A Novel Form of Prostate-specific Antigen Transcript Produced by Alternative Splicing

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Abstract

Molecular characterization of prostate-specific antigen (PSA) has not been well elucidated, despite a great deal of clinical study. We examined the heterogeneity of PSA using reverse transcription-PCR and direct sequencing. A novel, alternatively spliced variant of the PSA transcript was found in prostate cancer (PC), as well as in benign prostatic tissue. This alternative splicing leads to the deletion of 44 amino acid residues (amino acids 45–88) from mature PSA, resulting in the loss of asparagine 45, which is a binding site for a carbohydrate chain. By these nested reverse transcription-PCR systems, this novel, alternatively spliced PSA gene was recognized in 13 of 18 (72.2%) cases with noncancerous prostate tissue, 4 of 5 (80.0%) PC cases, and 3 of 12 (25.0%) blood samples from PC patients (noncancerous prostate tissue group versus blood sample group, P = 0.011). At present, the biological significance of this alternative splicing remains to be established.

Introduction

PC is currently the most common malignancy in elderly males in Western countries (1). The recent popularization of administering serum PSA tests as part of a regular health checkup or clinic visit has resulted in an increase in the number of newly diagnosed PC patients. However, PSA is produced not only by malignant cells but also by noncancerous prostate epithelial cells. Therefore, there is a substantial overlap in serum PSA levels between men with BPH and those with PC (2–4). Recently, different molecular forms of serum PSA have been characterized. The differences in the serum ratio between free (noncomplexed) and total PSA, including PSA complexes with α1-antichymotrypsin, have been introduced to assist in the differential diagnosis between BPH and PC (5–7). Although the basis for the heterogeneity of PSA using reverse transcription-PCR and direct sequencing; the other half was fixed with neutralized buffered formalin for routine histopathological examination. We also analyzed peripheral blood samples from 12 patients with PC who had clinically metastatic disease. Cells were isolated from blood samples (10 ml each) by a density separation method using Leucoprep (Becton & Dickinson, Franklin Lakes, NJ), washed once in PBS, and stored at −80°C (8).

Analysis of the PSA Gene by RT-PCR and Direct Sequencing. Total cellular RNA of tissues and blood samples was isolated using the TRIzol extraction kit (Life Technologies, Inc., Rockville, MD) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 5 μg of total RNA using 20 units of RAV-2 reverse transcriptase (Takara, Otsu, Japan) and random nonamers (Takara). Primer sequences were chosen within PSA gene regions that maximized the mismatches with other genes of the same family, such as the human kallikrein gene (9, 10), and these sequences are shown in Fig. 2A. Portions (1 μl) of the cDNA were amplified by PCR using the S1 and A1 primers. The reaction mixture (50 μl) consisted of 20 mM Tris-HCl (pH 7.5), 8 mM MgCl₂, 7.5 mM DTT, 12.5 μg of BSA, 0.2 μM deoxynucleotide triphosphates, 15 μM of activated calf thymus DNA, 1.25 units of Thermophilus aquaticus (KOD Dash) polymerase (Toyobo, Osaka, Japan), and 200 ng of each primer. Amplification was performed with 30 cycles of denaturation (98°C, 10 s), annealing (60°C, 2 s), and extension (74°C, 30 s). After amplification, 5 μl of the RT-PCR products were subjected to electrophoretic analysis on a 2% agarose gel with ethidium bromide. DNA sequencing of the PCR products was performed by the dyeoxy chain termination method (11) using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems; Perkin-Elmer, Foster City, CA; Ref. 8).

Analysis of the Expression of an Alternative PSA Gene by Nested RT-PCR. To verify the reliable expression of an alternative PSA gene, we synthesized the novel alternatively spliced PSA gene-specific primer (SS1, 5′-CTGCCCACTGCAATCATGGGAAGC-3′), which extends 2 bases past the splice junction, according to the nucleotide sequence of human alternative PSA (Figs. 2A and 3A). A normal PSA transcript cannot be amplified by the SS1 primer because this primer crosses over the spliced junction site (Fig. 2A). Subsequently, only alternatively spliced PSA transcript is amplified. Nested RT-PCR for 18 nonmalignant prostatic tissues and 5 PC tissues was performed with the first 30 cycles of denaturation (98°C, 10 s), annealing (65°C, 2 s), and extension (74°C, 30 s) using S1 and A1 primers, and the next 20 cycles of the same temperature profile using SS1 and A3 primers. Nested RT-PCR for blood samples from 12 PC patients was performed with the first 40 cycles followed by an additional 40 cycles using the same temperature profile and the primer pairs described above. To certify the existence of the PSA transcript, nested RT-PCR was performed using S1 and A1 primers followed by S4 and A2 primers, which can amplify the common part (nucleotides 619–917) of normal and alternatively spliced PSA transcripts.

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2 To whom requests for reprints should be addressed, at Department of Urology, Shiga University of Medical Science, Seta, Otsu, Shiga 520-2192, Japan. Phone: 81-77-548-2273; Fax: 81-77-548-2400; E-mail: yoshiki@belle.shiga-med.ac.jp. The abbreviations used are: PC, prostate cancer; PSA, prostate-specific antigen; BPH, benign prostatic hypertrophy; RT-PCR, reverse transcription-PCR.
Western Blot Analysis. To investigate the presence of PSA protein derived from alternatively spliced PSA transcript in prostate tissues, Western blot analysis was performed as described previously (12). BPH tissues were used as the source for this experiment. Anti-PSA polyclonal antibody was kindly supplied by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). In all gels, 2 μg of protein dissolved in sample buffer were loaded per lane. The proteins were separated by 16% SDS-PAGE under reducing conditions and then transferred onto a polyvinylidene difluoride membrane (TEFCO Corp., Tokyo, Japan). The polyvinylidene difluoride membrane was placed in Super Block (Pierce, Rockford, IL) to block nonspecific binding sites. The first antibody (diluted 1:500) was used to incubate the membrane overnight. Normal rabbit serum was used as a negative control instead of anti-PSA antibody. The immunoproducts were visualized using the Histofine streptavidin-biotin kit (Nichirei, Tokyo, Japan).

Results

Nucleotide Sequence of the Human PSA Gene. We amplified a portion of the human PSA gene from prostatic tissues by RT-PCR using the S1 and A1 primers. In addition to the major product, which corresponded in size to mature PSA, a faint minor product was shown more faintly by gel electrophoresis (Fig. 1). We determined the nucleotide sequence of each of the PCR products. The nucleotide

![Fig. 1. We amplified a portion of the human PSA gene from prostatic tissue by RT-PCR using the S1 and A1 primers. In addition to the major product, which corresponds in size to mature PSA, a faint minor product was seen at a lower position than the major product. Lane M, a 100-bp ladder (Toyobo).](image)

![Fig. 2. A, the nucleotide sequences of PSA and alternatively spliced PSA. Double underlining indicates the region that is absent in the alternatively spliced variant. PSA exons are boxed, and the oligonucleotide primer sequences used are underlined and designated. Arrows show the orientation of the primers. The dotted line shows the orientation of the alternative PSA-specific primer. B, parts of the deduced amino acid sequences of PSA and alternative PSA. *, asparagine 45, which is the only N-glycosylation binding site for the carbohydrate chain in PSA. Forty-four amino acid residues were deleted by alternative splicing, and lysine subsequently appeared.](image)
sequence of the major product was identical to that reported previously for the human PSA gene (10). However, the nucleotide sequence of one minor product was identical to that of the human PSA gene except for a 129-nucleotide deletion in the exon 3 (nucleotides 248–376; Fig. 2A). This result revealed that this minor product was a novel, alternatively spliced variant. By this alternative splicing, 44 codons (codons 45–88) were deleted from mature PSA composed of 237 amino acid residues (Fig. 2B), resulting in the loss of asparagine 45, which is a binding site of carbohydrate chains (13). This novel PSA product was calculated to have a molecular weight of 21,071.19.

Expression of the Alternative PSA Gene by Nested RT-PCR. We analyzed the expression of the alternative PSA gene in noncancerous prostatic tissues, in PC tissues, and in the peripheral blood of patients with advanced PC by nested RT-PCR using an alternative PSA-specific primer (SS1). We confirmed that the primer pair SS1/A3 amplified 366-bp products from the alternatively spliced variant, but not from the normal transcript (Fig. 3B). The alternative PSA gene was recognized by these nested RT-PCR systems in 13 of 18 (72.2%) noncancerous prostate tissues, 4 of 5 (80.0%) PC tissues, and 3 of 12 (25.0%) blood samples from PC patients. Of the five PC tissues, one lymph node metastasis tissue did not contain the alternatively spliced PSA transcript. The other four primary PC tissues expressed both mature PSA transcripts and spliced ones. There was a statistically significant difference in alternative PSA gene expression between the first group and the last group in these three groups (P = 0.011 by \( \chi^2 \) test; Fig. 3C).

Existence of an Alternatively Spliced PSA Gene Product. Western blotting analyses revealed multiple PSA protein bands. As predicted from the data regarding the deduced amino acid residues of variant PSA, a protein band corresponding to a molecular weight of approximately 21,000 was shown, in addition to a main band of M, 33,000 (Fig. 4).

Discussion

PSA is well known as the most powerful tool to diagnose and monitor patients with PC. However, its weak point has become apparent from numerous previous reports (2–4). Best characterized as a differential antigen, PSA is not a cancer-specific protein. To overcome this potential problem, various expedients have been invented such as PSA density, PSA velocity, free-total PSA ratios, and so on (5–7, 14, 15). In these modifications of the measurement of PSA, the differentiation of free-total PSA ratios would be caused by the variation of the molecular form.

We believed that the details of the PSA molecule should be investigated more intensively. Thus far, only a small number of studies have addressed this particular facet of PSA research. Among these studies, the products of Christensson et al. (5) are worthy of special mention. Christensson et al. (5) revealed the existence of binding proteins for PSA, which significantly affected the field of PC research. Their efforts bore fruit as the measurement of free-total PSA...
Further investigation should be done, and the question of whether this alternative PSA gene can be translated into a protein as efficiently as the more predominant form of PSA should also be investigated. In future studies, the clinical significance and utility of the splicing heterogeneity of PSA may be more fully discerned.

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References

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