Expression of Prostate-specific Membrane Antigen in Transitional Cell Carcinoma of the Bladder: Prognostic Value?1

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INTRODUCTION

The expression of Prostate-specific membrane antigen (PSMA) mRNA was assessed in the normal bladder urothelium (n = 9), transitional cell carcinoma (TCC) specimens (n = 52), TCC-derived cell lines (n = 3), and preoperative blood samples from TCC patients (n = 27). Specific PSMA mRNA was found in 100% of normal and malignant tissues and two cell lines. PSMA protein was detected in normal (n = 3) and malignant tissues (n = 4). Using a PSMA-specific substrate, PSMA enzymatic activity was found in two bladder cell lines and correlated with immunostaining. Seven of the 27 TCC preoperative blood samples were positive by reverse transcription-PCR. These preliminary results, obtained on a nonrandomized cohort of patients, correlated with tumor invasion (positive RT-PCR: 0% for pT ≤2 versus 41% for pT ≥3) and 2-year survival rate (81% in the PSMA-negative group versus 29% in the PSMA-positive group). Although the clinical usefulness of this assay requires confirmation in larger prospective randomized trials, current preliminary results suggest that a blood-borne PSMA mRNA PCR assay may be a useful tool to predict a poor outcome in TCC patients.

REFERENCES

1. Ninety % of bladder tumors are derived from the urothelium, the transitional epithelium that lines the urinary bladder and the ureter (1). The natural history of TCCs varies widely, although it is characterized by a high rate of recurrence with an aggressive clinical course (2). Many extensive studies have been performed to determine whether particular oncogenes, tumor suppressor genes, or chromosomal changes are involved in bladder tumors to identify tumors with a high risk of recurrence and progression. None of these markers, however, has been demonstrated to improve the diagnosis and treatment in individual patients (3). The depth of infiltration, the differentiation grade, and the presence of concomitant carcinoma in situ are still the most common parameters to predict tumor progression and survival (3). Although ~80% of TCCs are initially superficial, recurrence after localized therapy occurs in 30–90% of the cases. Despite significant improvement in localized therapy, intravesical chemotherapy, and immunotherapy, 15–20% of these recurrences evolve to invasive and/or metastatic stages that require radical and/or systemic therapies (2). There is clearly a subgroup of patient (i.e., <50%) who will survive after radical surgery and adjuvant therapy (4). The major problem is selecting those patients at risk of progression, who may benefit from earlier aggressive treatment (i.e., radiotherapy, chemotherapy, and immune therapy). Because no bladder urothelium-specific serum marker is currently available, the diagnosis of advanced TCC relies on conventional imaging technologies (i.e., computed tomography scan, ultrasound, and magnetic resonance imaging), the resolution sensitivities of which limit the detection to metastases >1 cm. Therefore, any biological marker capable of improving the detection of early metastatic disease would be of great clinical benefit.

PSMA is a Mr 100,000 type II membrane glycoprotein identified by Horoszewicz et al. (5) from crude membrane extract of the androgen-dependent prostate cancer cell line, LNCaP. The expression of PSMA is low in the normal prostatic epithelium, increases markedly in prostate cancer, and is maintained in poorly differentiated tumors and prostate cancer metastasis (6). In contrast to secreted prostate-specific antigen, PSMA is a membrane-bound antigen, the expression of which increases after androgen ablation protein (reviewed in Ref. 7). Several successful strategies have been developed to detect PSMA protein or mRNA to identify and target prostatic cells (8, 9).

9 The abbreviations used are: TCC, transitional cell carcinoma; PSMA, prostate-specific membrane antigen; RT-PCR, reverse transcription-PCR; NAALADase, N-acetylated-α-linked acidic dipeptidase; NAAG, N-acetyl-aspartyl-glutamate.

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Initially believed to be highly prostate specific, PSMA has been shown recently, however, to be expressed in nonprostatic tissues such as breast carcinoma, duodenum, and in normal and malignant renal tissues (10–12).

In the present study, the expression of PSMA mRNA, protein, and enzymatic activity is demonstrated in the normal bladder, in TCC specimens, and in primary cell lines established in vitro from TCC specimens. In addition, preliminary clinical data suggest that circulating PSMA-expressing cells can be detected in TCC and that detection of PSMA transcript seems to correlate with stage and survival.

MATERIALS AND METHODS

Human Bladder Specimens and Blood Samples. Bladder and blood samples were obtained after informed patient consent. Surgical specimens were collected from 9 normal bladders and 52 TCCs (25 = pT1, 27 = pT3). Specimens were snap-frozen in liquid nitrogen for RNA analysis and formalin-fixed/paraffin-embedded for pathological analysis and immunohistochemical detection of PSMA. Blood samples were obtained preoperatively from an additional series of 27 consecutive patients scheduled to undergo radical cystoprostatectomy for invasive or recurrent TCC. All patients were males, ages 49–82 (mean, 67) years. Preoperative stratification and follow-up of TCC disease were performed by chest, abdominal, and pelvic computed tomography scan, abdominal ultrasounds, and liver tests. None of the patients had detectable metastases preoperatively. Screening for concomitant prostate cancer was based on serum prostate-specific antigen tests. None of the patients had detectable metastases preoperatively. Screening for concomitant prostate cancer was based on digital rectal examination and serum prostate-specific antigen value. All patients diagnosed with ≥pT3 have received a standard adjuvant MVAC regimen (methotrexate, vinblastine, Adriamycin, cisplatin). Prostatic and bladder specimens were analyzed separately, and tumor invasion was stratified according to the Tumor-Node-Metastasis classification. Local and invasive node metastatic disease was investigated both by performing pathological analysis on tissues obtained at surgery as well as by using radiological and bone scan investigations. Venous blood (2 × 10 ml; Sarstedt, Nümbrecht, Germany) was collected in EDTA-treated tubes. All tubes were processed within 1 h of collection.

Cell Lines and Reagents. LB831, LB796, and LB905 bladder carcinoma cell lines are primary cultures established from invasive TCC tumors and were generously provided by the Ludwig Institute for Cancer Research (Brussels, Belgium). LB831 has been characterized recently (13). Cells were grown in Iscove’s modified Dulbecco’s medium containing 10% FCS, 116 µg/ml l-arginine, 36 µg/ml l-asparagine, 219 µg/ml l-glutamine, and antibiotics (200 units/ml penicillin and 100 µg/ml streptomycin) in a 95% air/5% CO2 incubator at 37°C. Androgen-dependent human prostate cancer cell lines LNCaP, androgen-independent human prostate cancer cell line PC-3, and myeloblastic leukemia cell line K562 were obtained from American Type Tissue Collection (Manassas, VA). An androgen-independent human prostate cancer cell line, TSU, was generously provided by Dr. John Isaacs (Johns Hopkins Oncology Center, Baltimore, MD). Cell lines were grown in RPMI 1640 supplemented with l-glutamine, nonessential amino acids, and 10% FCS (Life Technologies, Inc., Gent, Belgium) in a 90% air/5% CO2 incubator at 37°C.

PSMA RT-PCR Assay. Total RNA from bladder tissue was obtained by guanidinium/thiocyanate/phenol/chloroform extraction technique (14). Total RNA from cell lines and bloody coats obtained after centrifugation on Ficoll Hypaque of blood samples was extracted using Trizol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer’s instruction. Details regarding reverse transcription, amplification, and control measurements for preventing carryover have been described previously (15). To simplify the assay procedure, the previously described nested RT-PCR, with two rounds of 19 cycles each, was replaced by a single round of 32 cycles. Amplification was performed in a DNA thermal cycler 480 (Perkin-Elmer, Foster City, CA) by using the previously described internal PSMA primers (15). The limit of detection for PSMA with the single-round, 32-cycle PCR assay was assessed on serial dilution of LNCaP in blood, LNCaP in the PSMA-negative cell line K562, and finally with serial dilution of 1.2-kb PSMA cDNA plasmid pJR184, as detailed previously. The limit of detection was defined as the last dilution giving a positive PCR result with an inter-assay coefficient variation <20% (15). Blood specimens used as negative controls were obtained from 200 healthy blood donors (100 females and 100 males).

Northern Blotting. The pSPORT plasmid coding for the 2.6-kb PSMA cDNA probe is a generous gift from W. D. Heston (Memorial Sloan-Kettering Cancer Center, New York, NY). Northern blot and hybridization were performed on nylon membranes with UV-fixed poly(A+) RNA (2 µg/lane) extracted from normal adult tissues (Clontech, Palo Alto, CA) according to the manufacturer’s instruction. Gels were autoradiographed, and results were normalized for actin expression. Scanning densitometry was performed to semiquantitatively assess the level of expression.

DNA Sequencing and Analysis. The PSMA cDNA product from TCC lines (n = 3), TCC surgical specimens (n = 10), and normal bladder urothelia (n = 5) were sequenced in both orientations. The sequencing reactions were carried out on an automated ABI 373 A apparatus by using the Taq Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer’s instruction. The specificity of the remaining PSMA cDNA PCR products was assessed by Southern transfer, probe radiolabeling, hybridization, and autoradiography as described (15).

Immunohistochemical Detection of PSMA. Paraffin-embedded sections, 5-µm thick, from 3 normal and 4 malignant urothelial tissues, and 3 lymph node metastases from prostatic adenocarcinoma were dewaxed, rehydrated, and processed as described previously (16). Cells growing in culture were collected, cytospun onto glass slides, fixed in 4% paraformaldehyde, and permeabilized. Cells were incubated overnight at 4°C with an anti-PSMA monoclonal antibody, CYT-351 (dilution 1:300), generously provided by Cytogen Corp. (Princeton, NJ), and revealed with an anti-mouse EnVision-Peroxidase system (Dako, Gølstrup, Denmark). A prostate cancer lymph node metastasis and LNCaP cell line were used as positive controls. Myeloid K562 and prostate PC-3 cell lines were used as nega-
ative controls (8, 15). Staining intensity was semiquantitatively expressed as described (17).

**PSMA Enzymatic Assay.** In previous studies, PSMA has been demonstrated to express a folate hydrolase enzymatic activity (18) and an NAALADase activity (19). Non-PSMA γ-glutamyl hydrolases are ubiquitously found in the lysosomes of cells and are also secreted by many tumor cell types (20). To specifically determine PSMA enzymatic activity, the NAALADase activity of PSMA was assayed using [3H]NAAG, adapted as described previously by Tiffany et al. (21). The NAALADase activity of LNCaP human prostate cancer cells has been characterized previously, and membranes from these cells were used as positive controls. The prostate cancer cell lines TSU and PC-3, which do not express PSMA by immunohistochemical or enzymatic analysis, were used as negative controls (21).

Membranes were prepared and amount of [3H]NAAG hydrolysis determined for each cell type assessed. Briefly, LNCaP, TSU, PC-3, LB905, LB831, and LB796 cell lines were grown to 80–90% confluence in serum-containing media as described above. Ten to 50 million cells were scraped in cold HBSS and pelleted at 1000 × g at 4°C. The cell pellet was resuspended in cold 50 mM Tris (pH 7.4), sonicated three times for 5 min each, and then centrifuged at 100,000 × g for 10 min at 4°C. The pelleted membranes were resuspended in 50 mM Tris and assayed for protein content, and additional Tris buffer was added to bring the concentration of protein to 0.5 mg/ml. Samples were stored at −80°C before use.

Regarding the determination of NAALADase activity, 100 μg of protein (i.e., 200 μl) from each cell line were added to PSMA assay buffer (100 μl of 10 mM CoCl₂, 250 μl of 200 mM Tris, pH 7.4), and the volume was brought to 950 μl. To determine PSMA-specific activity, the highly potent PSMA inhibitor 2-(phosphonomethyl)-pentanedioic acid (Alexis, San Diego, CA; Ref. 22) was added to a final concentration of 1 μM to a second identical sample. After a 10-min incubation at room temperature, 50 μl of 1 μM [3H]NAAG (DuPont NEN, Boston, MA) were added, and reactions incubated for 28 h. For assays using LNCaP membranes, 5 μg of total protein were used per assay, and [3H]NAAG hydrolysis was assayed after only 3 h. At the end of the incubation period, 200 μl from each assay were loaded on a column constructed by placing a 3-mm solid glass bead into a 9-inch Pasteur pipette to which 5 cm of ion exchange resin preconditioned with distilled H₂O. The column was washed with 2 ml of 1 M formic acid into 10 ml of scintillation mixture, and total counts were determined using a Beckman LS 8100 scintillation counter. Specific PSMA activity was determined by subtracting activity in samples containing membranes plus inhibitor from samples containing membranes only. A standard curve was plotted using increasing concentrations of [3H]NAAG to convert measured counts to pmol [3H]glutamate released. Results were expressed in pmol [3H]glutamate released/(min × mg) of total protein and are the mean of three separate assays ± SE. Each experiment was done in duplicate. Activity was considered not detectable if <0.001 pmol/(min × mg).

**RESULTS AND DISCUSSION**

**Limit of Detection of the RT-PCR Assay.** The single-round assay consistently detected 10 LNCaP cells in 1 ml of venous blood or 1.9 LNCaP cells in 10⁶ peripheral blood mononuclear cells and 1 LNCaP cell in 10⁵ K562. One thousand copies of the cDNA coding PSMA plasmid, pJRD184, were consistently detected, whereas dilutions of 100 and 10 copies produced, respectively, a weaker consistent band or an inconsistent barely detectable band (data not shown). In comparison, the limit of detection of the TCC cell line LB905 diluted in the PSMA-negative K562 was 1:100. No positive signal was recorded in the 200 blood specimens from normal male/female blood donors. These results confirm our previous data demonstrating the absence of illegitimate transcript amplification at such a limit of detection (15).

**Expression of PSMA mRNA in a Panel of Normal Human Tissues.** Northern blotting from normal human tissues confirmed the expression of PSMA mRNA in several tissues. Besides a very strong prostate expression, PSMA transcripts were also clearly detected in RNAs extracted from the small intestine, the bladder (Fig. 1), and in extracts from several other tissues including kidney, brain, and liver (data not shown). Scanning densitometry of the detected products demonstrates that PSMA expression was ~500 times lower in the normal bladder than in the normal prostatic tissue.

**Expression of PSMA mRNA in Normal Urothelium, TCC Surgical Specimens, and TCC-derived Cell Lines.** Specific PSMA mRNA transcripts were detected in each of the 9 normal urothelia, the 52 TCC surgical specimens, and in 2 of the 3 TCC-derived cell lines (Fig. 2). The sequence analysis of the PCR products from 5 normal urothelia, 10 TCC specimens, and the 2 positive TCC-derived cell lines confirmed that these PSMA mRNA transcripts were 100% homologous in length and cDNA sequence to the PSMA mRNA transcripts extracted from the prostate (8). Southern blot on the remaining samples confirmed the specificity of PSMA PCR products (data not shown). These results demonstrate that PSMA mRNA is significantly expressed in the normal urothelium and TCC tissues or derived cell lines.
PSMA Protein Expression in the Normal Urothelium, TCC Surgical Specimens, and in TCC Cell Lines. A positive homogeneous staining intensity and pattern of expression were consistently detected in normal and malignant urothelia specimens (Table 1; Fig. 3, A and B). Heterogeneous staining was detected in two of the three TCC-derived cell lines, LB905 and LB831 (Table 1; Fig. 3C). In comparison, PSMA expression in LNCaP and prostatic adenocarcinoma specimens was more intense than any of the urothelial-derived tissues (Table 1; Fig. 3D).

Measurement of PSMA Enzymatic Activity in Bladder Cancer-derived Cell Lines. Using the described assay, a small amount of [3H]NAAG hydrolysis was consistently detected in LB905 [0.033 ± 0.003 pmol/(min x mg)] and LB831 [0.013 ± 0.001 pmol/(min x mg)] cell lines. In these two positive bladder cell lines, the amount of [3H]NAAG hydrolysis is 1000–2000-fold lower than the average hydrolysis obtained measured with prostate cancer cell line LNCaP [29 ± 2.6 pmol/(min x mg)]. In the PSMA negatively stained TCC-derived cell line LB796 and prostate cancer cell lines, TSU and PC-3, no PSMA activity was detected.

These results demonstrate that a weak but consistent expression of PSMA enzymatic activity can be found in the membrane of TCC-derived cell lines. In addition, the difference of the amount of enzymatic activity between these cell lines and the highly PSMA-positive prostate cancer cell line LNCaP accurately reflects the difference of intensity staining detected by immunohistochemistry.

Molecular Detection of PSMA mRNA in Blood Samples from Patients Treated for TCC of the Bladder. RT-PCR amplification of PSMA mRNA transcripts in blood is reported to improve early detection of advanced prostate cancer (7–9). Because PSMA expression has been demonstrated in bladder, it was legitimate to assess whether PSMA transcripts could be detected in bladder cancer patients. Screening for concomitant prostate cancer, based on digital rectal examination and serum prostate-specific antigen value, was negative in all cases. Cross-sectional pathological analysis of the prostate specimens revealed the existence of a concomitant prostatic adenocarcinoma in 10 patients. All of the adenocarcinomas were classified pT2 or less. Seven of the 27 (26%) preoperative blood samples tested positive for PSMA by RT-PCR. Only one of the PSMA-positive patients showed histological evidence of concomitant localized prostatic carcinoma (i.e., pT2a) and invasive bladder cancer (i.e., pT3b). Nine of 10 patients with both TCC and localized prostate cancer had no detectable PSMA mRNA in the blood (i.e., 5 TCC ≤ pT2 and 4 TCC ≥ pT3). These results confirm the previously published low rate of RT-PCR detection of PSMA transcripts in the blood of patients with localized prostate cancer (i.e., 15–25%; reviewed in Ref. 23). TCC specimens were classified as follows: pT1 (4 of 27), pT2 (6 of 27), pT3 (16 of 27), and pT4 (1 of 27). As shown in Table 2, no PSMA transcript was found in blood of patients presenting with TCC classified pT2 or less. In contrast, 7 of the 17 patients diagnosed with TCC ≥ pT3 tested positive for PSMA in blood. According to the Tumor-Node-Metastasis classification of TCC in the latter 7 PSMA-positive patients, 4 were M+ (2N+ M+ and N0 M+) and 3 were N0 M0. Among the 10 PSMA-negative patients ≥ pT3, 7 were N0 M0 and three were N+ M0.

Ten patients died during the follow-up period (range, 1–33 months; median, 15 months), all from metastatic TCC progression: 6 of 7 PSMA-positive patients versus 4 of 20 PSMA-negative patients. Conversely, 3 patients developed metastatic disease, and 2 died in the group of PSMA-negative patients with TCC ≥ pT3. In patients with TCC ≥ pT3, 1- and 2-year survival rates correlate with the detection of PSMA transcripts in the preoperative blood specimen. In patients with PSMA-negative assay, 1- and 2-year survival rates were 88 and 79%, respectively, whereas positive detection was associated with a drop of the survival rate to 75 and 29% at 1 and 2 years, respectively. Although confirmation requires larger prospective studies, these results suggest that PSMA-positive patients have a worse outcome. The possibility that molecular detection of PSMA transcripts could be used as a preoperative predictive marker of advanced disease, therefore, deserves further attention.

The present study demonstrates that full-length, PSMA-specific transcripts are consistently detectable in normal and malignant urothelium, as well as in peripheral blood from patients presenting with progressive TCC of the bladder. Although

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<th>Cell lines and tissular samples</th>
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<td>LNCaP</td>
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<tr>
<td>Adenocarcinoma</td>
<td>5+</td>
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Table 1 CYT-351 immunostaining of normal and malignant urothelial cells

Intensity and pattern of CYT-351 immunostaining are given for bladder tissues, including bladder-derived cell lines (LB905, LB831, and LB796) as well as normal urothelium and malignant TCC. Results are compared with negative (myeloid K562 and prostate PC-3 cell lines) and positive (prostate LNCaP cell line and surgical specimen of adenocarcinoma) controls.
PSMA expression is much weaker than in prostate-derived tissues, this transcript encodes a substantial amount of PSMA protein. In TCC-derived cell lines, PSMA is detectable by immunohistochemical staining and is enzymatically active, producing a level of enzymatic activity that correlates with the intensity of immunostaining.

Initially believed to be restricted to the prostatic epithelium, PSMA expression therefore appears to be more ubiquitous. Although this could theoretically impair prostate-specific anti-PSMA targeting strategies, it also generates new potential applications for this biological marker (24–26). In patients presenting with invasive TCC of the bladder and no evidence of a concomitant PSMA-positive tumor, current results suggest that preoperative PSMA mRNA blood transcripts are associated with advanced disease and progression after radical treatment. Whether PSMA assay can be considered as an individual diagnostic and prognostic marker in TCC patients, however, remains to be determined in a larger cohort of patients.

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**Table 2** Correlation between PSMA mRNA detection and 2-year survival in patients presenting with a TCC of the bladder

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<th>Correlation between molecular detection of PSMA-positive cells, pathological features of 27 cystoprostatectomy specimens, and 2-year survival of the patients: (a) global data in patients presenting with transitional cell carcinoma patients; (b) stratification according to the pathological TCC stratification; and (c) stratification according to the presence or absence of concomitant prostate cancer.</th>
<th>PMSA</th>
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ogy, Department of Clinical Biochemistry, Université Catholique de Louvain, Brussels, Belgium, for the quality of their technical assistance.

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