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# Prostate specific membrane antigen (PSM) is expressed in various human tissues: implication for the use of PSM reverse transcription polymerase chain reaction to detect hematogenous prostate cancer spread

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**Abstract** Detection of prostate-specific membrane antigen (PSM)-mRNA expression in blood samples using reverse transcription polymerase chain reaction (RT-PCR) is discussed as a new diagnostic marker of circulating micrometastases in prostate cancer patients. We applied the RT-PCR technique to different human tissues and obtained positive signals for PSM transcripts in human genital and multiple extra-genital tissue sites. The cDNAs were prepared from different human tissues and prostatic cell lines. RT-PCR and nested RT-PCR for PSM was performed with primers derived from the published PSM cDNA. The RT-PCR fragments obtained were cloned and showed 100% sequence homology to PSM. Southern blot hybridization with labeled probes was used to confirm the specificity of the amplicons. In addition to the known PSM expression in the human brain, PSM-mRNA was detected in cDNA isolated from human testis, epididymis and seminal vesicles and in the PC-3 prostatic cancer cell line. Furthermore, we found PSM-mRNA in heart, liver, lung, kidney, spleen, and thyroid gland. The results indicate that PSM expression is not restricted to the prostate gland, but represents a more general component of genital and extra-genital human tissues. This must be considered when RT-PCR and nested RT-PCR screening for PSM expression is performed as a diagnostic measure in blood from prostate cancer patients.

**Key words** Reverse transcription-polymerase chain reaction (RT-PCR) · Micrometastases · Prostatic cancer · Prostate membrane-specific antigen (PSM)

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## Introduction

Prostate cancer is one of the most common malignancies in males and is the second leading cause of cancer deaths in elderly men in the United States [16]. In addition to the established tumor markers prostate specific antigen and prostatic acid phosphatase, the prostate specific membrane antigen (PSM) has recently been introduced as a new diagnostic marker for screening and follow-up of prostate cancer [2, 4, 5, 22]. The PSM gene was cloned in 1993 by Israeli et al. [6], and the expression was thought to be restricted to the human prostate gland and the human LNCaP cell line. Very low levels of expression were detectable by ribonuclease protection assay in human brain, salivary glands and small intestine [7]. A recently published study, however, showed that PSM expression is detectable in breast carcinoma, kidney and liver [20]. Different groups have investigated the use of PSM-specific primers and the nested RT-PCR to detect circulating micrometastatic cells in blood samples of prostate cancer patients [8, 9]. Also, a monoclonal antibody ELISA and Western blotting was used to determine serum levels [13, 14, 17, 19]. In the present study, we applied RT-PCR and nested RT-PCR to screen for the expression of PSM-mRNA in the male reproductive tract, prostatic cells and extra-genital sites and found unexpected expression in these tissues. These findings are important for the possible use of PSM as diagnostic means in monitoring prostate (cancer) patients.

## Materials and methods

Different fresh human tissues (two independent samples from human brain, heart, kidney, liver, lung, spleen and thyroid gland, and three independent genital tissues from prostate, seminal vesicle, epididymis, and testis) were obtained from the Departments of Urology, Pathology and Gynecology and Obstetrics at the University Hospital of Marburg.

The LNCaP, Du-145, PC-3 and MCF-7 cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, Md.). Stromal prostatic cells were derived from benign prostatic hyperplasia, characterized immunohistochemically and termed P21. The cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 4 mM L-glutamine, 0.2% sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B (Gibco, Eggenstein, Germany) and were incubated with 5% $CO_2$ at 37°C. All cells were checked for mycoplasma contamination using the mycoplasma PCR ELISA (Boehringer Mannheim, Germany). Total RNA was isolated using TRIzol (Gibco) according to the manufacturer's protocol. The RNA was treated with DNase I (RNase-free, Boehringer Mannheim) and 1–3 μg were reverse transcribed using the M-MLV reverse transcriptase, oligo-dT [15] (Promega, Heidelberg, Germany) and 0.5 mM dNTPs (Boehringer Mannheim) in a final volume of 20 μl at 37°C for 90 min. All cDNAs were synthesized independently in duplicate. Intron-spanning primer pairs specific for human PSM were deduced from the GenBank DNA sequence (M99487) using PC/gene software (IntelliGenetics, Geneva, Switzerland), checked for homology and synthesized by MWG Biotech (Ebersberg, Germany). The outer primer pair amplifies a 471 bp fragment from nucleotides 1389 to 1860 and the nested primer pair spans nucleotides 1480–1782 yielding a 303 bp product:

outer primer pair for PSM
forward 1368 5′-TCACCGGGACTCATGGGTGT-3′
reverse 1860 5′-GCCTGAAGCAATTCCAAGTCG-3′
inner primer pair for PSM
forward 1480 5′-AAGGAAGGGTGGAGACCTAG-3′
reverse 1782 5′-ACTGAACTCTGGGGAAGGAC-3′

The integrity of the different transcribed cDNAs was checked using primer pairs specific for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplifying a 300 bp fragment from nucleotide 360 to 659 of the GAPDH-cDNA sequence:

forward GAPDH 360 5′-CGTCTTCACCACCATGGAGA-3′
reverse GAPDH 659 5′-CGGCCATCACGCCACAGTTT-3′

The PCR-master mixture consisted of 5 μl of 10× PCR buffer, 2 mM $MgCl_2$, 2 U Taq-polymerase (PanSystems, Nürnberg, Germany), 1 μl of 10 mM dNTPs, 50 pmol of each primer, and 1 μl cDNA in a final volume of 50 μl overlayed with 30 μl of mineral oil. Amplification was performed on a Hybaid Omnigene Thermocycler (MWG Biotech). After an initial denaturation at 95°C for 4 min the following PCR profile was used: 20 s 94°C, 30 s 60°C, 1 min 72°C for 30 cycles (27 for GAPDH). Thereafter, 1 μl of a 1:1000 dilution in $H_2O$ of the PSM-RT-PCR product was used as template in the nested RT-PCR reaction for additional 20 cycles under the same conditions. The master-mixture without cDNA was performed as a first negative control. As a second negative control 1 μl of the isolated total RNA omitting reverse transcription reaction was used to detect potential genomic DNA contaminations. The PCR products obtained from RT-PCR of LNCaP-cDNA were cloned in pCR 2.0 (outer 471 bp) and pCR 2.1 (inner 303 bp) using the TA cloning kit (Invitrogen, de Schelp, Netherlands) and transformed in *E. coli* strain inv-alpha F′. For plasmid preparations the plasmid kit from Quiagen (Hilden, Germany) was used. The plasmids were sequenced using a Lycor automatic sequencer (MWG Biotech). Sequence alignment was performed at the NCBI BLAST world wide web server. Ten μl of the PCR products were blotted from ethidium bromide-stained 1.5% agarose gels onto positively charged nylon membranes (BioSupport, Dreieich, Germany) according to Koetsier et al. [11] and UV-crosslinked. Non-radioactive labeling of cDNA probes for PSM and GAPDH was performed using the DIG-High Prime Kit (Boehringer Mannheim). Hybridization was carried out overnight at 65°C in 5 × SSC, 0.02% SDS, 5% blocking reagent (Boehringer Mannheim) and 0.1% *N*-lauroylsarcosine. Chemiluminescence detection was performed according to the manufacturer's recommendation using a 1:10 000 diluted anti-digoxigenin Fab fragment conjugated to alkaline phosphatase (Boehringer Mannheim) and CDP 1:100 (Tropix, Heidelberg, Germany) as substrate. Membranes were sealed in transparencies and exposed to Fuji RX new films (Siemens, Frankfurt, Germany).

## Results

Expression of PSM-mRNA was investigated in the prostatic cell lines LNCaP, PC-3, Du-145, and stromal cells derived from benign prostatic hyperplasia (BPH). The LNCaP cell line is known to express PSM and was used as positive control in all assays. In PC-3 cells, which were described earlier as PSM negative, we obtained a 471 bp PSM-specific fragment after the first amplification, using the outer primer pair. The nested RT-PCR reaction performed with a 1:1000 dilution of the outer PCR product confirmed this result and revealed a specific 303 bp fragment in PC-3 cells (Fig. 1). PSM RT-PCR performed with cDNAs derived from the Du-145 cell line, the stromal P21 cells, MCF-7 breast cancer cells and the RNA controls were always negative in three independent experiments (Fig. 1). The cloned and sequenced 471 and 303 bp PSM products showed a 100% identity to the published PSM cDNA sequence.

The cDNAs from human testis, epididymis, seminal vesicles and prostate were positive for PSM expression after 30 amplification cycles with the outer primer pair. The nested PCR revealed a positive 303 bp PSM product in all tissues, indicating the expression of PSM and its presence throughout the male genital tract. In addition to the male reproductive organs, we observed positive results in extra-genital tissues such as the thyroid gland (Fig. 2). Furthermore, we obtained positive RT-PCR results in varying intensity in liver, lung, kidney, heart, and spleen, while the MCF-7 control remained negative for PSM expression (Fig. 3).

RT-PCR of the corresponding total RNA preparations performed as a control, remained negative (Fig. 3). cDNAs prepared from LNCaP cells, benign hyperplasia of the prostate and prostate cancer served as positive controls and expressed the PSM signal at high abundance (Figs. 2, 3). Southern blotting of the obtained RT-PCR products and hybridization utilizing the DIG-labeled cDNA probe confirmed the results. Chemoluminescence detection revealed a specific signal in all positive PSM RT-PCRs (Figs. 1–3).

## Discussion

The use of RT-PCR of molecular markers such as PSA and PSM in the prediction and diagnosis of prostate

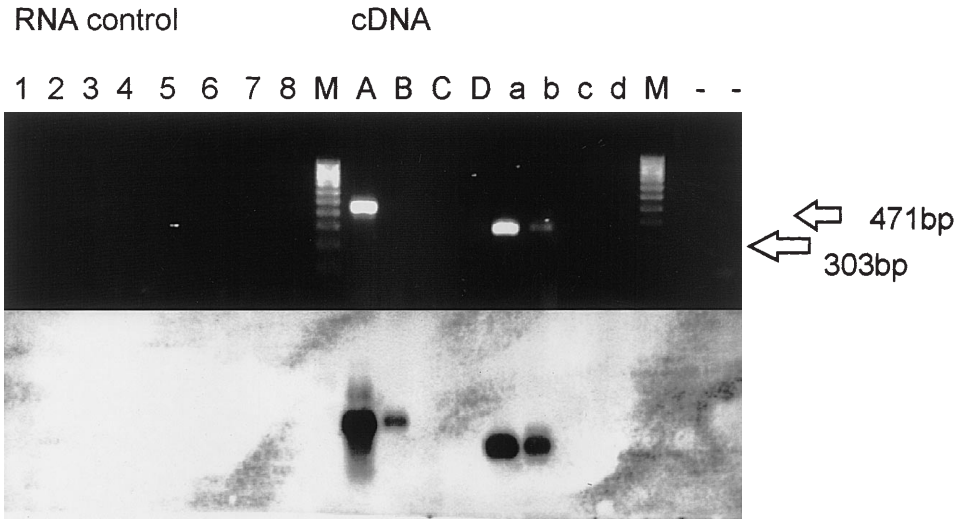
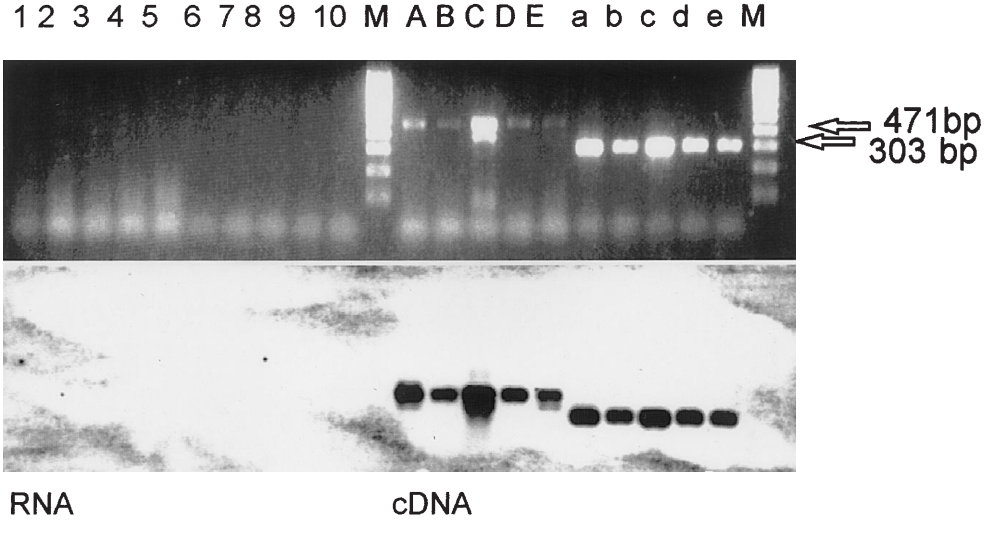



**Fig. 1** Ethidium bromide stained agarose gel of 10 µl prostate-specific membrane antigen (PSM) and nested PSM reverse transcription-polymerase chain reaction (RT-PCR) products of human prostatic cell cDNAs and RNA controls (*top*) and the corresponding DIG-labeled Southern blot hybridization (*bottom*). LNCaP cDNA (A,a) and PC-3 cDNA (B,b) are positive for PSM mRNA expression. Du-145 (C,c), stromal cells (D,d) and the RNA controls (lanes 1–8) remain negative. Lanes: *M* 100 bp marker, – negative controls, PSM RT-PCR, cDNA: *A* LNCaP, *B* PC-3, *C* Du-145, *D* stromal cells. Nested PSM RT-PCR, cDNA from *a* LNCaP, *b* PC-3, *c* Du-145, *d* stromal cells. PSM RT-PCR, RNA from: *1* LNCaP, *2* PC-3, *3* Du-145, *4* stromal cells. Nested PSM RT-PCR, RNA from: *5* LNCaP, *6* PC-3, *7* Du-145, *8* stromal cells

**Fig. 2** Ethidium bromide visualized agarose gel (*top*) and the corresponding DIG-labeled Southern blot hybridization (*bottom*) of 10 µl PSM and nested PSM RT-PCR products of human genital tissues cDNAs and RNA-controls. Lanes: PSM RT-PCR, cDNA from: *A* testis, *B* epididymis, *C* prostate, *D* seminal vesicles, *E* thyroid gland (control). Nested PSM RT-PCR, cDNA from: *a* testis, *b* epididymis, *c* prostate, *d* seminal vesicles, *e* thyroid gland. All tissues exhibited a PSM RT-PCR product even with the outer primer pair after 30 amplification cycles. The PSM RT-PCR RNA controls remained negative: *1* testis, *2* epididymis, *3* prostate, *4* seminal vesicles, *5* thyroid gland. Nested PSM RT-PCR; RNA from: *6* testis, *7* epididymis, *8* prostate, *9* seminal vesicles, *10* thyroid gland

disease is an important new tool in clinical research. The high sensitivity of these methods, however, is combined with a possible decrease in specificity. Therefore care is needed to exclude artificial results [10, 15, 24]. The puzzling findings in our study of prostate cancer cells in peripheral blood of prostate cancer patients using PSM RT-PCR prompted us to apply the same hypersensitive detection methods to different human genital and extra-genital tissues and prostatic cells. Using RT-PCR and nested RT-PCR primers, our results indicate that – contrary to initial claims – the distribution of PSM is not restricted to prostatic tissue. Israeli et al. [7] observed positive PSM RT-PCR results in blood of control group patients with a testis cyst and in one case of renal cell carcinoma, respectively, and could not explain their findings at that time. The question of the potential origin of these positive findings may be answered by our present studies, showing PSM message being present both in kidney and testis. It is known that two different alternatively spliced mRNA forms of PSM occur in the prostate: PSM and PSM′, which lacks the signal sequence close to the 5′ end of the cDNA [18]. In normal



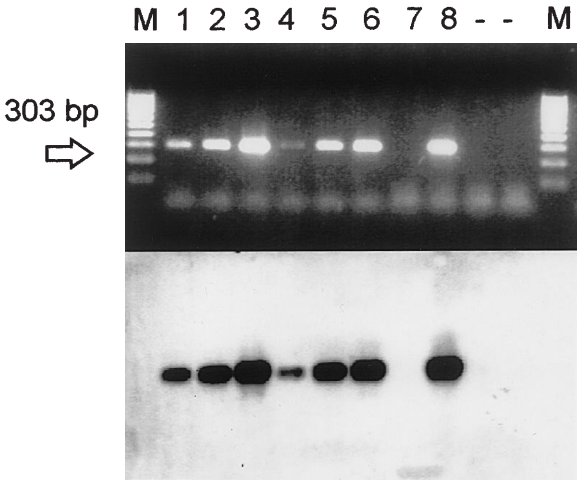



**Fig. 3** Ethidium bromide stain of an agarose gel loaded with 10 µl nested PSM RT-PCR products of cDNAs from different human tissues (*top*) and DIG-labeled Southern hybridization (*bottom*). Lanes: *M* 100 bp marker, nested PSM RT-PCR, cDNA from: *1* heart, *2* brain, *3* liver, *4* lung, *5* spleen, *6* kidney, *7* MCF-7, *8* LNCaP. All tissues and the LNCaP cells (*8*) revealed a 303 bp product, whereas the MCF (*7*) cells and the controls (- left: first RT-PCR round, - right: nested RT-PCR) remained negative

human prostate both forms occur in equal amounts, whereas in the prostate tumor state more PSM than PSM′ is found and it is known that other sequences on chromosome 11 in the human genome are closely related to PSM [12]. Nevertheless, our newly designed intron-spanning primer pairs are amplifying common regions of the PSM cDNA that have earlier been used for detection of micrometastases in blood [1, 8]. The cloned and sequenced PSM-PCR and nested RT-PCR fragments are identical with the published cDNAs of PSM and PSM′, and potential genomic contaminations of the cDNAs were excluded by controls using the corresponding total RNAs as templates. In addition, the PC-3 cell line, earlier reported to be negative for PSM expression [7], was constantly positive with our primer pairs. In contrast the Du-145, the stromal cells and the MCF-7 breast cancer cell lines used as control remained negative [9]. The stromal cells derived from benign prostatic hyperplasia (BPH) tissue are apparently composed of 40% fibroblastic and 60% smooth muscle cells, as determined by immunohistochemical staining, and were also negative for PSM expression. This is at variance with findings of Troyer et al. [19], who detected immunoreactivity of smooth muscle cells using a monoclonal antibody against PSM. Recently, Heston [3] localized PSM immunoreactivity in the brush border of the proximal small intestine. The results in measuring PSM serum levels are also controversial. Rochon et al. [17] published that in Western blot analysis PSM levels in serum of prostate cancer patients were significantly higher than in normal controls, while Troyer et al. [19] observed no elevated PSM levels in actively progressing metastatic prostate cancer in serum. However, the increasing PSM immunoreactivity described in prostate tissues after androgen deprivation might put PSM in favor as a biomarker for hormone-independent prostate cancer [23]. Nevertheless, a standardization of gene-specific primer pairs and optimal RT-PCR conditions and the determination of a possible threshold caused by cells of non-prostatic origin is necessary to avoid false-negative or false-positive results [21]. Although the results are hitherto controversial, a combination and quantification of RT-PCR results is suggested to improve the specificity for detection of disseminating prostate cancer.

In conclusion, the use of PSM as a novel marker for the molecular detection of circulating prostate cancer micrometastases in peripheral blood needs to be critically re-evaluated and the contamination with cells of non-prostatic origin must be considered in any case of positive signals.

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