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SHORT COMMUNICATION

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Novel polymorphisms of prostate-specific antigen (*PSA*) gene associated with PSA mRNA expression in breast cancer

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Abstract A growing body of evidence suggests that prostate-specific antigen (PSA) is a novel prognostic factor for breast cancer. The molecular mechanism of variant PSA expression in breast cancer has remained poorly understood in spite of intensive research. Previous studies have shown that the coding region of the *PSA* gene is not a target for mutations in prostate cancer and breast cancer. The purpose of this study was to analyze genetic variations in the promoter region of the *PSA* gene, and to detect whether such variations are correlated with PSA mRNA expression in breast tumors. We identified two polymorphisms in the proximal promoter region of the *PSA* gene. These polymorphisms are located at positions −252 (G or A) and −205 (A or AA), and generate three genotypes. The genotypes were associated with PSA mRNA expression. Our findings suggest that these polymorphisms identified in the proximal promoter region may affect the transcriptional activity of *PSA*.

Key words Prostate-specific antigen gene · Polymorphism · Promoter · Breast cancer · Japanese · Genotyping

Introduction

The prostate-specific antigen (*PSA*) gene codes for a serine protease that is a 240-amino acid glycoprotein (33 kDa) and is found in the serum as a 33-kDa monomer, as a 100-kDa complex with α_1-antichymotrypsin, and as an 800-kDa complex with α_2-macroglobulin (Armbruster 1993). PSA is useful in the detection, staging, and monitoring of prostate cancer (Smith et al. 1996; Partin et al. 1997; Pound et al. 1997). It was originally identified as a tissue-specific protein expressed exclusively by the epithelial cells of the prostate gland (Oesterling 1991). Recently, improved ultrasensitive methods and RNA analysis have shown that PSA is not exclusively synthesized by the human prostate gland, but is also produced by the breast, ovary, liver, kidney, adrenal, and parotid glands (Levesque et al. 1995; Smith et al. 1995).

PSA immunoreactivity and mRNA expression in breast cancer were first identified by Diamandis et al. (Diamandis et al. 1994; Monne et al. 1998). PSA positivity in primary breast tumors was found to be significantly associated with smaller tumors, steroid hormone receptor positivity, low cellularity, diploid tumors, low S-phase fraction, less advanced disease stage, younger patient age, lower risk of relapse, and longer overall survival (Yu et al. 1995; 1998). Therefore, PSA is considered as a novel indicator of favorable prognosis in breast cancer patients. However, the molecular mechanism of PSA variation in breast cancer has remained poorly understood. The *PSA* gene is under regulation by steroid hormones. Studies have shown that the wild-type *PSA* gene is regulated by the androgen receptor (AR), mediating transcription by binding to a cognate sequence on the *PSA* proximal promoter at positions −154 and −394 (Schuur et al. 1996). The third androgen response element (ARE) is located in the distal enhancer at position −4200 (Cleutjens et al. 1997). The proximal and the distal enhancers of the *PSA* gene interact in a cooperative manner and are required for optimal expression of the gene (Riegman et al. 1991; Schuur et al. 1996; Cleutjens et al. 1997). A G/A substitution polymorphism in the *PSA* promoter ARE1 region in prostate cancer has been reported recently (Xue et al. 2000). The present study was undertaken to analyze whether any other alterations are presented in the other AREs of the *PSA* gene.

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Subjects and methods

Samples

Whole blood and tumor specimens were obtained from 53 patients who had undergone surgery for breast cancer. All patients gave their informed consent prior to the collection of samples, according to institutional guidelines. Tumor specimens had been collected at the time of surgery, snap-fozen, and stored at −80°C after being histologically confirmed. Genomic DNA extraction was performed using a QIAamp Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Polymerase chain reaction (PCR) amplification

Based on the published sequence of the human *PSA* gene promoter region (EMBL, accession number U37672), PCR primers were designed to amplify ARE 2 (sense primer, 5′-TCTAGTTTCTGGTCTCAGAG-3′; antisense primer, 5′-GAGAGCTAGCACTTGCTGTT-3′) and ARE 3 (sense primer, 5′-ACCTGAGATTAGGAATCCTC-3′; antisense primer, 5′-CAGGCATCCTTGCAAGATG-3′). DNA amplification was performed using a Perkin-Elmer GeneAmpsystem 480 in a total volume of 50-μl reaction mixture containing 50ng genomic DNA (template), 200mM each of dATP, dTTP, dCTP, and dGTP, 0.5mM of each primer, 2.5 units of Taq DNA polymerase, and 2.0mM $MgCl_2$. Both paired normal and tumor DNA samples were amplified under the following reaction conditions: 94°C for 2min × 1 cycle, 94°C for 1min / 55°C for 30s / 72°C for 45s × 35 cycles, and 72°C for 5min × 1 cycle. The amplified fragment was electrophoresed in a 2% agarose gel to verify the reaction, and then subjected to a single-stranded conformational polymorphism (SSCP) study.

Non-radioisotopic SSCP analysis

After PCR, the solution (3μl) was mixed with same volume of loading buffer (99% deionized formamide, 20mM ethylenediamine tetraacetic acid [EDTA], 0.05% xylene cyanol FF, and 0.05% bromphenol blue), and the mixture was heated at 95°C for 5min and immediately cooled on ice. The whole volume of the mixture was applied to a GeneGel Excel 12.5/24 gel (Pharmacia Biotech, Uppsala, Sweden) and electrophoresed under 300 volts at 10°C on a GenePhor Electrophoresis Unit (Pharmacia Biotech) for 3h. The gels were stained by silver staining, using a PlusOne DNA Silver Staining Kit in a GeneStain Automated Gel Stainer (Pharmacia Biotech).

Direct sequencing

The PCR reaction products were purified by Qiagen quick purification columns (Qiagen, Clatsworth, CA, USA) to remove excess primers and deoxynucleosides. One μl of the solution was used for sequence reaction, using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). After purification using a Centri-Sep Spin Column, the sample was resuspended in 20μl of template suppression reagent (TSR) for sequence analysis in an Applied Biosystems Model 310 Sequencer (Applied Biosystems). Sequencing was performed on both strands of DNA of paired tumor and non-tumor specimens.

Restriction enzyme analysis

The −252 (G/A) and −205 (A/AA) polymorphisms in the proximal promoter of PSA create restriction sites for *Nsp*I and *Sfc*I, respectively. PCR amplicons obtained with the primer pair ARE 2 were incubated with *Nsp*I or *Sfc*I (New England, BioLabs, Beverly, MA, USA) according to the manufacturer's recommendations. An 8% polyacrylamide gel was used to separate and visualize the digested PCR products.

RNA extraction and reverse transcription (RT)-PCR

Total RNA was extracted from the 53 tumor tissue samples, using an RNeasy Kit (Qiagen). One μg of the RNA was reverse-transcribed in 20μl of reaction solution at 42°C, using the SuperScript Preamplification System (Life Technologies, Gaithersburg, MD, USA). Two μl of the transcript was subjected to the reverse transcription-polymerase chain reaction (RT-PCR). PSA2 (5′-GAG GTC CAC ACA CTG AAG TT-3′) and PSA 3 (5′-CCT CCT GAA GAA TCG ATT CCT-3′) oligonucleotide primers with high specificity were used for our RT-PCR amplification. PSA2 and PSA3 bind sequences that span intron III such that PCR amplification yielded a 360-base pair DNA and a 214-base pair RNA product. RT-PCR with β-actin primers (sense primer, 5′-GCT CGT CTG CGA CAA CGG CTC-3′ and antisense primer, 5′-CAA ACA TGA TCT GGG TCA TCT TCT C-3′) was used as an internal RNA control. PCR products were analyzed on 2% agarose gels.

Statistical analysis

Non-parametric analysis (Fisher's exact test, StatView 5; Abacus Concepts, Inc., Berkely, CA) was used for comparisons of PSA mRNA expression between the group of individuals possessing one or two alleles of the genotype and the group without that genotype. A *P* value of less than 0.05 was considered significant.

Results

Three types of SSCP pattern were found among the samples amplified with ARE 2 primer pairs (Fig. 1). By direct sequencing on both strands, we observed two polymorphic sites, at −252 (G/A) and −205 (A/AA); the numeric posi-

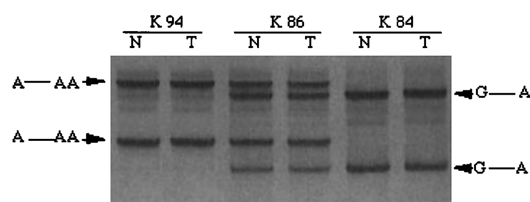

Fig. 1. Single-stranded conformational polymorphism (SSCP) analysis of androgen response element (ARE) 2 in the *PSA* gene promoter. Shown are three representative cases with different genotypes. *N*, Normal; *T*, tumor

Fig. 2. Restriction enzyme analysis of the −252 (by *Nsp*I, *left*) and −205 (by *Sfc*I, *right*) polymorphic sites. Shown are three representive samples, cases k94 (A/A—AA/AA), k86 (G/A—A/AA), and k84 (G/G—A/A)

tion is based on the published sequence (Genbank accession no. U37672). As predicted, by *Nsp*I or *Sfc*I treatment, the 271-bp PCR fragment with −252 A to −205 A was cleaved into two small fragments (by *Nsp*I, 172 and 99 bp; by *Sfc*I, 212 and 59 bp, respectively) (Fig. 2). Interestingly, we found that the −252 A polymorphic site was linked with the presence of −205 AA, and the −252 G was linked with the presence of −205 A. In the −252 to −205 genotype distributions, there were 29 G/A—A/AA heterozygotes, 4 A/A—AA/AA homozygotes, and 20 G/G—A/A homozygotes in the 53 breast cancer patients. By RT-PCR analysis, 21 of the 53 (40%) patients had PSA mRNA expression. Allelic frequencies and genotype distributions are presented in Table 1. The distribution of the A—AA (G/A—A/AA + A/A—AA/AA) and G—A (G/G—A/A) alleles and association with PSA expression are shown in Table 2. PSA mRNA expression was significantly more frequent in breast cancer patients carrying at least 1 A—AA alleles.

Table 1. Allelic frequencies and genotype distribution in breast cancers (n = 53)

Sites	Frequencies (%)
−252	
A	34.9
G	65.1
−205	
AA	34.9
A	65.1
−252 to −205	
A—AA	34.9
A—A	0
G—AA	0
G—A	65.1
Genotypes (−252 to −205)	
G/A—A/AA	54.7
A/A—AA/AA	7.5
G/G—A/A	37.8

Table 2. Association between PSA genotype and its mRNA expression

	Genotype		
PSA mRNA	A/A—AA/AA + G/A—A/AA	G/G—A/A	P
Positive	18	3	0.0082
Negative	15	17	

Discussion

By PCR-SSCP and direct sequencing analysis, we identified two novel polymorphic sites, at −252 (G/A) and −205 (A/AA), in the proximal promoter region of the *PSA* gene. Furthermore, we found that only the A—AA and G—A (−252 to −205) alleles were shown in breast cancer patients.

Subsequent analysis of PSA expression in breast cancers of all three genotypes revealed that the expression of this gene was markedly higher in tumors having the G/A—A/AA or A/A—AA/AA genotypes than in tumors with the homozygotic G—A genotype (G/G—A/A). This polymorphism may thus be biologically significant, and may be associated with increased transcriptional activity of *PSA*. The novel polymorphisms identified in our present study do not alter the known AREs of the *PSA* promoter region, but may affect previously unidentified sites. This will need to be confirmed using functional analysis in transient transfection studies. It has been suggested that the PSA repressed tumor growth and metastasis. Lai et al. (1996) have reported that PSA stimulates the conversion of the potent estradiol (E2) to the less potent estrone (E1), thereby inhibiting the growth of certain breast cancer cell lines in vitro. Antiangiogentic and antimetasatic properties have also been demonstrated recently (Balbay et al. 1999; Fortier et al. 1999). PSA proteolytically cleaves parathyroid hormone-related protein (PTHrP) (Iwamura et al. 1996), which is present in both seminal fluid and breast tissue. The cleavage of this protein by PSA may represent an inhibitory role in breast cancer progression, because PTHrP stimulates breast cancer cell proliferation in vivo and in vitro, and a role in breast cancer metastatis has been suggested because PTHrP is expressed in most osseous metastasis (Wulf et al. 1997). Furthermore, PSA may proteolytically generate growth inhibitory peptides from the BRCA 1 gene product (Bradley and Sharan, 1996). A clinical study should be undertaken in larger populations to investigate the correlation between the novel polymorphisms identified in our present study and the clinical behavior, particularly the prognosis, of individual breast cancers.

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