Detection of Hematogenous Micrometastasis in Patients with Prostate Cancer

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Abstract

The goal of this study was to determine if patients with stage D0-3 prostatic adenocarcinoma have detectable hematogenous micrometastasis. Polymerase chain reaction amplification of prostate-specific antigen mRNA, which is exclusively expressed by prostatic epithelial cells, was used to detect circulating prostatic cells. Peripheral venous blood was obtained from 17 control and 12 prostate cancer patients with stage D0-3 prostatic adenocarcinoma. Of the 12 cancer cases, four patients (stage D1-3) tested positive for prostate-specific antigen mRNA, indicating the presence of circulating micrometastasis. The 17 negative controls all tested negative. Contrary to a long held hypothesis, these data point to the possibility that hematogenous metastasis may be a relatively early event in the natural history of human prostate cancer. These findings may have an important impact on our understanding and treatment of prostate cancer.

Introduction

Prostate cancer metastasis will claim the lives of over 30,000 Americans this year (1). The mode of dissemination, however, remains very poorly understood. An almost dogmatic view of metastasis holds that prostate cancer cells first spread through the prostatic capsule and then into the lymphatics and eventually travel hematogenously to bone (2–9). This hypothesis has been based on histopathological studies which have significant limitations, and in actuality the sequence of metastatic events remains unknown. Solid tumor animal experiments suggest that only 0.01% of circulating cancer cells eventually create a single metastatic deposit (10–12). Ostensibly, a single bone metastasis from human PAC could be generated by 10,000 circulating cancer cells (2 cells/ml blood). In the past, the detection of such a low concentration of cells would be nearly impossible, but with the advent of the PCR and new tumor markers detection of micrometastasis would seem feasible. Recently, Wu et al. (13) and others used keratin-19 mRNA PCR to detect breast cancer micrometastasis in patient lymph nodes and bone marrow. In 1992 Miyomura et al. (14) also reported the detection of minimal residual acute lymphoblastic leukemia by PCR in patients harboring the Philadelphia chromosome.

The purpose of this study was to determine whether patients with stage D0-3 prostatic adenocarcinoma have directly detectable micrometastasis in peripheral venous blood using RT PCR targeted at the PSA gene. PSA is an important tumor marker produced exclusively by prostatic epithelial cells and is almost always expressed by prostate cancer (15). These data shed light on the molecular physiology of human prostate cancer metastasis, which ultimately may have important clinical implications. The entire PSA RNA gene assay consisted of four steps: (a) whole blood gradient isolation of nucleated cells; (b) total RNA extraction; (c) PSA RT PCR of total RNA; and (d) agarose gel electrophoresis of PCR products.

Materials and Methods

Patient Specimens. Selection of cases was based on the following criteria. Prostate cancer patients were chosen for analysis if they had (a) clinically and/or surgically staged D0-D2 disease (D0, elevated tumor markers with no demonstrable metastasis; D1, pelvic lymph node involvement; D2, disseminated disease to bone) without having received prior hormonal therapy and who had an elevated serum PSA or (b) stage D3 disease (D2 disease that is refractory hormonal therapy) with an elevated PSA. Negative control patients consisted of female volunteers, patients with BPH proven by biopsy, or men who were on a BPH study protocol. Patients who had had surgical manipulation of the prostate during the previous year were excluded from the study. Positive controls included a lymph node from a patient with known metastatic PAC tissue from pathologically proven BPH and a cDNA PSA plasmid provided by Henttu and Vihko (16). The protocol was approved by the institutional review board, and written consent was obtained. LNCAP and PC3 human cell lines were obtained from the American Type Culture Collection (Rockville, MD).

Blood Preparation for RNA Extraction. Approximately 6 ml of venous blood were obtained with a standard venipuncture technique using heparinized tubes. Whole blood was mixed with an equal volume of phosphate buffered saline, which was then layered over 8 ml of Ficoll (Pharmacia, Uppsala, Sweden) in a 15-ml polystyrene tube. The gradient was centrifuged at 200 × g for 30 min at 5°C. The lymphocyte and granulocyte layer (approximately 5 ml) was carefully aspirated and rediluted up to 50 ml with phosphate-buffered saline in a 50-ml tube, which was then centrifuged at 1800 × g for 20 min at 5°C. The supernatant was discarded, and the pellet containing nucleated cells was used for RNA extraction using the RNazol B method as described by the manufacturer (Tel-Test, Inc., Friendswood, Texas).

Oligonucleotide Primers and Probes. PSA2 (5'-GAGGTCCACA-CACTGAGGT) and PSA3 (5'-CCTCCCTGAAGAATCGATrCCT) oligonucleotide primers were custom designed with high specificity to the PSA gene. PSA2 and 3 bind exons 4 and 3, respectively. A Gene (Pharmacia, Uppsala, Sweden) in a 15-ml polystyrene tube. The gradient was centrifuged at 200 × g for 30 min at 5°C. The lymphocyte and granulocyte layer (approximately 5 ml) was carefully aspirated and rediluted up to 50 ml with phosphate-buffered saline in a 50-ml tube, which was then centrifuged at 1800 × g for 20 min at 5°C. The supernatant was discarded, and the pellet containing nucleated cells was used for RNA extraction using the RNazol B method as described by the manufacturer (Tel-Test, Inc., Friendswood, Texas).

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Polymerase Chain Reaction. The reverse transcriptase reaction and PCR amplification were performed sequentially without interruption in
confirmed with DNA sequence analysis of the amplified 214-containing ethidium bromide. All specimens were analyzed at least entirely sample was then run on a thin 3% Tris-borate-EDTA agarose gel were concentrated down to 10 μl with vacuum centrifugation, and the cycle 5 was 72°C for 15 mm (1 cycle); and the final cycle was a 4°C hold was 42°C for 15 min then 97°C for 15 s (1 cycle); cycle 2 was 95°C for 1 min, 60°C for 1 min, and 72°C for 30 s (15 cycles); cycle 3 was 95°C for 1 min, 60°C for 1 min, and 72 degrees for 1 min (10 cycles); cycle 4 was 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min (8 cycles); cycle 5 was 72°C for 15 min (1 cycle); and the final cycle was a 4°C hold until sample was taken out of the machine. The 50-μl PCR products were concentrated down to 10 μl with vacuum centrifugation, and the entire sample was then run on a thin 3% Tris-borate-EDTA agarose gel containing ethidium bromide. All specimens were analyzed at least twice to confirm a positive or negative outcome.

Sequencing. The 214-base pair product generated from PSA cDNA was purified with a Qiagen PCR Product Purification kit (Qiagen, Chatsworth, CA) as described by the manufacturer. One μg of the PCR product underwent a PCR sequencing reaction by using the Taq DyeDeoxy Terminator Cycle sequencing kit in a Perkin-Elmer 9600 PCR machine as described by Applied Biosystems (Foster, CA). The sequenced product was purified using Centri-Sep columns (Princeton Separations, Adelphia, NJ) as described by the company. This product was then analyzed with an ABI model 373A DNA sequencing system (Applied Biosystems) integrated with a Macintosh Ilci computer.

Results and Discussion

PCR Amplification of PSA mRNA. Several important factors were involved in custom designing and optimizing the RT PCR PSA reaction which produced very specific and sensitive results. The PSA2 and 3 primers were designed to flank PSA intron III such that PCR amplification of RNA and DNA produced 214- and 360-base pair fragments, respectively, eliminating the possibility of false positives from DNA contamination (Fig. 1). The specificity of these primers to the PSA gene was confirmed with DNA sequence analysis of the amplified 214-base pair fragment which in this segment had very little homology to the HMGK gene. The primers were designed to have significant mismatches with the HMGK gene, and in the unlikely event of amplifying HMGK mRNA, the PCR fragment would be approximately 100 bases smaller than the PSA PCR product. This point is important, since there is 75—85% homology between the PSA and HMGK genes (16). The potential risk of false positives from cross-contamination was avoided by performing RT PCR in a single tube without interruption and using filtered pipet tips. Sensitivity was enhanced by using high amounts of Taq polymerase, progressively increasing extension times, and analyzing the entire 50-μl PCR product on thin ethidium bromide agarose gels. These measures ensured a high-fidelity assay while maintaining technical simplicity.

Prostate human tissue specimens, tissue culture cell lines, and a PSA cDNA plasmid, cloned and described by Henttu and Vihko (16), were used as positive controls, and they demonstrated the 214-base pair bands as shown in Fig. 1. A pelvic lymph node with metastatic PAC, a primary prostate cancer, and a BPH specimen all produced strong PSA PCR signals. The LNCAP and PC-3 human prostate cell lines produced much weaker signals.

Detection of Circulating Hematogenous Micrometastasis. Twelve prostate cancer patients and 17 control patients underwent RT PCR analysis on PSA and actin RNA extracted from blood (Table 1). All cases demonstrated satisfactory RNA quality by actin PCR (Fig. 1). Of the 12 PAC patients with metastatic disease, four cases (33%) had positive PSA signals, indicating the presence of prostatic epithelial cells in the peripheral venous blood. These four cases consisted of stage D1 (n = 2), D2 (n = 1), and D3 (n = 1) prostate cancer patients (Fig. 1). Lanes 7 and 8 represent the same patient using a slightly different gradient method. The 17 negative controls, which consisted of 8 volunteer women and 9 men with BPH, all had undetectable PSA mRNA by PCR. These data indicate that RT PCR of the PSA RNA gene can be used to specifically detect circulating hematogenous micrometastasis in patients with stage D1–D3 pathology. These findings are in agreement with studies by Hamdy et al. (18), who detected circulating PSA-positive cells in patients with metastatic prostate cancer by flow cytology and immunohistology.

![Fig. 1. Top, representative gels A, B, and C, demonstrating amplification of PSA and actin RNA, respectively. Lanes 1–6, positive controls; Lanes 7–11, prostate cancer patients; Lanes 12–17, negative controls (bottom).](attachment:image.png)
Table 1 Hematogenous micrometastasis summary

<table>
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<tr>
<th>Stage</th>
<th>No. of patients</th>
<th>Positive PSA/PCR</th>
<th>Source</th>
<th>No. of patients</th>
<th>Positive PSA/PCR</th>
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<td>Females</td>
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<tr>
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<td>2</td>
<td>BPH</td>
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</tr>
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<td>D3</td>
<td>3</td>
<td>1</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>4 (33%)</td>
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</table>

Why Did Some Patients with Prostate Cancer Have Undetectable Hematogenous Micrometastasis? Overall, there were 8 of 12 prostate cancer patients who clearly had undetectable hematogenous micrometastasis consisting of stage D3 (n = 2), D1 (n = 2), and D0 (n = 4) patients. The negative results in stage D0 may truly represent an actual absence of circulating cancer cells. However, there may be two factors that could enhance the detection of cells that may be escaping detection. First, preliminary results indicate that the prostate cancer cells may be more concentrated in the buffy coat. The PSA signal was stronger in the RNA extracted from cells obtained only from the buffy coat (Lane 8) compared to those isolated from the entire Ficoll layer (Lane 7) in the same prostate cancer patient. These findings are in agreement with those of Harty et al., who found that prostatic epithelial cells migrate into the buffy coat (19). Thus, we are focusing on analyzing buffy coat cells. Second, circulating prostate cancer clones may be more poorly differentiated, thereby expressing lower levels of PSA. We are exploring the use of other markers which may be less influenced by malignant dedifferentiation such as keratin (13).

The Detection of Circulating Prostate Cancer Cells in Stage D1 Patients Raises Important Issues Regarding the Biology of Human Prostate Cancer Metastasis. These data support animal experimental work suggesting that only the fittest tumor cells survive the metastatic cascade (20). In other words, a large number of circulating cancer cells (greater than 10,000 cells in a 70-kg human) may be required in order for a small percentage (less than 0.01%) of cells to survive and create a metastatic deposit. The finding of cancer cells in the peripheral venous blood suggests that these cells circulate through all organ capillary beds while forming metastatic colonies almost exclusively in bone. These data lend credence to the theory that the distribution of prostate cancer metastasis is determined by organ tropism, and not strictly anatomic factors such as Batson’s vertebral venous plexus. Organ tropism may be determined by (a) preferential growth of cells in certain organs, (b) preferential adherence of cells to certain endothelial structures, and (c) chemotaxis. Finally, the discovery of prostate cancer cells in the circulation of patients with D1 pathology calls into question the hypothesis that blood metastasis is a terminal event in the natural history of prostate cancer metastasis. To the contrary, these data point to the possibility that prostate cancer cells may spread to the neurovascular structures and then into the blood circulation, and lymphatic spread may be an associated event that is not an essential step for dissemination (Fig. 2). This important hypothesis remains to be proven by applying further PCR cancer cell detection in a larger number of patients with varying stages of disease.

In summary, we describe a PCR-based technique which can detect circulating prostate cancer cells in some patients with stage D1-3 disease. These data suggest that hematogenous metastasis may not be a terminal event in the natural history of human prostate cancer. These findings may have an important impact on our understanding and treatment of prostate cancer as well as other common cancers.

References
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