Advances in Brief

Detection of Micrometastatic Prostate Cancer Cells in Lymph Nodes by Reverse Transcriptase-Polymerase Chain Reaction

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Abstract

We have developed a highly sensitive method for detecting prostate cancer cells using reverse transcriptase-polymerase chain reaction (RT-PCR) with primers specific for prostate-specific antigen gene. Forty-four lymph nodes obtained from 22 patients with prostate cancers were analyzed by RT-PCR to detect metastatic prostate cancer cells. RT-PCR could detect prostate-specific antigen mRNA in five lymph nodes with histologically and immunohistochemically identifiable metastases and in four lymph nodes with negative histological and immunohistochemical analyses for metastases. RT-PCR was a more sensitive method than histology and immunohistochemistry in detecting metastatic prostate cancer cells and could be applied for diagnosing micrometastases of prostate cancer to lymph nodes. This highly sensitive RT-PCR will be a relevant tool to allow a more accurate clinical assessment of lymph node metastases of prostate cancer and to understand lymphatic dissemination of prostate cancer biologically.

Introduction

Total prostatectomy with pelvic lymphadenectomy is performed in patients with prostate cancer clinically localized to the prostate. Among patients with prostate cancer histologically confined to the prostate and with no evidence of the disease in lymph nodes, some will suffer from distant metastases or local recurrences postoperatively. In these patients, the numbers of metastatic or residual cancer cells are so small that occult metastases or local minimal spread of the disease cannot be detected by conventional radiological imaging or traditional histopathological examinations. Methods for detection of occult metastases or local minimal spread of the disease need to be more sensitive and specific. An immunohistochemical technique using antibodies against tumor-specific antigens or tissue-specific antigens is a potential tool in the identification of the primary site of metastatic cancer and helpful for the recognition of the presence of small foci of metastatic disease. Molecular probes are also used to detect a small number of neoplastic cells in a large number of nonneoplastic cells (1). It is by PCR that the sensitivity of this approach is dramatically increased. A PCR-based method analyzing mRNA expression specific for neoplastic cells, RT-PCR, has been applied for detecting micrometastases or minimal residual diseases of malignancies which are difficult to be discovered by traditional morphological and immunohistochemical analyses (2–7). In this study, we attempted to detect PSA mRNA expression by means of RT-PCR in order to diagnose micrometastases of prostate cancer to lymph nodes.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; PSA, prostate-specific antigen; cDNA, complementary DNA; PBMC, peripheral blood mononuclear cell.

Materials and Methods

Cell Lines. LNCAp (8) and PC-3 (9) human prostate cancer cell lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum, penicillin, and streptomycin. The ACHN human renal cell cancer line and the HT-1376 human bladder cancer cell line were maintained in modified Eagle’s medium supplemented with 10% fetal calf serum and kanamycin.

Surgical Specimens. Fifty-five specimens of prostate tumors, 5 specimens of bladder cancers, and 5 specimens of renal cell cancers obtained by biopsy or surgical operation and 14 lymph nodes collected from 2 patients with benign prostate hyperplasia, 2 patients with renal cell cancer, and 3 patients with bladder cancer were analyzed to determine the specificity of RT-PCR for detecting PSA mRNA expression in prostate tumors. To compare RT-PCR with histological and immunohistochemical analyses in detecting metastatic prostate cancer cells in lymph nodes, 44 lymph nodes were analyzed in this study. One neck lymph node was obtained from one patient with stage D2 prostate cancer and 43 pelvic lymph nodes were collected from 21 patients with clinically localized prostate cancer undergoing total prostatectomy with pelvic lymphadenectomy. Lymph nodes were selected at operation and each of them was divided into two specimens, one of which was analyzed by RT-PCR and the other by histology and immunohistochemistry. Twenty-one prostate cancers obtained by prostatectomy were analyzed by RT-PCR and immunohistochemistry for detecting PSA mRNA and PSA expression.

Normal blood was obtained from a female volunteer and PBMC were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density centrifugation.

Extraction of mRNA. Direct isolation of polyadenylated RNA was carried out from the cell lines and the surgical samples by QuickPrep Micro mRNA Purification Kit (Pharmacia) following the manufacturer's instructions. The mRNA in the final elute was precipitated with ethanol, dried, and resuspended in 20 µl of 10 mM Tris-HCL pH 7.4, including 1 mM EDTA. The concentration of mRNA was determined by spectrophotometry and 0.2 µg of mRNA was used for RT-PCR to detect PSA mRNA and β-actin mRNA.

RT-PCR. Synthesis of cDNA from the isolated mRNA was carried out with a First-Strand cDNA Synthesis Kit (Pharmacia) including Moloney murine leukemia virus reverse transcriptase and pd(N)_6 primer in a final volume of 15 µl. Two oligonucleotides used as primers for detecting PSA expression were devised from the published sequence of PSA gene (10, 11): PSA-A, 5'-GGGAAAGTCCACGCTTACA-3' (nucleotides 858–875, PSA cDNA sequence); and PSA-B, 5'-CCCTCTCTCTCTTCTAC-3' (nucleotides 1592–1610). The PCR with PSA-specific primers produced a DNA fragment of 754 base pairs. The integrity of mRNA isolated from the experimental samples was checked by RT-PCR with primers for human β-actin. The primers for β-actin (12) were 5'-ACAATGAGCTGCGTGTGGCT-3' (β-A,) and 5'-TCTTACGTGCTGGGTGCT3' (β-B,). The PCR mixture consisted of 10 µl of 10× PCR buffer (500 mM KC1-100 mM Tris-HCL, pH 8.3–15 µM MgCl2-0.01% gelatin), 1 µl of deoxynucleotide triphosphate mixture (20 mM concentrations each of dATP, dCTP, dGTP, and dTTP), 5 µl of each oligonucleotide primer (4 µM), 0.5 µl of AmpliTaq DNA polymerase (5 units/µl) (Perkin Elmer Cetus, Norwalk, CT), one-half of the cDNA synthesis solution (7.5 µl) including 0.1 µg of the mRNA, and 71 µl of distilled water. Finally 100 µl of paraffin oil was added to prevent evaporation. Thirty of the following incubation cycles were performed, as follows: 60 s denaturation step at 95°C, 30 s annealing step at 55°C and 30 s extension at 72°C. PCR products were elec-
trophosed on 3% NuSieve 3:1 agarose gel (FMCS BioProducts, Rockland, ME) with HaeIII-digested 
X174 DNA molecular weight standards and visualized by ethidium bromide staining. Negative control reactions for the RT-
PCR were performed using all of the reagents as for the experimental samples, but without added mRNA, in each of the assays. To minimize contamination, 
the sample preparations and the RT-PCR were performed by precautionary procedures as suggested by Kwok (14).

Southern Blot Hybridization. PCR products electrophoresed on agarose gel were transferred onto Hybond-N+ (Amersham International plc, Buckingham-
shire, United Kingdom) by Southern blot method. An oligonucleotide, 
5'-GGAAACCTTGGAAATGACCAG-3' (nucleotides 1126–1145) internal to
the PCR primers (PSA-A, and PSA-B,) was used as a probe for Southern blot hybridization. The probe was labeled with fluorescein by an enhanced chemi-
luminescence 3'-oligolabeling system (Amersham) and allowed to hybridize the membrane. The hybridized probe on the membrane was detected by the enhanced chemiluminescence detection system (Amersham).

Determination of Detection Sensitivity. LNCaP cells were treated with trypsin and passed through with a 26-gauge needle to be single cells. The number of cells was determined by hemocytometer and cell suspension was prepared. Sensitivity of RT-PCR in detecting prostate cancer cells was determined by the following methods: (a) mRNA was isolated from 10^6 LNCaP cells, serially diluted, and used for RT-PCR; (b) serial dilutions of the cell suspension of LNCaP cells were mixed with 10^6 PBMC and then mRNA was isolated from the mixtures and used for RT-PCR to determine the sensitivity in detecting prostate cancer cells mixed in PBMC.

Histological Examinations. The lymph nodes and 21 prostate cancers were fixed in formalin, embedded in paraffin, and sectioned for histological studies. The sections were stained with hematoxylin and eosin and observed under a light microscope. Immunohistochemical staining for PSA was performed on formalin-fixed, paraffin-embedded sections with rabbit anti-PSA antibody (Biomeda Corp., Foster City, CA) (15). Localization of PSA was formed on formalin-fixed, paraffin-embedded sections with rabbit anti-PSA antibody (Biomeda Corp., Foster City, CA) (15). Localization of PSA was visualized with diaminobenzidine and nuclei were stained with methyl green.

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<th>Patient</th>
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\( ^a \) A_2, incidental multifocal or diffuse adenocarcinoma or incidental carcinoma of moderately differentiated or poorly differentiated adenocarcinoma; B_2, palpable carcinomas involving only one lobe of the prostate with diameter of less than 1.5 cm; B_3, palpable carcinoma involving both lobes of the prostate or being over 1.5 cm in diameter; C, palpable carcinoma extending through the prostatic capsule without metastases; D_2, prostate carcinoma with metastases to lymph nodes beyond regional lymph nodes and/or to distant organs. Clinical staging was performed according to the criteria proposed by the Japan Urological Association (1992).

Table 1 Data on patients with positive RT-PCR assays

Fig. 1. a, sensitivity of RT-PCR in detecting PSA mRNA isolated from LNCaP cells; 1.84 × 10^{-2} \mu g (Lane 1), 1.84 × 10^{-3} \mu g (Lane 2), 1.84 × 10^{-4} \mu g (Lane 3), 1.84 × 10^{-5} \mu g (Lane 4), 1.84 × 10^{-6} \mu g (Lane 5), 1.84 × 10^{-7} \mu g (Lane 6), or 1.84 × 10^{-8} \mu g (Lane 7) of the mRNA prepared from LNCaP cells were used for RT-PCR. Marker lane (M) was loaded with HaeIII-digested X174 DNA. b, sensitivity of RT-PCR in detecting LNCaP cells mixed in 10^6 PBMC; 10^6 (Lane 1), 10^5 (Lane 2), 10^4 (Lane 3), 10^3 (Lane 4), 10^2 (Lane 5), 10^1 (Lane 6), or 10^0 (Lane 7) of LNCaP cells were mixed with 10^6 PBMC and mRNA was prepared from each of the mixtures to be used for RT-PCR. Positive control reactions (\( \beta \)-actin) for each mRNA preparation was performed using primers specific for \( \beta \)-actin and amplified a 372-base pair (bp) DNA fragment. Marker lane (M) was loaded with HaeIII-digested X174 DNA.

Fig. 2. Detection of mRNA from the prostate cancer (Lanes 1 and 2), the right internal iliac node (Lanes 3 and 4), the right external iliac node (Lanes 5 and 6) and the left obturator node (Lanes 7 and 8) in patient 2. RT-PCR was performed on each of these samples, using primers specific for PSA mRNA (Lanes 1, 3, 5, and 7) or for \( \beta \)-actin mRNA (Lanes 2, 4, 6, and 8). Negative control reactions for RT-PCR using primers for PSA (Np) and for \( \beta \)-actin (Na) were performed without added mRNA. Marker lane (M) was loaded with HaeIII-digested X174 DNA. bp, base pair.

Results

Specificity of RT-PCR for Detecting PSA mRNA Expression. RT-PCR with primers specific for PSA gene amplified a 754-base pair DNA fragment from mRNA isolated from LNCaP prostate cancer cells and Southern blot hybridization analysis confirmed the identity of the fragment as cDNA of PSA. A faint band of a 754-base pair DNA fragment was also amplified from PC-3 prostate cancer cells. No amplified DNA fragments were observed in non-prostatic cancer cell lines (ACHN and HT-1376). In clinical specimens, a 754-base pair DNA fragment of PSA was amplified from 55 prostate tissues analyzed in this study, but the DNA fragment was not amplified from any bladder cancers, renal cell cancers, or lymph nodes obtained from non-prostate cancer patients.
DETECTION OF LYMPH NODE MICROMETASTASES BY RT-PCR

Sensitivity of RT-PCR in Detecting PSA mRNA Expression. mRNA (1.84 μg) was isolated from 10⁶ LNCaP prostate cancer cells. The 754-base-pair DNA fragment was amplified from 1.84 × 10⁻⁷ μg of mRNA or more by RT-PCR (Fig. 1a). The amount of mRNA (1.84 × 10⁻⁷ μg) corresponded to 0.1 cell of LNCaP. From the mixtures of LNCaP cells and PBMC, PSA mRNA expression was detected by RT-PCR at concentrations as low as a single cell of LNCaP mixed in 10⁶ PBMC (Fig. 1b).

Fig. 3. Histology and immunohistochemistry in the right internal iliac node (a and b), the right external iliac node (c and d), and the left obturator node (e and f) in patient 2 × 100. Metastatic prostate cancer cells were observed in the right internal iliac node (a) and PSA was localized in the cancer cells (b). A few atypical cells were seen in the right external iliac lymph node (c) and confirmed to be metastatic prostate cancer cells by immunohistochemistry (d). No metastatic prostate cancer cells were identified by histology (e) or immunohistochemistry (f) in the left obturator lymph node.
Detection of PSA mRNA Expression in Lymph Nodes by the RT-PCR. Forty-four lymph nodes from one patient with stage D2 prostate cancer and 21 patients undergoing total prostatectomy with pelvic lymphadenectomy were analyzed by RT-PCR to detect PSA mRNA expression. PSA mRNA expression was detected by RT-PCR in 9 lymph nodes from 6 patients. Table 1 presents data from the 6 patients. The neck lymph node obtained from patient 1 with disseminated bone metastasis and lymph node metastasis had PSA mRNA expression detected by RT-PCR and had histologically detectable prostate cancer cells, the PSA expression of which was confirmed by immunohistochemistry. The right internal iliac, the right external iliac lymph nodes, and the left obturator lymph node in patient 2 had PSA mRNA expression detected by RT-PCR (Fig. 2). The right internal iliac lymph node had histologically detectable metastatic prostate cancer cells containing PSA (Fig. 3, a and b). Although the right external lymph node contained a small number of atypical cells, diagnosing lymph node metastasis of prostate cancer was difficult for conventional microscopic study. These cells were confirmed to be micrometastatic prostate cancer cells by immunohistochemistry (Fig. 3, c and d). In the left obturator lymph node, no prostate cancer cells were detected by histology or immunohistochemistry (Fig. 3, e and f). Patient 3 had two lymph nodes with positive PSA mRNA expression detected by RT-PCR. One of them had histologically detectable metastatic prostate cancer cells, but the other had no histologically or immunohistochemically proved prostate cancer cells. Patient 4 had the right internal iliac lymph node with positive PSA mRNA expression detected by RT-PCR and the left internal iliac lymph node with negative PSA mRNA expression. In this patient, RT-PCR analysis yielded concordant results of histological and immunohistochemical analyses. Each of patients 5 and 6 had one lymph node with positive PSA mRNA expression detected by RT-PCR, in which no prostate cancer cells were detected by histology or immunohistochemistry. The remaining 32 lymph nodes obtained from 16 patients undergoing total prostatectomy with pelvic lymphadenectomy had neither PSA mRNA expression detected by RT-PCR nor prostate cancer cells identified by histology or immunohistochemistry, although the prostate cancers in these patients had PSA mRNA and PSA expression confirmed by RT-PCR and immunohistochemistry.

Discussion

We have demonstrated that RT-PCR with primers specific for PSA gene could amplify a 754-base pair DNA fragment only from mRNA isolated from prostate cancer cell lines and clinical samples of the prostate. We have demonstrated that RT-PCR had the ability to detect PSA mRNA expression from the amount of mRNA corresponding to 0.1 LNCaP prostate cancer cell and to detect a single LNCaP cell mixed in 10^8 PBMC. Detection sensitivity is reflective of mRNA copy numbers present in the tumor cells, so that sensitivity would be different in assays using clinical samples. RT-PCR, however, could have higher sensitivity than conventional microscopic or immunocytochemical study in detecting prostate cancer cells (6).

The clinical applicability of RT-PCR was documented by evaluating 44 lymph nodes obtained from 22 patients with prostate cancer undergoing biopsy or total prostatectomy with lymphadenectomy. In four lymph nodes, where metastatic prostate cancer cells were observed under a microscope, PSA mRNA expression was detected by RT-PCR. In one lymph node, where a small number of prostate cancer cells containing PSA were identified by immunohistochemistry, PSA mRNA expression was detected by RT-PCR. In four lymph nodes, where no metastatic prostate cancer cells were detected by histological or immunohistochemical analysis, PSA mRNA expression was detected only by RT-PCR. These results demonstrated that analyzing PSA mRNA by means of RT-PCR could be applied for diagnosing lymph node metastasis of prostate cancer and was a more sensitive technique than immunohistochemistry in detecting micrometastatic prostate cancer cells. Although it has not yet been concluded that a small number of PSA-containing cells detected only by RT-PCR within lymph nodes could always constitute clinically important metastatic prostate cancer, the presence of prostate cancer cells within lymph nodes could be the first and essential step for lymphatic dissemination and it could be speculated that cancer cells present in distant organ sites had the capacity to grow and proliferate to a level of clinical significance. It has been reported that immunocytochemical detection of bone marrow micrometastasis in patients with operable breast, gastrointestinal, and non-small cell lung cancer is of clinical value as a prognostic indicator of early relapse (16–19). Thus, in patients with prostate cancer, detecting PSA mRNA expression by means of RT-PCR to discover micrometastatic prostate cancer cells could potentially develop into a screen for determining the possibility of curative surgery or for identifying patients requiring more intensive therapy postoperatively.

In addition, RT-PCR analysis demonstrated that in patients with histologically detectable lymph node metastasis, a small number of prostate cancer cells had already spread into some lymph nodes with negative histology and immunohistochemistry. These findings may be helpful for understanding possible patterns of lymphatic dissemination of prostate cancer as well as for treating prostate cancer.

Certainly we are fully aware that, because only a small number of lymph nodes were analyzed in this study, further studies with a large number of lymph nodes should and will be performed to ascertain these encouraging results. Nevertheless, this study provides sufficient promising, albeit preliminary, data suggesting that a highly sensitive RT-PCR will be a relevant tool clinically to offer a more accurate assessment of lymph node metastases of prostate cancer and biologically to understand lymphatic dissemination of prostate cancer.

References


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