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## Original Paper

# Frequent Detection of Mutations in the 5′ Flanking Region of the Prostate-specific Antigen Gene in Female Breast Cancer

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**Prostate-specific antigen (PSA) is expressed in normal, hyperplastic and cancerous female breast tissue. Expression is regulated by steroid hormones. Some breast tumours produce very high levels of PSA, while others do not express any PSA. In this study, we selected three primary breast tumours which overexpressed PSA (PSA protein concentration in tumour cytosols > 4300 ng/l) and three tumours which were negative for PSA (< 1 ng/l). We extracted DNA and sequenced all five exons of the *PSA* gene. No mutations were found in the PSA coding sequence in any of the tumours. We identified only two polymorphic sites in exon 2. We also sequenced parts of the 5′ flanking region of the *PSA* gene in five tumours. All tumour DNAs contained abnormalities which consisted of point mutations and deletions of 1–7 base pairs. Except for one tumour which had only a 3 base pair deletion, all other tumours had multiple abnormalities (up to seven in one tumour). The deletions occurred adjacent to direct repeats similarly to deletions seen in the *p53* gene. Our data suggest that the coding sequence of *PSA* is not mutated in breast cancer. However, the 1.4 kb 5′-flanking region was mutated in all five tumours tested. The importance of this observation in relation to *PSA* gene regulation and breast cancer pathobiology remain to be determined.** © 1997 Elsevier Science Ltd.

**Key words: prostate-specific antigen, *PSA* gene mutations, breast cancer, steroid hormone-regulated genes, *PSA* gene regulation in breast cancer**
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## INTRODUCTION

THE GROWTH and differentiation of the mammary gland is a result of complex interactions of a number of endocrine hormones which act alone and in combination. The development and progression of breast cancer is also strongly influenced by hormonal status, yet the molecular mechanisms underlying the complex patterns of multihormone regulation of mammary epithelium are not well understood. This understanding can be greatly facilitated by analysing a suitable multihormone regulated gene.

The *PSA* gene codes for a serine protease which is a member of the kallikrein gene family, KLK3 [1,2]. It 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 $\alpha_1$-antichymotrypsin and as an 800 kDa complex with $\alpha_2$-macroglobulin [3]. Prostate-specific antigen (PSA) has been known as an important clinical marker in prostate disease for many years [4,5]; however, a growing body of literature has described a number of non-prostatic sources of PSA [6–9]. PSA was originally described as expressed in the epithelial lining of the ducts of the prostate gland [10] and has recently been found in ovarian, breast, colon, liver, kidney, adrenal and parotid tumours, as well as many of the cell lines corresponding to these tumours [11,12]. Approximately 30% of breast cancer cytosolic extracts have measurable PSA levels and PSA has been associated with hormone-responsive tumours and early stage disease. Finally, a number of human breast cancer cell lines have been described to express this glycoprotein in a hormone-dependent manner [13,14].

In prostate cancer, high PSA levels are associated with a poor prognosis and disease relapse [3,10] but PSA has been considered a favourable prognostic indicator for breast cancer [15]. In some breast cancer patients, a high level of PSA has been found in the serum, while in other patients there is no

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*Table 1. PCR and sequencing primers used in this study**

| | 5′ primer | 3′ primer |
|---|---|---|
| PCR primers | | |
| 5′ Flanking region (SE) | TGATTTTGCATGCCACC | GAATTCCAATAACCCCC |
| 5′ Flanking region (EH) | GGGAATTCCACATTGTTTGCTGCTGCA | GTGCAGGTGGTAAGCTTGG |
| Exon 1 | ATGGGGAGGGCCTTGGTCAG | TGTCTGGGCTGGGGTGCTG |
| Exon 2 | CCCCCGTGTCTTTTCAAACC | GCCTCCCCATGTGACCTGA |
| Exon 3 | CCCAACCCTGTGTTTTTCTC | GGCCCTCCTCCCTCAGA |
| Exon 4 | GGAGGAGGGGACAGGACTC | TCTAGACCCCAGCCCAGAAT |
| Exon 5 | GTCGGCTCTGGAGACATTTC | AACTGGGGAGGCTTGAGTC |
| Sequencing primers† | | |
| Exon 1 | GAGTCCTGGGGAATGAA | GAAAGAGCCTCAGCTTG |
| Exon 2 | TCCATCTCCTATCCGAG | CAGAACTTTCCCTCTCT |
| Exon 3 | TCTCTTTTGGAGCCTCC | AGGAGTCCTGTCCCCTC |
| Exon 4 | TGAGGGAGGAGGCCCAA | TTAAGGTCCCCACTCAC |
| Exon 5 | TCCAAAGCTGGGAACTG | GGCCTGGTCATTTCCAA |

*All presented in the 5′–3′ direction. †Labelled with Cy5 at the 5′-end.

PSA expression. In order to understand the variable levels of PSA in human breast disease, we decided to characterise the *PSA* gene in several breast tumours at the molecular level.

*PSA* gene regulation has been studied extensively in the LNCaP prostate carcinoma cell line and has been found to be positively regulated by a number of steroid hormones, such as R5020, oestradiol and epitestosterone [16]. However, the androgen receptor in this cell line has been found to have a point mutation which leads to a relaxed affinity for steroid hormones and as such, the LNCaP cell line may not be the ideal cell model with which to study the regulation of this gene. We have discovered more appropriate cell lines with which to study the tissue-specific expression of PSA in breast cancer. In the human breast cancer cell lines T-47D and BT-474, progestins and androgens are potent inducers of PSA expression. Also, oestradiol has been observed to inhibit the androgen-stimulated expression of PSA [14]. These steroidal effects on PSA expression are inversely correlated with their effects on cell growth. Oestradiol is a potent mitogen in these hormone-responsive human breast cancer cells, whereas androgens and to a lesser extent, progestins (in the presence of oestradiol) and glucocorticoids, cause an inhibition of cell growth [17]. *PSA* gene expression, therefore, could be used as a marker for growth inhibitory and differentiating agents. Recently, Lai and associates postulated that PSA acts as a negative growth regulator by stimulating the conversion of oestradiol to oestrone [18].

In this study, we sequenced the *PSA* gene of several breast tumours, some which were PSA-negative and some which expressed PSA highly, to determine if a mutation in the *PSA* gene could be responsible for the variable levels of PSA. In addition, we sequenced 1.4 kb pairs of the 5′ flanking region of the *PSA* gene in these tumours to see if any mutations or rearrangements occurred in the promoter region which might affect the regulation of PSA.

*Table 2. PSA type and PSA concentration in tumour extract*

| Tumour ID | PSA type* | PSA ng/l |
|---|---|---|
| 3785-5 | PSA1 | 26000 |
| 3790-1 | PSA1 | 6200 |
| 3797-2 | PSA1 | < 1 |
| 3797-11 | PSA1 | < 1 |
| 3641-1 | PSA2 | 4300 |
| 3799-10 | PSA2 | < 1 |

*Please see Figure 1.

## MATERIALS AND METHODS

### *Breast tumours*

We previously screened 200 breast tumour cytosolic extracts for PSA protein levels [19], and from these selected three tumours with a PSA level < 1 ng/l (PSA negative) and three tumours with the highest PSA concentration i.e. 4300, 6200 and 23 000 ng/l. Genomic DNA was isolated from these six tumours according to the standard proteinase K digestion and phenol:chloroform extraction procedure [20].

### *Sequencing*

The exons of the *PSA* gene were amplified by polymerase chain reaction and sequenced directly, following digestion with shrimp alkaline phosphatase and exonuclease I to remove excess primers and deoxynucleosides. The PCR primers are described in Table 1. The sequencing primers were obtained with Cy5 fluorescent dye labelled at the 5′ end and were used to sequence the exons with the Thermo Sequenase kit (Amersham International, U.K.). The sequencing primers are also described below. Sequencing reactions were resolved with the ALF Express Automatic Sequencer (Pharmacia Biotech, Sweden). Sequences were verified by sequencing both strands at least twice.

### *PCR protocols*

PCR (polymerase chain reaction) was performed in 0.2 ml thin-walled MicroAmp reaction tubes on a Perkin-Elmer (Palo Alto, California, U.S.A.) Gene Amp 2400 System. Total volume was 50 µl. The reaction mixture contained PCR buffer (50 mmol/l KCl, 10 mmol/l Tris buffer, pH 8.3,

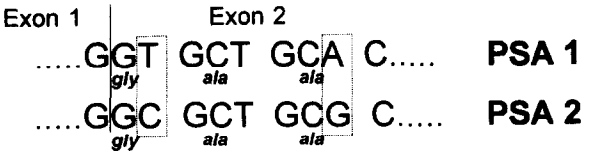


**Figure 1. Polymorphism in the *PSA* gene sequence. PSA 1 was found in four tumour DNAs and PSA 2 in two tumour DNAs. PSA 1 corresponds to the sequence published by Schulz and associates [22] and PSA 2 corresponds to the sequence published by Lundwall [21].**

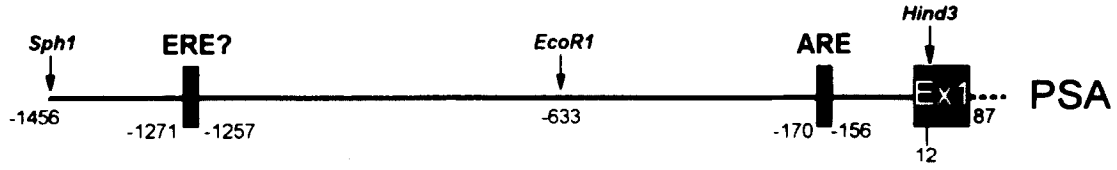


**Figure 2. 5′-flanking region of the *PSA* gene. Numbers refer to nucleotides; the first nucleotide in the coding sequence is considered as 1. SphI, EcoRI and HindIII are restriction sites; ERE, oestrogen response element; ARE, androgen response element; Ex1, exon 1. For more details see text.**

5′---TTT GCT **GC**---3′ **Deletion A**

5′---TGGCTC **CTAAAGG** CTAAAGG **CTAAA**---3′ **Deletion B**

**Figure 3. Deletions in the 5′-flanking region of the *PSA* gene. Deletion A was identified in 4/5 tumours; deletion B in 3/5 tumours. Underlined are sequences deleted; bold are adjacent repeated sequences.**

1.5 mmol/l $MgCl_2$, 10 mg/l gelatin), 200 μmol/l of dNTPs, 1 μmol/l of PCR primers, 2 U of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, Indiana, U.S.A.) and 5 μl of target DNA (added last). The PCR was performed with 1 cycle at 94°C for 5 min; 30 cycles with denaturation at 94°C for 30 s, annealing for 30 s and extension at 72°C for 30 s; and 1 cycle at 72°C for 7 min. The annealing temperature was 70°C for PSA exons 1, 2 and 4, 68°C for exons 3 and 5 and 65°C for the 5′ flanking regions.

*Constructs*

The PSA 5′ flanking region constructs were made by PCR of genomic DNA isolated from the six breast tumours, described above. Two separate fragments were amplified, from a *Sph*I restriction site at position −1456 to an *Eco*RI restriction site at position −633 (fragment SE) and from the *Eco*RI site to a *Hind*III site at position +12 (fragment EH). The PCR primers are described in Table 1. The two fragments were subcloned separately into the corresponding restriction sites in the multiple cloning region of the plasmid vector, pVZ, and sequenced using T3 and T7 primers.

## RESULTS

In order to examine the possibility that PSA was not expressed in the three PSA-negative tumours due to a gene defect, we amplified and sequenced the five PSA exons of the six tumours we analysed. We then compared the derived sequence to two published sequences found in Genbank [21, 22]. Except for a polymorphism in exon 2, which did not affect protein translation, the coding regions of the six tumours were identical (Table 2 and Figure 1). Two of the six tumour sequences were in agreement with the Lundwall exon 2 sequence [21] and the remaining four tumour sequences were in agreement with the Schulz and associates exon 2 sequence [22]. Additionally, we amplified and sequenced various 5′-flanking regions (Figure 2) of the breast tumours, as shown in Table 3. We identified a number of alterations based on comparisons of our data with published sequences of the 5′ flanking regions of the *PSA* gene [21, 23]. The alterations included base substitutions and deletions ranging from one to seven bases (Table 3 and Figure 3).

## DISCUSSION

Since the discovery that PSA is not expressed exclusively in the prostate, as previously believed, and the important observation that PSA may have some clinical implications in the prognosis of breast cancer, it has become crucial to elucidate the mechanism by which *PSA* gene expression is regulated in a tissue-specific manner. In order to address this question, we first studied the sequence of the PSA coding region of several breast tumours with varying levels of PSA expression from very high to none. This sequencing was done to determine if any defects in the coding region of the mRNA might be responsible for the lack of PSA expression in PSA-negative tumours. Aside from a polymorphic area in exon 2 (two polymorphic sites) which did not affect translation of the PSA protein, we did not find any differences between the tumours which expressed PSA highly and the tumours which were negative for PSA (Table 2 and Figure 1).

We also sequenced the 5′ flanking region of the *PSA* gene in these tumours to determine if there were any obvious differences or consensus between the two subgroups of breast tumours. Although we found several point mutations and deletions in the 5′ flanking regions of the *PSA* gene in these tumours, we did not find any consensual changes between the two subgroups of tumours (Table 3). In addition, none of

*Table 3. Mutations in the 5′-flanking region of the* PSA *gene in breast tumours*

| Tumour ID | Region sequenced* | Position of mutation† | Type of mutation |
|---|---|---|---|
| 3641-1 | −633→12 | −285 | G→A |
| | | −355 | C→T |
| | | −614→−615 | GCT deletion |
| 3797-2 | −633→12 | −614→615 | GCT deletion |
| 3799-10 | −1456→−633 | −766→−733 | GTAAAGG deletion |
| | | −1248→−1249 | CC→T |
| 3790-1 | −1456→12 | −205→−206 | A deletion |
| | | −252 | A→G |
| | | −614→−615 | GCT deletion |
| | | −668 | T→C |
| | | −766→−773 | CTAAAGG deletion |
| | | −906 | T→C |
| | | −1090 | C→T |
| 3797-11 | −1456→12 | −252 | A→G |
| | | −614→−615 | GCT deletion |
| | | −668 | T→C |
| | | −766→−773 | CTAAAGG |
| | | −787 | deletion |
| | | | C→T |

*Please refer to Figure 2 for definitions. †As compared to published sequences [21, 23].

these changes occurred within the characterised promoter area [22] or the characterised androgen response elements ARE I and ARE II [21, 24]. One interesting observation was that in the two deletions we discovered, the pattern of deletion followed a direct repeat. At position −614 in four of the tumours, a GCT was deleted just prior to a GC. At position −766 in three sequenced tumours, a CTAAAGG was deleted between CTAAAGG and CTAAA (Figure 3). This phenomenon has been previously observed in the *p53* gene in human cancers [25, 26] and a mechanism to account for it has been proposed. It is believed that mutations involving deletions or insertions are caused by a slippage mechanism during DNA replication [27].

In conclusion, we have provided evidence that the five exons of the *PSA* gene are not mutated in breast cancer. We found only one polymorphism in exon 2 which does not affect the PSA protein sequence. However, we found multiple mutations in the 5′-flanking region of the *PSA* gene in all tumours sequenced. The roles of these mutations in *PSA* gene regulation and in breast cancer are currently under investigation.


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