Advances in Brief

Sensitive Nested Reverse Transcription Polymerase Chain Reaction Detection of Circulating Prostatic Tumor Cells: Comparison of Prostate-specific Membrane Antigen and Prostate-specific Antigen-based Assays


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Abstract

A highly sensitive nested reverse transcriptase-PCR assay, with primers derived from the prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSM) cDNA sequences, has been used to detect occult hematogenous micrometastatic prostate cells. In 77 patients with prostate cancer, PSM and PSA primers detected circulating prostate cells in 48 (62.3%) and 7 (9.1%) patients, respectively. In treated stage D disease patients, PSM primers detected cells in 16 of 24 patients (66.7%), while PSA primers detected cells in 6 of 24 (25%). In post-radical prostatectomy patients with negative serum PSA values, PSM primers detected metastases in 21 of 31 patients (67.7%), whereas PSA primers detected cells in only 1 of 33 (3.0%), indicating that micrometastatic spread may be a relatively early event in prostate cancer. The analysis of 40 individuals without known prostate cancer provides evidence that this assay is highly specific and suggests that PSM expression may predict the development of cancer in patients without clinically apparent prostate cancer. Using PSM primers, we detected micrometastasizes in 4 of 40 controls, 2 of whom had known benign prostatic hyperplasia and were later found to have previously undetected prostate cancer. The clinical significance of detection of hematogenous micrometastatic prostate cells using PSM primers and potential applications of this molecular assay, as well as the assay for PSA, merit further study.

Introduction

Prostate cancer is an increasingly prevalent health problem in the United States. The number of newly diagnosed cases has markedly increased over the past decade, with approximately 200,000 new cases expected to be diagnosed in the United States in 1994 (1), making prostate cancer the most common malignancy in American males, excluding skin cancer. Estimates of 38,000 deaths in 1994 as a result of this disease (1) indicate that prostate cancer is second only to lung cancer as the most common cause of death in the same population. There is an urgent need for improved methods of detection of organ-confined "curable" disease. Despite the improved and expanded arsenal of modalities available to clinicians today, including sensitive serum PSA assays, computed tomography scans, transrectal ultrasonography, endorectal coil magnetic resonance imaging, etc., many patients are still found to have metastatic disease at the time of pelvic lymph node dissection and radical prostatectomy. We have developed a highly sensitive reverse transcription PCR assay capable of detecting occult hematogenous micrometastatic prostate cells that would otherwise go undetected by presently available staging modalities. This assay is a modification of similar PCR assays performed in patients with prostate cancer and other malignancies (2, 3, 4, 5). The assay uses PSM primers derived from the cDNA sequences of prostate-specific antigen (6) and the prostate-specific membrane antigen (6) and the prostate-specific membrane antigen recently cloned and sequenced in our laboratory (7).

Materials and Methods

Cells and Reagents. LNCaP and MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD). Details regarding the establishment and characteristics of these cell lines have been published previously (8, 9). Cells were grown in RPMI 1640 and supplemented with 1-glutamine, nonessential amino acids, and 5% FCS (GIBCO-BRL, Gaithersburg, MD) in a 5% CO2 incubator at 37°C. All cell media was obtained from the MSKCC Media Preparation Facility. Routine chemical reagents were of the highest grade possible and were obtained from Sigma Chemical Co. (St. Louis, MO).

Patient Blood Specimens. All blood specimens used in this study were from patients seen in the outpatient offices of urologists on staff at MSKCC. Two anticoagulated tubes/patient were obtained at the time of their regularly scheduled blood draws. Specimens were obtained with informed consent of each patient according to a protocol approved by the MSKCC Institutional Review Board. Samples were promptly brought to the laboratory for immediate processing. Seventy-seven specimens from patients with prostate cancer were randomly selected and delivered to the laboratory "blinded," along with samples from negative controls for processing. These included 24 patients with stage D disease (3 with D0, 3 with D1, 11 with D2, and 7 with D3), 31 patients who had previously undergone radical prostatectomy and had undetectable postoperative serum PSA levels (18 with P0 lesions, 11 with P1, and 2 P2), 2 patients with locally recurrent disease following radical prostatectomy, 4 patients who had received either external beam radiation therapy or interstitial implants (10 patients with untreated clinical stage T1-T3 disease, and 6 patients with clinical stage T4 disease on antiandrogen therapy). The 40 blood specimens used as negative controls were from 10 healthy males, 9 males with biopsy-proven benign prostatic hyperplasia and elevated serum PSA levels, 7 healthy females, 4 male patients with renal cell carcinoma, 2 patients with prostatic intraepithelial neoplasia, 2 patients with transitional cell carcinoma of the bladder and a pathologically normal prostate, 1 patient with acute prostatitis, 1 patient with acute promyelocytic leukemia, 1 patient with testicular cancer, 1 female patient with renal cell carcinoma, 1 patient with lung cancer, and 1 patient with a cyst of the testicle.

Blood Sample Processing/RNA Extraction. Four ml of whole anticoagulated venous blood was mixed with 3 ml of ice-cold PBS and then carefully layered atop 8 ml of Ficoll (Pharmacia, Uppsala, Sweden) in a 15-ml
polystyrene tube. Tubes were centrifuged at 200 × g for 30 min at 4°C. The buffy coat layer (approximately 1 ml) was carefully removed and rediluted to 50 ml with ice-cold PBS in a 50-ml polycarbonate tube. This tube was then centrifuged at 2000 × g for 30 min at 4°C. The supernatant was carefully decanted and the pellet was allowed to drip dry. One ml of RNAzol B was then added to the pellet and total RNA was isolated according to manufacturer’s directions (Cinna/Biotex, Houston, TX.). RNA concentrations and purity were determined by UV spectroscopy on a Beckman DU 640 spectrophotometer and by gel analysis.

**Determination of PCR Sensitivity.** RNA was isolated from LNCaP cells and from mixtures of LNCaP and MCF-7 cells at fixed ratios (i.e., 1:100, 1:1000, etc.) using RNAzol B. Nested PCR was then performed as described below with both PSA and PSM primers in order to determine the limit of detection for the assay. LNCaP:MCF-7 (1:100,000) cDNA was diluted with distilled water to obtain concentrations of 1:1,000,000. The human breast cancer cell line MCF-7 was chosen because they had been tested previously by us and shown not to express either PSM or PSA by both immunohistochemistry and conventional and nested PCR.

**PCR.** The PSA outer primer sequences are nucleotides 494–513 (sense) in exon 4 and nucleotides 960–979 (antisense) in exon 5 of the PSA cDNA. These primers yield a 486-base pair PCR product from PSA cDNA that can be distinguished from a product synthesized from possible contaminating genomic DNA.

PSA-494 5′-TAC CCA CTG CAT CAG GAA CA-3′
PSA-960 5′-CCT TGA AGC ACA CCA TTA CA-3′

The PSA inner upstream primer begins at nucleotide 559 and the downstream primer at nucleotide 894 to yield a 355-base pair PCR product.

PSA-559 5′-ACA CAG GCC AGG ATT TTC AG-3′
PSA-894 5′-GTC CAG CCT CCA GCA CAC AG-3′

All primers were synthesized by the MSKCC Microchemistry Core Facility. Five μg of total RNA was reverse transcribed into cDNA using random hexamer primers (GIBCO-BRL) and Superscript II reverse transcriptase (GIBCO-BRL) according to the manufacturer’s recommendations. One μl of this cDNA served as the starting template for the outer primer PCR reaction. The 20-μl PCR mix included 0.5U Taq polymerase (Promega), Promega reaction buffer, 1.5 mM MgCl2, 200 μM deoxynucleotide triphosphates, and 1.0 μM of each primer. This mix was then transferred to a Perkin Elmer Cetus 9600 DNA thermal cycler and incubated for 25 cycles. The PCR profile was as follows: 94°C for 15 s; 60°C for 15 s, and 72°C for 45 s. After 25 cycles, samples were placed on ice, and 1 μl of this reaction mix was used as the template for another 25 cycles with a new reaction mix containing the inner PSM primers. cDNA quality was verified by performing control reactions using primers derived from the β-2-microglobulin gene sequence (10), a ubiquitous housekeeping gene. These primers span exons 2–4 and generate a 620-base pair PCR product. The sequences for these primers are:

β-2 (exon 2) 5′-AGC AGA GAA TGG AAA GTC AAA-3′
β-2 (exon 4) 5′-TGG TGA TGG ATA AGA GAA T-3′

The entire PSA mix and 7–10 μl of each PSM reaction mix were run on 1.5–2% agarose gels, stained with ethidium bromide, and photographed in an Eagle Eye Video Imaging System (Stratagene, Torrey Pines, CA). Assays were repeated at least twice to verify results.

**Cloning and Sequencing of PCR Products.** PCR products were cloned into the pCR II plasmid vector using the TA cloning system (Invitrogen). These plasmids were transformed into competent Escherichia coli cells using standard methods (11) and plasmid DNA was isolated using Magic MiniprepIs (Promega) and screened by restriction analysis. Double-stranded TA clones were then sequenced by the dideoxy method (12) using [32P]dCTP (DuPont New England Nuclear) and Sequenase (United States Biochemicals, Inc.). Sequencing products were then analyzed on 6% polyacrylamide–7 M urea gels, which were fixed, dried, and autoradiographed as described.

**Southern Analysis.** PCR products were transferred from ethidium-stained agarose gels to Nytran nylon membranes (Schleicher & Schuell) by pressure blotting with a Posi-blotter (Stratagene) according to the manufacturer’s instructions. DNA was cross-linked to the membrane using a UV Stratalinker (Stratagene). Blots were prehybridized at 65°C for 2 h and subsequently hybridized with denatured, 32P-labeled, random-primed (13) cDNA probes (either PSA or PSM) (6, 7). Blots were washed twice in 1× SSC-0.5% SDS at 42°C and twice in 0.1× SSC-0.1% SDS at 50°C for 20 min each. Membranes were then air dried and autoradiographed for 1–3 h at room temperature with Hyperfilm MP (Amersham).

**Results**

**PSA- and PSM-nested PCR Assays.** The application of nested PCR increased our level of detection from an average of 1:10,000 using outer primers alone, to better than 1:1,000,000. Dilution curves demonstrating this added sensitivity are shown for PSA and PSM-PCR in Figs. 1 and 2, respectively. Fig. 1 shows that the 486-base pair product of the PSA outer primer set is clearly detectable with ethidium staining to 1:10,000 dilutions, whereas the PSA inner primer 355-base pair product is clearly detectable in all dilutions shown. In Fig. 2, the PSA outer primer 647-base pair product is also clearly detectable in dilutions to only 1:10,000 with conventional PCR, in contrast to the PSM inner nested PCR 234-base pair product which is detected in dilutions as low as 1:1,000,000. Southern blotting was performed on all controls and most of the patient samples in order to confirm specificity. Southern blots of the respective dilution curves confirmed the primer specificities but did not reveal any significantly increased sensitivity.

**PCR in Negative Controls.** We performed nested PSA and PSM PCR on 40 samples from patients and volunteers as described in “Materials and Methods.” Fig. 3 reveals results from 4 representative negative control specimens, in addition to a positive control. Each specimen in our study was also assayed with the β-2-microglobulin control, as shown in this figure, in order to verify RNA integrity. We obtained negative results on 39 of these samples using the PSA primers; however, PSM-nested PCR yielded 4 positive results. Two of these “false positives” represented patients with elevated serum PSA values and an enlarged prostate who underwent a transrectal prostate biopsy, revealing stromal and fibromuscular hyperplasia. In both of these patients the serum PSA level continued to rise, and a repeat prostate biopsy performed at a later date revealed prostate cancer. One patient who presented to the clinic with a testicular cyst was noted to have a positive PSM-nested PCR result which we have thus far been unable to explain. Unfortunately, this patient never returned for fol-
PSM-PCR DETECTION OF OCCULT PROSTATIC MICROMETASTASES

Fig. 1. Ethidium bromide-stained gel depicting PCR detection of PSA in LNCaP cells diluted with MCF-7 cells. Lanes A, products of outer primers; Lanes B, inner primer products.

Fig. 2. Ethidium bromide-stained gel depicting PCR detection of PSM in LNCaP cells diluted with MCF-7 cells. Lanes A, products of outer primers; Lanes B, inner primer products.

Fig. 3. Ethidium bromide-stained gel depicting representative negative and positive controls used in this study. Samples 1–5 were from a male with prostatitis, a healthy female volunteer, a male with benign prostatic hyperplasia, a control 1:1,000,000 dilution of LNCaP cells, and a patient with renal cell carcinoma, respectively. Below each sample is the corresponding control reaction performed with β-2-microglobulin primers to assure RNA integrity. No PCR products were detected for any of these negative controls. bp, base pairs. Lanes A, products of outer primers; Lanes B, inner primer products.

Discussion

Improved and more sensitive methods for the detection of minimal, occult micrometastatic disease have been reported for a number of malignancies with the use of immunohistochemical methods (14), as well as PCR (3, 4, 5). The application of PCR to detect hematogenous micrometastases in prostate cancer was first described by Moreno et al. (2) using conventional PCR with PSA-derived primers. We have subsequently reported the application of a nested PCR approach to improve the level of detection (15, 16) of prostatic cells, as well as the use of primers derived from the PSM antigen cloned and characterized by our laboratory (7). Several other groups have recently reported the application of PSA-PCR to identify micro-

low-up; thus, we have not been able to obtain another blood sample to repeat this assay. We obtained a positive result with both PSA and PSM primers in a 61-year-old male patient with renal cell carcinoma. This patient has a normal serum PSA level and a normal digital rectal examination. Overall, if we exclude the two patients in whom a positive PCR, but no other clinical test, accurately predicted the presence of prostate cancer, 36 of 38 (94.7%) of our negative controls were negative with PSM primers, and 39 of 40 (97.5%) were negative using PSA primers.

Patient Samples. We assayed in a blinded fashion, in which the laboratory staff were unaware of the nature of each specimen, 117 samples from 77 patients mixed randomly with 40 negative controls. The patient samples represented a diverse and heterogeneous group as described earlier. Several representative patient samples are displayed in Fig. 4, corresponding to positive results from patients with both localized and disseminated disease. Patients 4 and 5, both with stage D prostate cancer, exhibited positive results with both the outer and inner primer pairs, indicating a large circulating tumor cell burden, as compared to the other samples. Although our PSM and PSA primers yielded similar sensitivities in LNCaP dilution curves as shown previously, PSM primers detected micrometastases in 62.3% of our patient samples, whereas PSA primers only detected 9.1%. In patients with documented metastatic prostate cancer (stages D0–D3) receiving antiandrogen treatment, PSM primers detected micrometastases in 16 of 24 (66.7%), whereas PSA primers detected circulating cells in only 6 of 24 (25%). In our study 6 of 7 patients with hormone-refractory prostate cancer (stage D3) were positive. PSA primers revealed micrometastatic cells in only 1 of 15 (6.7%) patients with either pT3 or pT4 (locally advanced) prostate cancer following radical prostatectomy. PSM primers detected circulating cells in 9 of 15 (60%) of these patients. Interestingly, we detected circulating cells in 13 of 18 (72.2%) patients with pT2 (organ-confined) prostate cancer following radical prostatectomy using PSM primers. None of these patient samples were positive by PSA-PCR.
metastatic cells in peripheral blood and bone marrow, as well as lymph node tissues (2, 17–19, 20, 21). We find an increased detection rate of micrometastases using PSM primers. When human prostate tumors and prostate cancer cells in vitro were studied by immunohistochemistry and mRNA analysis, PSM appeared to be highly expressed in anaplastic cells, hormone-refractory cells, and bony metastases (22, 23, 24), in contrast to PSA. If cells capable of hematogenous micrometastasis represent the more aggressive and poorly differentiated cells, they may express a higher level of PSM per cell as compared to PSA, enhancing their detectability by reverse transcriptase PCR.

Our nested reverse transcriptase PCR assays are both sensitive and specific. Results have been reliably reproduced on repeated occasions. Long-term testing of both cDNA and RNA stability is presently under way. Both assays are capable of detecting one prostatic cell in at least 1 million non-prostatic cells of similar size. This confirms the validity of our comparison of PSM versus PSA primers. We can expect a similar level of PSM expression in both human prostatic cancer cells in vivo and LNCaP cells in vitro, as we have shown previously (22). The specificity of our PSM-PCR assay was supported by the finding that two “negative control” patients with positive PSM-PCR results were both subsequently found to have prostate cancer. This suggests an exciting potential application for this technique for use in cancer screening. In contrast to recently published data (18), we fail to identify any significant ability for PSA primers to accurately detect micrometastatic cells in patients with pathologically organ-confined prostate cancer, despite the sensitivity of our assay. Our data revealing the surprisingly high percentage of patients with localized prostate cancer that harbor occult circulating prostate cells following “curative” radical prostatectomy suggest that micrometastasis is an early event in prostate cancer. It must be noted, however, that these results derive from a small number of patients. Prospective long-term follow-up of these patients will be required to determine the potential prognostic value and clinical significance of these unexpected findings. Our results with PSA primers in stage D patients are similar to results reported previously by Moreno et al. (2), despite the fact that our primer sequences and method of PCR (conventional versus nested) differed. In our study, 6 of 7 patients with hormone-refractory prostate cancer (stage D2) were positive with both PSA and PSM primers. All 6 of these patients died within 2–6 months of their assay, despite aggressive cytotoxic chemotherapy, in contrast to the 1 patient that tested negatively in this group and is alive 15 months after his assay. From data in this paper, therefore, it appears that PSA-PCR positivity serves as a predictor of early mortality.

The application of this powerful new modality to potentially stage and/or follow the response to therapy in patients with prostate cancer certainly merits further investigation. In comparison to molecular detection of occult tumor cells, current clinical modalities for the detection of prostate cancer spread appear inadequate. Since it is well established that only a minute fraction of circulating tumor cells will eventually go on to form a solid metastasis, and that a certain minimal tumor cell burden appears to be necessary (25), we again emphasize that the clinical significance of occult hematogenous micrometastases in prostate cancer will require analyzing larger numbers of samples, as well as longitudinal assessment of the clinical course of these patients.

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References


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