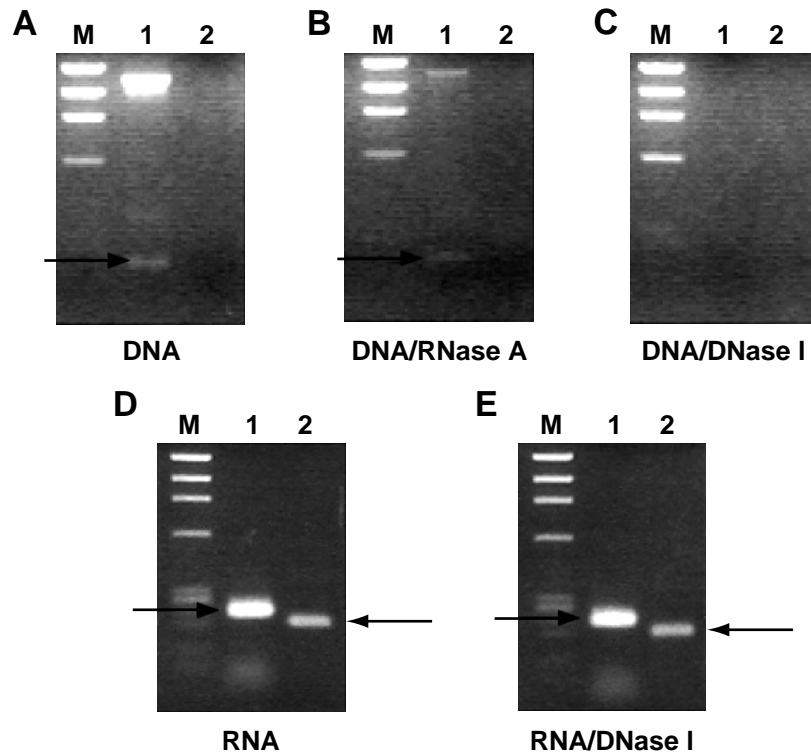


Fig. 5. Comparison of the PCR products detected with PSM primers-1 and PSM primers-2 in LNCaP genomic DNA and total RNA treated with RNase A or DNase I. A, untreated genomic DNA; B, genomic DNA treated with RNase A; C, genomic DNA treated with DNase I were amplified by PCR; D, untreated total RNA; E, total RNA treated with DNase I were amplified by RT-PCR. Lane 1, inner PCR products amplified with PSM primers-1; lane 2, amplified with PSM primers-2. M, molecular weight marker ϕ X174/*Hae*III. Arrows indicate PCR products derived from the *PSM* gene.

detected when the PSM primers-1 set was used to amplify total RNA extracted from the peripheral blood of BPH patients following DNase I treatment (data not shown). These results indicated that the PCR product from the peripheral blood of BPH patients originated from genomic DNA contamination. No PCR product was detected when PSM primers-2 was used to amplify genomic DNA treated with RNase A or DNase I (Fig. 5, B and C; lane 2). RT-PCR products were generated by both PSM primers for total RNA, even when it was treated with DNase I before the assay (Fig. 5, D and E).

DISCUSSION

The RT-PCR assay for detection of circulating prostate cancer cells in peripheral blood from cancer patients is expected to have higher sensitivity for stage diagnosis than other tumor markers or imaging diagnostic methods. The detection of circulating hematogenous tumor cells in a prostate cancer patient by PCR assay was first reported by Moreno *et al.*¹⁴ The results in their study were in agreement with studies by Hamdy *et al.*,¹⁵ who detected

circulating prostate-specific antigen (PSA)-positive cells in patients with metastatic prostate cancer by flow cytology and immunohistology.

Since PSM is naturally expressed only by prostate cells, binds to the cell membrane and is not secreted into the bloodstream, PSM expression in peripheral blood indicates that metastatic prostate cancer cells may exist in the blood circulation. Israeli *et al.* first reported the detection of PSM in the peripheral blood of prostate cancer patients and not of normal male and female subjects or BPH patients.¹⁰ Other groups confirmed that PSM detection from peripheral blood was specific to metastatic prostate cancer.¹¹⁻¹³ Although the full DNA sequence of PSM has not been established, Loric *et al.* attempted to design new primers of PSM-specific oligonucleotides for RT-PCR (PSM primers-2 in this report) which might include a certain length of intron.¹¹ A major problem of the PSM RT-PCR assay is the appearance of false-positive results; the false-positive rate is rather high in RT-PCR assays using the oligonucleotide primers set designed by Israeli *et al.* Loric *et al.* reported that their new oligonucleotide primers specifically amplified the *PSM* gene fragment of pros-

tate cancer patients, but did not generate a product from normal donors or BPH patients.¹¹⁾ In the present study, we compared the new primers with the primers designed by Israeli *et al.* using the same blood samples in order to compare the specificity and the sensitivity of these assays.

Our data confirmed that the specificity and sensitivity for detecting the *PSM* gene in blood samples differ with the design of the primers. Surprisingly, we observed a positive PCR product for *PSM* in the blood samples of 3 of 4 BPH patients when *PSM* primers-1 was used. In contrast, no false-positives were obtained with *PSM* primers-2 in normal donors or BPH patients, showing that the assay was highly specific to prostate cancer.

A possible explanation for the false-positive results for BPH patients is that an unknown template had contaminated the samples used for the amplification by *PSM* primers-1. As shown in Fig. 5, the template gave a positive PCR when genomic DNA extracted from LNCaP cells was amplified using *PSM* primers-1, whereas it gave a negative PCR when the genomic DNA was treated with DNase I. The origin of the upper PCR product in Fig. 5A has not been clarified. Recognition sites of *PSM* primers-1 may exist on genomic DNA sequences. The PCR product derived from the *PSM* gene (196 bp band) was confirmed by the digestion pattern with restriction enzymes (data not shown). In contrast, all assays were negative when the samples from normal donors were amplified using *PSM* primers-1, suggesting that genomic DNA might have accidentally contaminated total RNA during the extraction procedure from BPH patients.

Genomic DNA contamination may occur in a situation where multiple blood samples are processed at the same time. DNase I treatment is thought to decrease the false-positive rate. Since no false-positive result was obtained in the peripheral blood samples from normal donors and BPH patients, *PSM* primers-2 seemed to have higher specificity with acceptable accuracy for detecting prostate cancer cells in the peripheral blood compared to *PSM* primers-1. Lintula and Stenman reported that an RT-PCR assay for *PSM* using the same primers as *PSM* primers-1 showed positive results in the blood of 12 of 13 normal young male and female donors, suggesting that the RT-PCR might have been detecting *PSM* mRNA in the leuko-

cyte fraction of normal blood.¹²⁾ However, our results using *PSM* primer-1 did not show any *PSM* mRNA expression at all in peripheral blood from normal donors but showed positive products in the blood of some BPH patients (Figs. 2 and 3), which is contrary to Lintula's report. They indicated that these variations could probably be explained by differences in the sensitivity of the PCR methods. One possible reason for their frequent false-positive results is contamination of genomic DNA in total RNA samples, since they did not treat total RNA with DNase I. In fact, when the total RNA samples were treated with DNase I, no false-positive was detected in the samples of BPH patients in the present study (data not shown).

Comparisons of sensitivity for detecting circulating prostate cancer cells in the peripheral blood of metastatic prostate cancer patients by PCR using *PSM* gene or *PSA* gene have been reported.^{10-13, 16-18)} Israeli *et al.* indicated that *PSM* detection by PCR is superior to *PSA* detection,¹⁰⁾ while Cama *et al.* obtained an opposite result.¹³⁾ Other groups have made similar comparisons of the *PSM* gene and other genes for the detection of prostate cancer cells in the blood by the RT-PCR method. However, no reports have discussed the differences in the sensitivity and specificity of RT-PCR assays depending upon the design of primers for *PSM* gene amplification. The design of *PSM* primers for RT-PCR assay is known to be critical. We used two different sets of *PSM* primers and compared their sensitivity for prostate cancer cell detection in the peripheral blood of patients with prostate cancer. With the same blood samples, we found that the sensitivity differed with the different primers. The reason for this is still unknown, but easily amplified regions and hardly amplified regions may exist in *PSM* mRNA as a consequence of its complicated three-dimensional structure.

In conclusion, our investigation demonstrated that the *PSM* primer set reported by Loric *et al.* was superior to that of Israeli *et al.* (in terms of high sensitivity and low false-positive rate) for detecting *PSM* mRNA in the peripheral blood of prostate cancer patients.

(Received September 9, 1998/Revised November 5, 1998/
Accepted November 10, 1998)

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