Human Prostatic Carcinoma Oncogene PTI-1 Is Expressed in Human Tumor Cell Lines and Prostate Carcinoma Patient Blood Samples

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Abstract

Rapid expression cloning and differential RNA display identifies a gene, named prostate tumor inducing gene-1 (PTI-1), that is differentially expressed in prostate cancer versus normal prostate and benign prostatic hypertrophy. PTI-1 encodes a truncated and mutated human elongation factor 1α, and its 5' untranslated region (UTR) shares significant homology with the 23S rRNA gene of Mycoplasma hyopneumoniae. PCR with human genomic DNAs, using PTI-1 5' UTR-specific primers, suggests that this sequence is part of the human genome. Furthermore, reverse transcription (RT)-PCR, with one primer specific to the 5' UTR region and the other to the elongation factor 1α coding region, amplifies PTI-1 transcripts from total RNA of various human tumor cell lines and blood samples from prostate carcinoma patients. RT-PCR products with the predicted size and sequence of PTI-1 are detected in RNAs from cell lines of human prostate, breast, and colon carcinomas. This RT-PCR product is shown by Southern blotting and sequence analyses to contain the junction sequence between the 5' UTR and the coding region of the PTI-1 gene. Furthermore, RT-PCR analysis indicates that the PTI-1 gene is also expressed in prostate carcinoma patient-derived blood samples. On the basis of serial dilution experiments, PTI-1 can detect 1 prostate carcinoma cell in 10⁶ cells not expressing PTI-1. In this context, PTI-1 represents a sensitive marker for detecting human prostate cancer in the bloodstream. This study confirms the authenticity of the PTI-1 gene and documents its potential clinical utility as a sensitive and specific indicator of prostate cancer progression.

Introduction

Adenocarcinoma of the prostate is presently the most prevalent internal cancer of men in the United States and the second most frequent cause of cancer-related deaths. Current methodologies for the early detection of prostate cancer, including physical examination, monitoring PSA levels, tissue biopsy, and ultrasound and bone scans, are restricted in both sensitivity and specificity (1–3). In addition, present testing modalities do not permit a distinction between cancers that will remain indolent and those that will prove aggressive and life threatening (1–4). Using DNA transfection approaches with a novel acceptor cell line, CREF-Trans 6 (5), and the molecular approach of rapid expression cloning and differential RNA display (6), a novel putative prostatic carcinoma acceptor cell line, CREF-Trans 6 (5), and the molecular approach of P. B. F. is a Chernow Research Scientist in the Departments of Pathology and Urology, Columbia University, College of Physicians and Surgeons, Columbia University, New York, New York 10032.

Materials and Methods

Cell Lines. This study incorporated the following human cell lines: prostate carcinoma (LNCaP and DU-145), breast carcinoma (T47D), and colon carcinoma (SW480). Additional cell types studied include CREF-Trans 6 cells and nude mouse tumor-derived CREF-Trans 6 cells transplanted with LNCaP DNA (CREF-Trans 6:4 NMT; Ref. 5). Cells were grown in DMEM supplemented with 5% (rodent cells) or 10% (human cells) fetal bovine serum at 37°C in a 95% air/5% CO₂-humidified incubator. All cell lines used in the present study were tested for Mycoplasma contamination using the GenProbe Mycoplasma test kit (Gaithersburg, MD) and found to be Mycoplasma free.

Genomic DNA Extraction and PCR. Human brain and kidneys were frozen in liquid nitrogen, ground into powder, and digested with 100 μg/ml proteinase K at 50°C overnight, followed by phenol/chloroform extraction and ethanol precipitation (7, 8). Oligonucleotides were synthesized for PCR amplification corresponding to nt 147 to 167 UU and nt 550 to 570 UU of PTI-1 (GenBank accession no. L41490). Primer pair UU and UL will generate a 424-bp product. PCR was performed in a 50-μl volume, with 1 μl of brain or kidney genomic DNA, 0.5 μM each of primer (UU and UL), 400 μM deoxyribonucleotide triphosphates, 2 mM MgCl₂, and 1 unit of Taq DNA polymerase (Life Technologies, Inc.). Forty cycles of amplification were performed with each cycle consisting of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C on a programmable thermal cycler (MJ Research). “Hot start” PCR technique was applied. PCR products were analyzed on a 2% agarose gel by ethidium bromide staining.

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1 The abbreviations used are: PSA, prostate-specific antigen; RT-PCR, reverse transcription PCR; PTI-1, prostate tumor inducing gene-1; BPH, benign prostatic hypertrophy; EF-1α, elongation factor 1α; UTR, untranslated region; nt, nucleotides; PSM, prostate-specific membrane antigen; U5, 5' UTR upper; UL, 5' UTR lower; BU, bridge upper; BL, bridge lower; BSB, bridge-specific probe.
RNA Isolation from Cultured Cells and RT-PCR. Total cytoplasmic RNA was isolated from logarithmically growing cell cultures as described previously (9, 10). One μg of total RNA extracted from various tumor cell lines was reverse transcribed into cDNA with 150 ng of random primers and 200 units of Superscript II RNAse H^{-} Reverse Transcriptase (Life Technologies, Inc.) in the presence of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl_2, 10 mM DTT, and 500 μM deoxynucleotide triphosphates. The reaction mixture (20 μl) was incubated at 42°C for 90 min and terminated by heating at 70°C for 15 min. Oligonucleotides were synthesized for RT-PCR amplification corresponding to nt 537 to 557 BU and nt 768 to 788 BL of the PTI-1 gene (GenBank accession no. L41490). Primer pair BU and BL will generate a 252-bp product. PCR with 2 μl of the reverse transcription reaction mixture was similar to the genomic DNA protocol with some modifications. Fifteen cycles of amplification were performed in the first round of PCR with primer BU (0.5 μM) alone, and then BL (0.5 μM) primer was added for another 40 cycles of amplification. PCR direct sequencing (New England Biolabs) was performed with [γ-32P]ATP-labeled primers following the manufacturer's recommendations.

Southern Blotting Analysis of RT-PCR Products of PTI-1 Gene Transcripts. Oligonucleotides were synthesized for Southern blotting analysis with the BSP 5'-AAAATTTAAGCTTATCCAGTCCGC-3'. Ten pmol of the BSP oligonucleotide was incubated with 5 μl [γ-32P]ATP (10 mCi/ml) and 20 units of T4 polynucleotide kinase (Life Technologies, Inc.) at 37°C for 60 min in the presence of 70 mM Tris-HCl (pH 7.6), 10 mM MgCl_2, 100 mM KCl, and 1 mM β-mercaptoethanol. The reaction was terminated by heating at 70°C for 10 min, and labeled oligonucleotide probe was purified by ethanol precipitation. RT-PCR products amplified by the BU and BL primer pair were transferred onto nylon membrane (Hybond film; Amersham Corp.) according to standard capillary blotting protocols. After fixation at 80°C for 2 h, the membrane was incubated at 56°C overnight in the presence of 1% SDS, 1 mM NaCl, and 100 μg/ml sonicated salmon sperm DNA. Hybridization was performed the next day by incubating with 1% SDS, 1 mM NaCl and labeled probe at 56°C overnight. The membrane was washed twice with 100 ml 2X SSC (1X SSC = 0.15 M sodium chloride, 0.015 M trisodium citrate, pH 7.0) at room temperature for 5 min each, once with 200 ml 2X SSC, 1% SDS at 56°C for 30 min, and once with 200 ml 0.1X SSC at room temperature. The blot was exposed with Kodak X-OMAT film for 30 min at room temperature.

Determination of PCR Sensitivity. RNA was isolated from LNCaP cells and from mixtures of LNCaP and CREF-Trans 6 cells ranging from 1:1,000 to 1:1,000,000 as described previously (9, 10). PCR was performed using PTI-1 5' UTR-specific primers (5'-GAGTCTGAAATGGGCAGCTT-3' and 5'-AGTCTGATCGATTGCC-3'; Ref. 7) and PSA-specific primers (5'-TACCCACGTGATCGAACA-3' and 5'-CTGGGAAGCCACCATCATA-3'; Refs. 11 and 12). The CREF-Trans 6 cell line was chosen to dilute LNCaP cells because this cell line does not express PTI-1 or PSA (7, 10—12). PTI-1 expression was evaluated using the same 5' UTR primer pair used for determining PCR sensitivity (7). Positive and negative PTI-1 expression was also confirmed in samples using PCR with the UU and UL and the BU and BL primer pairs.

Results

The 5' UTR Sequence of the PTI-1 Gene Is Present in the Human Genome. If PTI-1 is truly an etiological agent in human prostate cancer, then this gene or related DNA sequences must be a component of human genetic material. Demonstration that the 5' UTR of PTI-1, which displays a strikingly high degree of homology with prokaryotic rRNA sequences, is indeed present in the human genome was deemed a priority for future mechanistic studies of the PTI-1 gene. Moreover, this information is required before and irrespective of the mechanism by which activation of this sequence occurs during cancer development.

As shown in Fig. 2, primer pair UU and UL (representing sequences in the 5' UTR of PTI-1) generated a specific PCR product with the expected size (424 bp) from human brain genomic DNA. The size of the PCR product from genomic DNA (Fig. 2, lanes 1 and 2) is the same size as that of the PTI-1 cDNA, suggesting that it is an intron-free region. It is well-documented that both prokaryotic and eukaryotic RNA genes are without introns, and our findings are consistent with this conclusion (13). RNase digestion of the template did not affect the detection of this product. The amplification of this product also required the simultaneous presence of all the following components in the PCR reaction mixture: Taq polymerase, both primers, and the template DNAs (Fig. 2). Similar experimental results occur when human kidney genomic DNA is substituted for brain genomic DNA (data not shown). Although the present studies cannot rule out definitively the possibility of a minute quantity of bacterial DNA in our genomic DNA preparations, thereby generating false-positive results, it is recognized that the probability of Mycoplasma contamination is less likely in tissue samples than in cell cultures (14). The possibility that Myco-
from Tumor Cell Lines. It is hypothesized that even if there is Mycoplasma or related bacterial contamination in the cell cultures studied, this contamination alone could not explain the presence of both prokaryotic RNA sequences and human EF-1α sequences contiguous on the same RNA molecule. Thus, if such a junction point can be demonstrated to exist in total RNA, it is unlikely that the identification of the PTI-1 gene was due to an experimental artifact.

As shown in Fig. 3A, primer pair BU (consisting of sequences within the 5' UTR of PTI-1) and BL (consisting of sequences within the EF-1α region of PTI-1) generates a RT-PCR product with the expected size (252 bp) in total RNAs from CREF-Trans 6:4 NMT (nude mouse tumor-derived CREF-Trans 6 clone transfected with LNCaP HMW DNA), T47D (human breast carcinoma), SW480 (human colon carcinoma), and LNCaP and DU-145 (human prostate carcinoma) cells. This PCR product was not detected in RNAs extracted from CREF-Trans 6 cells. This expression pattern is identical to that reported previously using Northern blotting analysis and probing with a PTI-1 5' UTR-specific probe (7). As shown in Fig. 3B, this PCR product also hybridizes with a PTI-1 cDNA-specific oligonucleotide BSP. BSP consists of 20 nucleotides, with 10 nucleotides on either side of the junction point between the 5' UTR and the coding region of the PTI-1 cDNA (Fig. 1). The highly stringent hybridization and washing conditions (see “Materials and Methods”) used in Southern blotting analysis demonstrates that this PCR product contains the PTI-1 cDNA sequences, specifically the sequence surrounding the junction point between the 5' UTR and coding region of the PTI-1 gene. This conclusion was further supported by direct sequencing of one of the PCR products obtained from the DU-145, LNCaP, and CREF Trans 6:4 NMT cell lines (data not shown), which documents that they consist of both prokaryotic ribosomal-like RNA sequences and human EF-1α sequences. These sequences were the same as those published previously for the PTI-1 gene (7).

It was also important to rule out the possibility that the PTI-1
plasmid might be a source of potential contamination-generating artifactual results. The PTI-1 gene was initially identified as a 1.8-kb insert from an LNCaP cDNA library. Subsequently, the remaining 215 bp at the 5' end was obtained by the rapid amplification of cDNA ends procedure (7). Because the missing region of PTI-1 was located in the 5' UTR, a full-length PTI-1 cDNA was never generated. Therefore, any PCR or RT-PCR products produced with primers constructed from the first 215-bp and remaining 415-bp regions of the 5' UTR of PTI-1 could not be derived from a plasmid template but could only result from RNA or genomic DNA. As shown in Fig. 3C, primer pair UU (located inside the first 215-bp region that is missing in the PTI-1 cDNA clone) and UL (designed within the 5' UTR of PTI-1 that is present in the PTI-1 cDNA clone) permitted the amplification of the 5' UTR sequences of PTI-1 from the same total RNAs of tumor cell lines that were positive for the bridge region. Moreover, the pattern of expression of this sequence is identical to that of the junction sequences of the PTI-1 gene (Fig. 3, A and B). These results document that the PTI-1 gene is an authentic putative human oncogene that is expressed in specific human tumor cell lines derived from appropriately transfected CREF-Trans 6 (CREF-Trans 6:4 NMT) and prostate and additional human carcinomas.

Expression of the PTI-1 Gene in Prostate Carcinoma Patient Blood Samples. To determine the sensitivity of PTI-1 as a gene-based marker for detecting prostate carcinoma cells, PTI-1-expressing LNCaP cells were serially diluted with non-PTI-1-expressing CREF-Trans 6 cells; total RNA was isolated, and samples were compared by RT-PCR for PTI-1 expression (Fig. 4A). Using primers designed in the unique 5' UTR region of PTI-1, a positive PTI-1-specific amplified fragment (280 bp) was detected when 1 LNCaP cell was diluted in 10^6 CREF-Trans 6 cells. In contrast, when primer sequences corresponding to PSA were used in the amplification, a weaker signal (corresponding to a 486-bp fragment) was obtained that represented 1 LNCaP cell diluted in 10^6 CREF-Trans 6 cells. The efficiency of detection of PSM (12) in serially diluted cells using a single pair of PSM-specific primers (generating a 647-bp fragment) was even less sensitive than PSA detecting 1 prostate carcinoma cell diluted in 10^5 CREF-Trans 6 cells (data not shown). These results demonstrate that RT-PCR of PTI-1 is currently a sensitive detector of human prostate carcinoma cells, significantly exceeding the sensitivity of PSA and PSM.

The sensitivity of PTI-1 in detecting prostate cancer cells in diluted samples (Fig. 4A) suggested that monitoring PTI-1 transcripts might also prove useful as a direct screening test for the detection of prostate carcinoma cells in the circulatory system of prostate cancer patients. To determine if this assumption was correct, RT-PCR was performed using primers specific for the 5' UTR of PTI-1 with RNAs isolated from blood samples confirmed as positive or negative for PSA expression (Fig. 4B and data not shown). PTI-1 was able to detect carcinoma cells in two of two samples from patients with confirmed metastatic disease (Fig. 4B and data not shown). In contrast, blood samples from six confirmed negative volunteer females and males and four patients with prostate cancer that had not spread past the margin of the prostate gland (also found negative for PSA) were negative for PTI-1 expression (Fig. 4B and data not shown).

A second test of PTI-1 involved a study using nine random RNAs isolated from coded blood specimens (Fig. 4C). These samples were analyzed for PSA and PTI-1 expression by RT-PCR using PSA-specific primers (11) and the primer pair UU and UL (within the 5' UTR of PTI-1), respectively. Of these nine samples, two were found to be positive for PTI-1 transcripts (Fig. 4C). These two samples were also positive for PTI-1 when using the BU and BL primer pair (data not shown). One patient (Fig. 4C, Lane B), determined to have metastatic prostate cancer, was positive for PTI-1 expression but negative for PSA expression. However, the other PTI-1-positive patient (Fig. 4C, Lane F) was assumed by pathology to have localized cancer in the prostate gland, and this patient's blood sample was also negative for RT-PCR of PSA. In the remaining seven patients, seven were confirmed as having nonmetastatic prostate cancer, and they were all negative for PTI-1 expression, whereas five of seven were negative and two were positive for PSA expression (Fig. 4C). The only patient who was suspicious for metastatic prostate cancer based on very high serum PSA protein levels was negative by RT-PCR for expression of both PTI-1 and PSA (Fig. 4C, Lane I).

Although further studies are needed with a larger number of patient samples, including patients with and without confirmed metastatic prostate cancer, the present studies provide compelling evidence suggesting that RT-PCR of PTI-1 may prove useful as a sensitive methodology for monitoring extraprostatic disease in patients prior to surgery, staging prostate cancer, and evaluating patients' response to chemotherapy and radiation therapy.

Discussion

The ability to accurately monitor the aggressiveness of human prostate cancer is a priority for defining the appropriate means of therapeutically intervening in the progression of this disease. Recent studies document that the identification of blood-borne PSA-expressing cells by RT-PCR can be achieved in patients with localized as well as metastatic prostate cancer and that this detection provides a dependable marker for predicting local invasion of a prostate tumor.
proceeding surgical procedures (11, 12, 15). PSA, a Mr 34,000 glyco-protein, is a prostate-associated serine protease with predominant expression by prostate epithelial cells, the cells most often associated with prostatic oncogenesis (16, 17). Recently, assays used to monitor this protein in the blood have changed the management of prostate cancer patients by permitting the early detection of prostate tumors as well as by providing a more efficient means to follow the progression of this disease. The specificity of PSA for prostate cells has permitted the development of an RT-PCR-based assay that can detect as few as 1 PSA-synthesizing cell in 10^6 non-PSA-expressing blood cells (11, 12, 15). A further enhancement of the PSA assay involves the addition of digoxigenin-modified nucleotide to the PCR reaction (11, 15). A Southern blot made from such electrophoresis reaction products can then be analyzed by sensitive immunostaining techniques that greatly enhance the detection of the specific PSA-derived cDNA product. When this enhanced assay was applied previously to peripheral blood specimens taken from prostate cancer patients with confirmed metastases, it was positive for the majority (77.7%) of patients in this category that were studied (9, 13). PTI-1 is not expressed in normal prostate or BPH, but PTI-1 expression is apparent in both PSA-positive, hormone-sensitive LNCaP and PSA-negative, hormone-refractive DU-145 human prostate carcinoma cells (7). The current study demonstrates a ≥100-fold increase in the sensitivity of RT-PCR of PTI-1 versus PSA and PSM in detecting prostate cancer cells. Moreover, PTI-1 RT-PCR of blood RNA can detect cancer cells from patients with metastatic prostate cancer. In these contexts, an RT-PCR-based assay using PTI-1 may permit the detection of prostate cancer cells in the circulation that would not be detected using RT-PCR with PSA or PSM. In addition, the specificity of PTI-1 for prostate carcinoma versus normal prostate or BPH eliminates the possibility of generating false-positives that could occur using RT-PCR of PSA or PSM.

Important questions that we have experimentally addressed include the nature of the 5' UTR region of PTI-1 that displays homology to bacterial ribosomal 23S RNA and the genuineness of the PTI-1 gene (7). The PTI-1 gene was cloned from an LNCaP human prostate cancer cDNA library using a 214-bp fragment detected by differential RNA display in LNCaP DNA-transfected, tumor-derived CREF-Trans 6 cells (7). Sequence analyses confirm that the 214-bp sequence with homology to prokaryotic rRNA is located within the 5' UTR of the PTI-1 gene (7). Although all of the cell lines used in the original study were tested using the GenProbe Mycoplasma test kit and found to be free of Mycoplasma contamination, it is still possible that the PTI-1 gene arose from a cloning artifact generated by a low level of undetected bacterial contamination present in the CREF-Trans 6:4 NMT or LNCaP cell lines. Several lines of evidence are presently provided that argue against this possibility and validate the authenticity of the PTI-1 gene. Using PCR-based approaches with genomic DNAs isolated from human brain and kidney, we documented the presence of the prokaryotic rRNA homologous sequences in the human genome. Using an RT-PCR-based strategy with primers corresponding to the 5' UTR and EF-1α coding region of PTI-1, an appropriate junction sequence is amplified from total RNA from CREF Trans 6:4 NMT cells, various human tumor cell lines, and blood samples from patients with confirmed metastatic prostate cancer. Even if our tissue samples and cell lines contained Mycoplasma or related bacterial contamination, these adventitious organisms alone would not be capable of generating the unique junction sequence in a population of total cytoplasmic RNAs. Our ability to detect such a junction sequence in total RNAs from specific cancer cells provides compelling evidence for the authenticity of the PTI-1 gene and its encoded message.

The present study raises a number of important issues that require resolution to define the role of PTI-1 in human cancer etiology and evolution. Documentation of the presence of the 5' UTR Mycoplasma homology region in the human genome forces one to examine the potential origin of such prokaryotic gene sequences in the eukaryotic genome. Although no mechanism is provided, a recent study suggests a potential relationship between persistent chronic infection with Mycoplasmas and malignant transformation (18). Unlike retroviruses and DNA tumor viruses that can incorporate their genetic material into the host genome, no evidence is currently available indicating that Mycoplasma gene sequences can integrate into the human genome as part of their infectious cycle. However, it is possible that Mycoplasma, or more likely one of its ancestors, may randomly insert its genetic material into human or one of its ancestor's genome. This integration may occur by a mechanism that is similar to that by which foreign gene sequences insert into the genome of transgenic animals. The presence of sequences that are highly homologous to prokaryotic genes in the human genome has been reported previously, although the functions of these sequences are not known (19). It is very tempting to speculate that, based on the high degree of homology between the 5' UTR of PTI-1 and Mycoplasma gene sequences, such an event may have occurred recently in evolution, thereby generating the PTI-1 gene.

Additional important issues are the mechanism by which PTI-1 expression is activated in human tumor cells and the role of PTI-1 in mediating the cancer phenotype. Differential expression of PTI-1 in cancer cells may occur by activation of transcription from an upstream promoter from the EF-1α or another target gene, resulting in transcription of the 5' UTR and EF-1α region of PTI-1. Alternatively, gene activation could result from genome rearrangement, including gene deletion, inversion, and translocation, which are common occurrences in many cancers (20, 21). Elucidation of the genomic structure, including the promoter region, of PTI-1 is necessary to shed light on this question and to define the molecular basis for the differential expression of PTI-1 in human prostate and additional cancers versus normal cells. Further studies are also necessary to determine the functional relevance of PTI-1 expression in determining the cancer phenotype. If PTI-1 expression is shown to be causally related to cancer development or progression, then this gene could serve as a potential target for inhibiting the neoplastic process.

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References

PROSTATIC CARCINOMA ONCOGENE PTI-1 EXPRESSION


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