Contribution of allelic variability in prostate specific antigen (PSA) & androgen receptor (AR) genes to serum PSA levels in men with prostate cancer

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Background & objectives: Wide variability in serum prostate specific antigen (PSA) levels exists in malignant conditions of the prostate. PSA is expressed in normal range in 20 to 25 per cent of prostate cancer cases even in presence of high grade Gleason score. This study was aimed to assess the influence of genetic variants exhibited by PSA and androgen receptor (AR) genes towards the variable expression of PSA in prostate cancer.

Methods: Pre-treatment serum PSA levels from 101 prostate cancer cases were retrieved from medical record. PSA genotype analysis in promoter region and AR gene microsatellite Cytosine/Adenine/Guanine (CAG) repeat analysis in exon 1 region was performed using DNA sequencing and fragment analysis techniques.

Results: A total of seven single nucleotide polymorphisms (SNPs) in the PSA promoter region were noted. Only two SNPs viz., 158G/A (P<0.001) in the proximal promoter region and -3845G/A (P<0.001) in enhancer region showed significant association with serum PSA levels. The carriers of homozygous GG genotype (P<0.001) at both of these polymorphic sites showed higher expression of PSA whereas homozygous AA genotype (P<0.001) carriers demonstrated lower PSA levels. The combination effect of PSA genotypes along with stratified AR CAG repeats lengths (long, intermediate and short) was also studied. The homozygous GG genotype along with AR long CAG repeats and homozygous AA genotype along with AR short CAG repeats at position -3845 and -158 showed strong interaction and thus influenced serum PSA levels.

Interpretation & conclusions: The genetic variants exhibited by PSA gene at positions -3845G/A and -158G/A may be accountable towards wide variability of serum PSA levels in prostate cancer. Also the preferential binding of G and A alleles at these polymorphic sites along with AR long and short CAG repeats may contribute towards PSA expression.

Key words Androgen receptor - polymorphism - prostate cancer - prostate specific antigen - SNP
Prostate specific antigen (PSA) is one of the most widely used markers for screening of prostate cancer and for assessment of patients’ response to therapeutic interventions. Many clinicians consider serum PSA levels above 4 ng/ml as an indicator of prostate cancer. It is recommended that men with PSA levels beyond this threshold should undergo prostatic needle biopsy for further confirmation of malignancy. However, there are certain limitations associated with the PSA testing. It is known that 20 to 25 per cent of prostate cancer cases show low PSA levels even in presence of high grade prostate cancer. This false negativity leads to misdiagnosis or delayed diagnosis of prostate cancer. Similarly, higher PSA values seen due to conditions of the prostate besides cancer, result into unnecessary biopsies. Thus it was recommended that an extended diagnostic gray zone of PSA up to 20 ng/ml be applied for accurate diagnosis of cancer.

Many supplementary tests such as free/total PSA ratio, PSA velocity, PSA density, etc. have been recommended to improve diagnostic performance of PSA but with limited utility. Using genotype specific cut-off values of PSA for screening prostate cancer has also been suggested.

Prostate specific antigen gene contains a 6 kb promoter region with proximal and distal enhancer regions which contribute equally towards its expression. PSA gene expression is largely under the androgenic control via the action of androgen receptor (AR). Androgen activates the AR, which in turn binds to androgen responsive element (ARE) in the promoter region of PSA gene triggering the expression of PSA protein. In PSA gene three AREs are noted, viz. ARE I, ARE II and ARE III at positions -170, -394 and -4200 bp, respectively from the transcriptional start site. The single nucleotide polymorphisms (SNPs) present in these regions of the PSA gene may interact with the androgen receptor (transcription factor) with varying affinities resulting in either low or high expression of PSA protein. In AR gene, the CAG (Cytosine/Adenine/Guanine) microsatellite repeat length in the exon 1 region that encodes for variable-length glutamine repeats in the N-terminal domain of the AR protein has been found to associate with the transcriptional activity of AR protein. Shorter the CAG (cytosine/Adenine/Guanine) repeat length, greater is the transcriptional activity of AR protein. With this view, we aimed to study the genetic variants exhibited by PSA and AR genes and their contribution to variability of serum PSA levels in prostate cancer.

**Material & Methods**

This study was conducted over a period of five years from April 2007 to December 2011 at the Lokmany Tilak Municipal Medical College (LTMMC), Mumbai in collaboration with the National Institute for Research in Reproductive Health (NIRRH), Mumbai, and Bhabha Atomic Research Centre (BARC) Hospital, Mumbai, Maharashtra, India. Enrolment of patients was done at the department of Urology, LTMMC and department of Surgery, BARC Hospital. DNA extraction, quality, quantity and integrity analysis of DNA and estimation of serum PSA by enzyme linked immunosorbent assay was done at the department of Biochemistry, LTMMC, Mumbai. DNA sequencing and DNA fragment analysis were done at NIRRH.

According to National Cancer Registries at Mumbai, Ahmedabad, Chennai, and Bangalore, prostate cancer was the fourth, ninth, and fifth most common cancer, respectively, amongst males, highlighting the fact that prostate cancer is not a major health problem in India. A total of 101 histologically proven cases of prostate cancer were included in this study. The patients selection was both prospective as well as retrospective. Of these, 62 cases were selected retrospectively. The patients were selected prospectively when they came to the out patient department of LTMM and BARC hospitals for check up. The detailed clinical findings of prostate cancer cases such as age, digital rectal examination, pre-treatment PSA levels detected by enzyme linked immunosorbent assay, Gleason score and clinical stage of prostate cancer determined according to the Tumor- Node- Metastasis (TNM) system were noted from the medical records. The research protocol was ethically approved by the Institutional Review Board (IRB) of Lokmany Tilak Municipal Medical College and BARC hospital. A written informed consent was obtained from all subjects to ensure voluntary participation in the study. From each patient 2 ml of blood in EDTA bulb was collected for DNA extraction by QIAamp DNA mini kit (Qiagen Inc, Germantown, MD).

**PSA genotyping**: DNA sequencing of entire androgen responsive region of PSA gene was performed. A 813bp of proximal promoter region encompassing ARE I and II and 651bp of enhancer region encompassing ARE III were amplified by PCR and further sequenced. PCR amplification was performed using the commercially available KAPA HiFi PCR kit (KAPA Biosystems, Inc., USA). This kit contained a novel DNA polymerase,
engineered for fast and versatile high-fidelity PCR. In addition, reaction time for this kit was significantly shorter than those required for wild-type DNA polymerases.

Genomic DNA (50 ng) was used in a 50 µl of reaction with the components as per KAPA HiFi PCR kit instruction. The sequence of the forward and reverse primers specifically designed for this study (Sigma-Aldrich Pvt. Ltd., Bangalore, India) were 5’ TGGATTTTGCATGAGGATGA 3’ and 5’ GGACAGGGTGAGGAAAGACAA3’ for 813 bp of ARE I and II region and 5’ GTGAAATGCTGGCAGAGTCCA 3’ and 5’ TCGATCGGGACCTAGAACCTT 3’ for 651 bp of ARE III region. The PCR reaction conditions were: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 98°C for 20 sec, annealing at 62°C for ARE I and II and 63.5°C for ARE III both for 15 sec followed by extension at 68°C for 30 sec. Final extension was performed at 68°C for 30 sec. The amplified PCR product was further purified using Gel Extraction Kit (Sigma-Aldrich, USA) and used for further DNA sequencing.

**DNA sequencing:** The DNA sequencing reaction was performed using Big Dye Termination reaction kit (Applied Biosystems, USA). In each 10 µl of reaction mixture, 150-200 ng of purified PCR product with Ready reaction premix 1 µl, Big Dye sequencing buffer (5x) 1.5 µl, primer (3.2 pmol/µl) 1 µl was added. PCR cycling conditions were: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 10 sec, annealing at 50°C for 5 sec and extension at 60°C for 4 min. The amplified PCR product was purified using Ethanol/EDTA/sodium acetate precipitation method (Big Dye V 3.1 Chemistry, Applied Biosystems, Foster City, USA). Further, the purified PCR product was denatured at 95°C in 10 µl of formamide. The samples were then loaded on ABI 3130XL genetic analyzer (Applied Biosystems, USA) with POP-7 polymer. Analysis of DNA sequence was done using Sequence analysis 5.0 software from Applied Biosystems. The following SNPs in the PSA gene with minor allele frequency >5 per cent were noted viz: -158G/A, -205D/I, -252G/A in ARE I and II; -4330 polyC, -4216C/A, -3845G/A, -3774G/A in ARE III region.

**AR CAG repeats:** Fluorescently (6FAM) labelled primers were used for PCR amplification of ~ 290 bp fragment in AR gene containing varying CAG repeats16. The PCR amplification was performed in 50 µl reaction volume containing 40 ng of genomic DNA, 10 pmol/µl of primers (Forward 5’- 6FAM TCC AGA ATC TGT TCC AGA GCG TGC 3’ and Reverse 5’ GCT GTG AAG GTT GCT GTT CCT CAT 3’) (Applied Biosystems, Foster City, USA)16 with 10x PCR buffer (Fermentas Canada, Inc.), 1.5 Mm MgCl2, 3 U Taq polymerase (Fermentas Canada, Inc.) and dNTP (Fermentas, Canada Inc.). PCR conditions were: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 59°C for 1 min and extension at 72°C for 1 min, final elongation was at 72°C for 10 min. 1 µl of amplified product was mixed with 10 µl of formamide containing 0.3 µl of Genescan Liz 500 size standard (Applied Biosystems, USA) and denatured at 95°C for 5 min. The denatured PCR product was run on Avant 3100 genetic analyzer (Applied Biosystems, USA) containing POP4 polymer for determining the size of PCR product using Genescan software. During each run an internal control sample of known AR CAG repeat number was electrophoresed to determine the number of repeats in unknown samples.

**Statistical analysis:** The PSA genotype frequency distribution for each SNP was determined using online SNPstats software (http://bioinfo.iconcologia.net/SNPstats). Normality test for serum PSA data was performed using 1-sample KS test. As the PSA data assumed non normal distribution, log transformation of PSA data was performed using transform computing variable programme from SPSS 16 software (SPSS Inc., Chicago, IL, USA). Log- transformed PSA data were expressed as mean ± SE. Univariate analysis from SPSS 16.0 was used to study the association of each PSA SNP with serum PSA levels. Levene’s test of homogeneity of variance was performed for PSA levels across genotypes of each SNP. Multiple pairwise comparison of serum PSA levels between the genotypes in each SNP was performed after adjusting the confidence interval by Bonferroni. Generalized linear model was used for studying the interaction between the SNPs in the PSA gene and AR CAG repeats. P<0.05 was considered significant.

**Results**

Mean age at diagnosis was 68.1 ± 7.1 yr with digital rectal examination revealing positive for 87 of 101 diagnosed prostate cancer cases. In our study, 54 (53%) cases of prostate cancer presented with high-grade prostate cancer (Gleason score >7) and 47 (47%) cases with low grade prostate cancer (Gleason score ≤7).
Wide variability in PSA levels was observed in prostate cancer cases. It ranged from 0.15 to 1098 ng/ml with mean ± SD of 88.3 ± 159 ng/ml. High standard deviation was noted in PSA levels because 20 per cent of cases presented had PSA levels less than 20 ng/ml. The values for serum PSA were log-transformed.

**PSA genotype and serum PSA:** Log - transformed serum PSA levels in the prostate cancer group in relation to the PSA genotypes are presented in Table I. The univariate analysis of variance performed for each SNP showed a significant difference in serum PSA levels only for SNP at position -3845G/A (**P**<0.001) and -158G/A (*P*<0.003). All other SNPs in the PSA gene did not show difference in the serum PSA levels.

Further, to assess the effect of each genotype arising from SNPs on serum PSA levels, multiple pair-wise comparison analysis was done. The estimated mean difference in serum PSA levels for each genotype was compared after adjusting the confidence interval by Bonferroni. This demonstrated significant difference for the genotypes at position -3845 and -158. The homozygous AA genotype carriers (**P**<0.001) at both the positions showed significantly lower PSA levels as compared to homozygous GG genotype carriers whereas heterozygous genotypes demonstrated intermediate serum PSA levels (Table I).

**Combined effect of variants in PSA & AR gene on the serum PSA levels:** To understand the contribution of AR CAG repeats along with PSA gene variants on serum PSA levels, stratification of AR CAG repeat lengths into short, intermediate and long was done. This grouping in the CAG repeat lengths was obtained by stratifying CAG repeats length into three equal percentile groups (33.3%) using binning method from SPSS 16.0 software package. The CAG repeat length of ≤ 21 was defined as short, 22-24 as intermediate and ≥25 as long.

**Interaction of AR CAG repeats with each SNPs in PSA gene:** The interaction of AR CAG repeats with each of the seven SNPs in the PSA gene is presented in Table II. It was observed that only two SNPs viz., -158G/A and -3845G/A interacted strongly with AR CAG repeats, thus significantly influencing serum PSA levels (**P**<0.02 and *P*<0.001, respectively).

| SNP ID      | Genotype | n (%) | Log_{10}PSA (ng/ml) | P value |
|-------------|----------|-------|---------------------|---------|
|             | G/G      |       | Mean | SE |       |       |
| -4330polyC  | 8C/8C    | 36 (36)| 1.421 | .120 | Ref |
|             | 8C/9C    | 50 (50)| 1.547 | .104 | 1.00 |
|             | 9C/9C    | 15 (15)| 1.951 | .193 | 0.06 |
| -4216C/A    | C/C      | 74 (73)| 1.561 | .086 | Ref |
|             | C/A      | 26 (26)| 1.489 | .157 | 0.73 |
|             | A/A      | 1 (1) | 2.299 | .675 | 0.84 |
| -3845G/A    | G/G      | 68 (67)| 1.689 | .077 | Ref |
|             | G/A      | 28 (28)| 1.519 | .116 | 0.67 |
|             | A/A*     | 5 (5) | -1.196 | .320 | 0.001 |
| -3774G/A    | G/G      | 23 (23)| 1.454 | .171 | Ref |
|             | G/A      | 55 (54)| 1.623 | .097 | 1.00 |
|             | A/A      | 23 (23)| 1.484 | .161 | 1.00 |
| -252G/A     | G/G      | 66 (65)| 1.491 | .095 | Ref |
|             | G/A      | 35 (35)| 1.654 | .120 | 0.28 |
| -205D/A     | D/D      | 66 (65)| 1.491 | .095 | Ref |
|             | D/A      | 35 (35)| 1.654 | .120 | 0.28 |
|             | G/G      | 35 (35)| 1.791 | .116 | Ref |
| -158G/A    | G/A      | 53 (52)| 1.518 | .090 | 0.2 |
|             | A/A**    | 13 (13)| .369 | .323 | 0.001 |

SNP, single nucleotide polymorphism; Ref, Reference

Univariate analysis of variance: *P*<0.001; **P**<0.003

Multiple pair-wise comparison analysis: *P*<0.001; **P**<0.001
In both the SNPs, the homozygous G/G genotype carriers showed higher expression of PSA in presence of long CAG repeats whereas the homozygous A/A genotype along with short CAG repeats showed higher expression of PSA levels. This ascertains that short CAG repeats has a high binding affinity towards A allele whereas long CAG repeats has a high binding affinity towards G allele, thus leading to higher expression of PSA levels for both the alleles. However, it was also observed that the PSA levels of G allele carriers were several times higher compared to that of A allele carriers.

**Effect of both -158G/A + -3845G/A + AR CAG repeats on PSA levels:** The combination effect of genotypes of the two SNPs along with stratified AR CAG repeats was observed on serum PSA levels using univariate analysis of variance (Table III). With increased number of G allele copies at -3845 and -158 along with long CAG repeats, there was a corresponding increase in the serum PSA levels. Similarly, A allele copies along with long CAG repeats at both the position demonstrated comparatively lower PSA levels ($P < 0.001$). This showed that the presence of G allele at either of the ARE I or ARE III region along with long CAG repeats was essential for the higher expression of PSA in prostate cancer.

### Discussion

Our study focused on analyzing the PSA and AR genotype - phenotype association in prostate cancer. The knowledge of this may aid substantially in refining the diagnostic efficacy of PSA testing. Previous studies have reported the association of SNP at position -158G/A with serum PSA levels\(^9,17-19\). However, many contradictory findings have also been reported associated with this SNP\(^20-23\). The dominance of -158A or -158G allele in influencing the serum PSA levels is still contentious\(^22\).

In this study two SNPs viz. 158G/A (rs266882) in ARE I and -3845G/A (rs266864) in ARE III of PSA gene showed association with serum PSA levels. Further, these two SNPs (-158G/A and -3845G/A) of PSA gene carrying homozygous G allele showed strong association with higher expression of PSA whereas homozygous A allele carriers demonstrated low PSA levels. Genetic variant at position -158G/A is one of the most studied polymorphisms in the PSA gene for its association with serum PSA levels. Xue *et al.*\(^17\) demonstrated higher PSA levels for normal healthy men carrying PSA -158AA genotype along with short AR CAG repeats. In contrast to this finding, Xu *et al.*\(^9\) demonstrated lower PSA levels...
for men carrying -158AA genotype pattern. Further study by Medeiros et al\textsuperscript{18}, in prostate cancer patients demonstrated strong association between -158AA genotype pattern and higher pre-treatment serum PSA. dos Santos et al\textsuperscript{19} showed association between -158AA genotype and lower pre-treatment serum PSA levels in prostate cancer. Other studies\textsuperscript{20-22} did not demonstrate any association of -158G/A genotype with serum PSA levels in both normal healthy controls and prostate cancer patients.

Apart from SNPs in the androgen responsive regions, SNPs in the distal enhancer region (-4643 A/G, -5412T/C and -5429T/G) have been reported. Cramer et al\textsuperscript{24} demonstrated significant association of -4643 A/G, -5412T/C and -5429T/G with serum PSA levels. They also showed significantly higher PSA levels for carriers of G allele at -4643, C allele at -5412 and G allele at -5429 in PSA gene. In our previous study on effects of SNPs in distal enhancer region in prostate cancer cases, we found no significant difference in PSA levels corresponding to these alleles\textsuperscript{25}. A study conducted in African American men also revealed no significant association of these SNPs with serum PSA levels\textsuperscript{26}.

This is perhaps the first study to demonstrate the collective influence of variants in the PSA gene along with AR CAG repeats lengths on the serum PSA levels in prostate cancer. Overall, our results demonstrate that variability exists in the PSA expression pattern in prostate cancer with respect to both the allelic variants at positions -158G/A and -3845G/A along with polymorphic AR CAG repeats. The low PSA levels

### Table III. Combined effect of SNPs at positions -158G/A and -3845G/A on serum PSA levels in prostate cancer

| -3845G/A (ARE III) | -158G/A (ARE I) | AR CAG repeats | Serum PSA level (ng/ml) Mean ± SD |
|--------------------|----------------|----------------|----------------------------------|
| A/A \*             | A/A            | Long           | NCA                              |
|                    |                | Intermediate   | NCA                              |
|                    |                | Short          | NCA                              |
| G/A                | Long           | NCA            |
|                    | Intermediate   | -0.27±0.38     |
|                    | Short          | 0.59±0.38      |
| G/G                | Long           | NCA            |
|                    | Intermediate   | 0.27±0.54      |
|                    | Short          | 0.55±0.54      |
| G/A                | A/A            | Long           | -0.55±0.54                       |
|                    | Intermediate   | 1.01±0.38      |
|                    | Short          | 1.10±0.19      |
| G/A                | Long           | 0.93±0.38      |
|                    | Intermediate   | 1.54±0.19      |
|                    | Short          | 1.87±0.2       |
| G/G                | Long           | NCA            |
|                    | Intermediate   | 1.85±0.2       |
|                    | Short          | 2.39±0.54      |
| G/G                | A/A            | Long           | NCA                              |
|                    | Intermediate   | 1.23±0.38      |
|                    | Short          | 1.47±0.19      |
| A/G                | Long           | 1.69±0.18      |
|                    | Intermediate   | 1.62±0.15      |
|                    | Short          | 1.65±0.16      |
| G/G                | Long           | 2.22±0.27      |
|                    | Intermediate   | 1.51±0.55      |
|                    | Short          | 1.56±0.18      |

NCA, no cases available (with this combination); \*P<0.001; univariate analysis of variance
demonstrated by the A allele at both the polymorphic sites may be the factor leading to the false negative findings in prostate cancer. Thus the knowledge of \( PSA \) genotype pattern may help in predetermining the PSA levels in prostate cancer and thus the diagnostic sensitivity of PSA test could be improved. However, \( PSA \) genotype analysis is not required in all patients suspected to have prostate cancer but patients with clinically suspicious lower PSA levels only may be PSA genotyped. The combination study of PSA A allele along with short CAG repeats and G allele with long CAG repeats resulted in corresponding increase in PSA for both the alleles. Further studies are required to determine the genotype specific PSA range with a large number of patients.

In conclusion, genetic variants in \( PSA \) and \( AR \) genes at positions -3845 G/A and -158 G/A may influence the serum PSA levels and thus may also impact the risk and severity of prostate cancer.

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