Enhanced Expression of Prostate-specific Membrane Antigen Gene in Prostate Cancer as Revealed by in Situ Hybridization

Masako Kawakami and Jun Nakayama

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Materials and Methods

Patients. Needle biopsy prostate specimens from 15 patients with prostate cancer were obtained at Shinshu University Hospital and used in this study. The clinicopathological features of the patients are shown in Table 1. All specimens were reviewed and graded according to the system of Gleason (6).

Cell Line. LNCaP, a human prostate carcinoma cell line, was obtained from the American Type Culture Collection (Rockville, MD; Ref. 8). Monolayer cultures were maintained on plastic dishes in complete medium composed of RPMI 1640 and 10% FCS. The cell line was routinely passaged every 4–5 days and harvested when 75% confluent for isolating total cellular RNA.

Preparation of a PSM-specific Transcript Probe for in Situ Hybridization. Total cellular RNA was isolated from 5 × 10^6 LNCaP cells according to the acid guanidinium thiocyanate-chloroform extraction method using an Isogen kit (Nippon Gene, Tokyo, Japan; Ref. 9). Subsequently, double-stranded cDNA was synthesized by reverse transcription of the extracted RNA. Briefly, total RNA (1 μg) was reverse-transcribed with 0.25 μl of Moloney murine leukemia virus reverse transcriptase (200 units/μl) in a total volume of 20 μl containing 10× reverse transcription buffer, 150 μM of each deoxynucleotide triphosphate, 0.5 μg of oligo(dT)15 primer, 10 mM DTT, and 1 μl of RNase inhibitor, RNasin (10 units/μl; Promega, Madison, WI). The reaction was performed at 42°C for 1 h.

Using this double-stranded cDNA as a template, a PSM-specific nucleotide sequence surrounding the initiation methionine (nucleotides +101 to +37; the first nucleotide of the initiation codon is +1) was amplified by PCR (Fig. 1). The 5’ and 3’ primers were designed to be 5’-GCTCTAGAGATT-GAGAGACTTTAC-3’ and 5’-GGGTTACACACGCGACTGGT-3’, respectively, underlined. According to the published sequence (4), the XhoI and Asp718 sites are

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The abbreviations used are: PSA, prostate-specific antigen; PSM, prostate-specific membrane antigen.
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PSM-SPECIFIC TRANSCRIPTS IN PROSTATE CANCER

Fig. 1. Diagram of PSM and PSM' cDNAs. The translation initiation site of PSM cDNA is denoted as +1. Both PSM and PSM' are generated through alternative splicing, and the nucleotides from -147 to +119 are lacking in PSM' cDNA. The RNA probe was designed to identify the nucleotide sequence from -101 to +37 of PSM cDNA, which is absent from PSM' cDNA. Box, open reading frame. TM, transmembrane region.

as described previously (10, 11). Similarly, a sense probe was prepared for negative control experiments by using an Asp718-cut template and SP6 RNA polymerase with the same kit.

In Situ Hybridization of PSM-specific Transcripts. Tissue specimens were subjected to in situ hybridization to detect PSM-specific transcripts using a nonradioactive system (10, 11). After the tissue sections were deparaffinized in xylene, hydrated slides were immersed in 0.2 M HCl for 20 min and then digested with 100 µg/mL proteinase K at 37°C for 20 min, followed by postfixation with 4% paraformaldehyde. Theses slides were rinsed with 2 mg/ml glycine and subsequently acetylated for 10 min in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0). The hydrated slides were then dewaxed with chloroform and air-dried. After prehybridization with 50% deionized formamide and 2× SSC for 1 h at 45°C, the slides were hybridized with 0.5 mg/ml antisense or sense probe in 50% deionized formamide, 2.5 mM EDTA (pH 8.0), 300 mM NaCl, 1 x Denhardt's solution, 10% dextran sulfate, and 1 mg/ml brewer's yeast tRNA at 45°C for 16 h.

After hybridization, the slides were washed in 50% formamide and 2× SSC for 1 h at 45°C and digested with 10 mg/ml RNase A at 37°C for 30 min. After washing with 2× SSC and 50% formamide at 45°C for 1 h, 1× SSC and 50% formamide at 45°C for 1 h, and 1× SSC and 50% formamide at room temperature for 30 min, the sections were subjected to immunohistochemistry for detection of the hybridized probes using an alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim). The alkaline phosphatase reaction was visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. A control study using the sense probe showed no specific reactivity. The intensity of the signal in the carcinoma cells was graded on a scale of - to +++ (+, no reactivity; +, weak reactivity; ++, moderate reactivity; ++++, strong reactivity).

Immunohistochemistry for PSA. Deparaffinized tissue slices were also subjected to immunohistochemical staining for the detection of PSA. A rabbit polyclonal antibody directed against human PSA was purchased from Dako (Glostrup, Denmark), and immunohistochemical detection was performed by the indirect method followed by counterstaining with hematoxylin (12). A control experiment was done by omitting the primary antibody from the staining procedure, and no specific staining was found. The intensity of the staining in the carcinoma cells was graded on a scale of - to +++ (-, no reactivity; +, weak reactivity; ++, moderate reactivity; +++, strong reactivity).

Results and Discussion

Expression of PSM-specific Transcripts in Prostate Cancer. We have successfully demonstrated PSM-specific transcripts in prostate tissues fixed with a standard fixative, 20% neutral formalin. The transcripts could be detected in most of the carcinoma cells from all 15 cancer specimens examined irrespective of the clinical stage, Gleason score, preoperative serum PSA value, and response to hormonal therapy, as shown in Table 1. Although the level of transcripts in the carcinoma cells varies among patients, transcription of the PSM gene was markedly increased in carcinoma cells compared to that of normal prostate glands (Fig. 2B). In normal prostate glands, PSM-specific transcripts were restricted to the basal cells (Fig. 2B, inset). As background activity, weak signals for the transcripts were also noted in the prostatic stromal cells. These results clearly show that the PSM-specific transcripts were closely associated with malignant transformation of the prostate. This finding is also consistent with the result of an immunohistochemical study by Lopes et al. (13) using monoclonal antibody 7E11-C5, which was originally used for molecular cloning of PSM cDNA. They showed that 7E11-C5 antibody reacted more strongly with all of the prostate carcinoma cells examined than it did with normal prostate tissue. The same study also revealed that immunostaining with 7E11-C5 could not distinguish basal cells from secretory cells in the normal prostate gland, and there was weak staining of both types of glandular epithelia. Taking into account that the antigenic determinant of 7E11-C5 is the first 6 amino acids from the amino-terminal of PSA (14), whereas the RNA probe we used contains the nucleotide sequence corresponding to these particular 6 amino acids, it may be possible that the PSM gene is transcribed in the basal cells but not in the secretory cells of the normal prostate gland, although the secretory cells express PSA.

Interestingly, the level of expression of PSM-specific transcripts was dependent on resistance to hormonal therapy as well as on the histological differentiation of the carcinoma. As shown in Table 1, carcinoma cells from hormone-refractory patients expressed higher levels of the transcripts (Fig. 2J) compared to cells from patients who showed a response to hormonal therapy (Fig. 2F). Recently, Israel et al. (15) showed that the expression of PSA by LNCaP cells was highest in steroid-depleted medium and was down-regulated in the presence of dihydrotestosterone and progesterone. Further study is required to investigate the transcriptional regulation of PSA by various hormones. In addition, increased expression of PSM-specific transcripts was found in poorly differentiated adenocarcinoma rather than in well-differentiated adenocarcinoma (Table 1). These results, taken together, indicate that enhanced expression of PSM-specific transcripts could be closely associated with the progression of prostate cancer.

Comparison of PSM-specific Transcripts and PSA. PSA is a distinct molecule present in the prostate gland and is also expressed in the vast majority of prostate cancers (2). The pattern of PSA expression was compared to that of PSM-specific transcripts in the present study. As reported previously (3) and confirmed here, PSA was most strongly expressed in the normal glandular epithelia (Fig. 2D). In prostate carcinomas, PSA was preferentially expressed in well-differentiated adenocarcinoma (Fig. 2H) rather than in poorly differentiated adenocarcinoma (Fig. 2L). In contrast to PSA, the expression of PSM-specific transcripts was more restricted to carcinoma cells (Fig. 2B) and thus was more specific for prostate cancer. Moreover, the PSM-specific transcripts and PSA were expressed in a reciprocal manner, as shown in Table 1. The carcinoma cells that abundantly expressed PSM-specific transcripts showed relatively weak PSA expression (Fig. 1, J and L) compared to those expressing low levels of PSM-specific transcripts (Fig. 2, F and H). In situ hybridization for PSM-specific transcripts could therefore be a useful tool for the diagnosis of prostate cancer.

In routine pathological examination of prostate cancer, it is sometimes difficult to diagnose adenocarcinoma with minimal cytological atypia (Fig. 2E), and it is also hard to recognize that the tumor is of prostatic origin when the carcinoma cells express little or no PSA (Fig. 2L). In such cases, in situ hybridization for PSM-specific transcripts could make an effective contribution to the diagnosis of prostate cancer.

In summary, the present study demonstrated the increased expression of PSM-specific transcripts in prostate cancer and the value of in...
### Table 1 Clinicopathological features and expression of the PSM-specific transcript and PSA

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a Staining intensity: +, weak; ++, moderate; ++++, strong.
b Normal range is below 2.8 ng/ml.
c D, responded to hormonal therapy; ID, refractory to hormonal therapy.

situ hybridization for the transcripts in the histological diagnosis of prostate cancer.

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### References

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