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Analysis of AR, PSA (KLK) and ER-β Genetic Variants and Benign Prostate Hyperplasia (BPH) Pathogenesis in Indian Population

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Benign Prostate Hyperplasia (BPH) pathogenesis exhibitsinter-individual variation in the genome as polymorphisms in the steroid hormone genes AR, PSA (KLK) and ER-β with profound effects in altering BPH disease progression rate. Single nucleotide polymorphisms (SNPs) designated 1754 A/G exon-1 in AR, Promotor-158 A/G in PSA(KLK) and 1730 A/G 3′UTR in Exon-8 in ER-β have been associated with BPH pathogenesis. In the current study, AR-1754 A/G exon-1, PSA-ARE1 Promotor-158 A/G and 1730 A/G 3′UTR in Exon-8 in ER-β were analysed in Indian population.The polymorphisms in BPH patients and healthy individuals were evaluated by PCR, RFLP–PCR and genotype–phenotype correlation. In the study AR and ER-β SNPs demonstrated significant association [55.7% (OR 3.0 (95% CI 1.67–5.46) (*p* 0.0002)] and [52.6 % (OR 6.5, 95% CI 3.27–12.74) (*p* 0.0001)] with BPH pathogenesis in patients as compared to control. With both the polymorphisms indicating a trend towards an association of the G allele with an increased risk of BPH pathogenesis. The A/G genotype frequency of PSA was 54 % in patients and was not associated with BPH pathogenesis. Further genotype–phenotype correlation study has provided evidence that gene–gene interactions play an important role in the etiology of BPH. Although susceptibility to pathogenesis cannot be dependent on a single or small number of genetic variants, it is noteworthy that AR, PSA and ER-β variants have been correlated globally with BPH pathogenesis. Hence, the higher frequency of AR and ER-β variants in the Indian population may be critical in BPH pathogenesis.

INTRODUCTION

The prostate gland is a hormonally regulated organ with growth accelerated at sexual maturity due to the action of androgen on both stromal and epithelial cells. In men over the age of 40–50 years, prostate gland may often show benign prostate hyperplasia (BPH) and convert to prostate cancer (PCa). Steroid hormones are involved in normal prostate growth and carcinogenesis,

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maintaining the homeostasis of cell survival and cell death in the prostate gland. Various factors are attributed to the pathogenesis of BPH. The principle hypothesis for hyperplasic condition of the gland is (a) steroid hormones-mediated cellular proliferation and (b) inflammatory response to local infections (Konwar *et al*., 2008). Androgen and other steroid metabolites along with estrogen have been considered as major risk factors in the pathogenesis of prostate enlargement (Geller *et al*., 1976; Rohrmann *et al*., 2007; Winter and Liehr, 1996). The proper understanding of the aetiology and pathogenesis of prostate enlargement are not clear as yet. Epidemiological studies indicatethat the incidence of both diseases are increasing globally (Orsted and Bojesen, 2013; Suzuki, 2009).

Clinically BPH is characterized as microscopic BPH and macroscopic BPH. Although BPH is uncommon before age 40, about 50% men develop BPH-related symptoms at 50 years of age. The incidence of BPH increases by 10% per decade and reaches 80% around 80 years of age. An estimated 75% men > 50 years of age show symptoms arising from BPH (Briganti *et al*., 2009).

Several attempts have been made during the last decade to obtain a thorough understanding of the BPH pathogenesis, where symptomatic treatment is the only option and treatment is often not successful resulting in repetitive Transurethral Resection of the Prostate (TURP). Genetic targets such as single nucleotide polymorphisms (SNPs) are promising genetic markers for a better understanding of the genetic basis for various complex diseases including cancers of breast, lung, etc. (Hajiloo *et al*., 2013). However, early diagnostic genetic marker for identification of BPH pathogenesis are not identified. Genetic variations represented by SNPs in prostate specific genes and role in progression and pathogenesis of prostate enlargement is not clear.

Genetic polymorphisms and association in pathogenesis of BPH have been reported (Konwar *et al*., 2008). Several studies have been conducted to evaluate AR polymorphisms in BPH. Giovannucci *et al*. (1999) reported an association of short CAG repeat length in BPH patients. Further, studies also supported the hypothesis that the shorter CAG repeat length of AR gene was

related to prostate enlargement (Roberts *et al*., 2004; Shibata *et al*., 2001). Polymorphisms in AR have also been linked to differences in rates of PSA production (Xu *et al*., 2002). It was also noted that two copies of the PSA (G) allele was associated with three-fold increased risk of prostate cancer. Further, the risk was elevated in men with short AR CAG alleles (Xue *et al*., 2000). Four SNPs (rs298793 T/C, rs1887994 G/T, rs1256040 C/T and rs1256062 A/G) in ER-β gene and a SNP in the promotor region (13950 T/C) (rs2897983) are associated with BPH and PCa risk (Thellenberg-Karlsson *et al*., 2006). An additional SNP in 3′UTR region of ER-β gene at position 1730 A/G was found significantly associated with PCa in African population (Zhao *et al*., 2004). However, biological functional studies to support the epidemiological findings, and analyses of gene–gene and gene–environment interactions are rare. Identifying the environmental factors that may modify the relationship between genetic polymorphisms and disease may provide a clue to possible functions of the genetic polymorphisms.

Hence, we investigated polymorphisms in candidate genes Androgen receptor (AR), Prostate Specific Antigen (PSA)(KLK) and Estrogen Receptor-β (ER-β) with BPH risk in Western part of Indian population (Table 1). Moreover, genotype–phenotype correlation studies have also been elucidated in the BPH patients for the disease progression in western part of the India.

MATERIALS AND METHODS

Study Subjects

The study comprised 200subjects with 80 BPH patients who underwent TURP (Transurethral Resection of the Prostate) and 120 healthy age matched control subjects from Gujarat, during the period of 2011–2014. Two milliliters of blood in sterile EDTA vial and 1–5 g TURP biopsy of the patients(in the age group 40–80 year) in transport medium were collected. Healthy individuals between 40–80 years with history of BPH selected as per AUASI (American Urological Association Symptom Index) guidelines formed the control group, and 2 ml blood was collected in sterile EDTA vial. The study was approved by The Maharaja Sayajirao University of Baroda Institutional Human Ethics Committee. Detailed demographic and anthropometric data were collected in structured questionnaire which included age, genetic pre-disposition, clinical

complications, socio-economic status, smoking habits (history of tobacco use) and AUASI guideline questions. Study subjects were evaluated by uroflow and digital rectal examination (DRE) for prostate volume prediction by urologist. Further patients' prostatic tissue samples were processed for histopathological evaluation to assess Benign Prostate Hyperplasia or Prostate cancer by the consultant pathologist.

Chemicals

The gene(s) specific primers were synthesized from Integrated DNA Technologies, India. PCR Master Mix and gel electrophoresis reagents, western blotting antibodies and reagents were obtained from Sigma-Aldrich, USA. Reverse Transcriptase-PCR reagents, genomic DNA isolation kit and restriction endonuclease enzymes were procured from Fermentas, GMBH, Germany. Other required reagents were obtained from local distributors. All the chemicals were of molecular biology grade**.**

Isolation of Genomic DNA and PCR

Genomic DNA was extracted from 200 µl blood using Fermentas pure extreme DNA purification kit according to the manufacturer's instructions. PCR was performed for AR, PSA(KLK) and ER-β genes with specific primers (Table 1). Twenty nanogram genomic DNA was used to amplify the genes in a 50 µl system using 0.30 µm of each Primer, 1.5 units of Taq DNA Polymerase, 200 µM dNTP, 1.5 mM $MgCl_2$, and 5 µl of 10X buffer (500 mM KCl, 200 mM Tris-HCl) (PCR Master Mix).

The PCR products were subjected to electrophoresis on agarose gel (2%) or polyacrylamide gel (15%) stained with ethidium bromide (EtBr) along with 1000 bp DNA ladder.

Restriction Pattern for Polymorphism

The amplified PCR product was incubated with appropriate restriction endonuclease enzyme (*NheI* for PSA(KLK), *Stu1* for AR and *Alu1* for ERβ) (Table 1) and the digestion pattern was analysed. The digested products were then analyzed by 15% DNA-PAGE after staining with EtBr to find out the SNPs specific to this population (Fig. 1).

RNA Extraction and cDNA Synthesis

For the prostate candidate gene(s) expression, total RNA was isolated from TURP biopsy sample and suspended in RNA stabilizing solution (Amresco LLC,

Table 1. Gene specific primers, restriction endonucleases and their recognition sites used for SNP study

Prostatic Gene and Polymorphism (Reference Sequence number)		PrimerSequences	Restriction enzyme, Recognition site and PCR product size post digestion
Androgen Receptor*(AR)* A/G at position 1754 in Exon-1 (rs 6152)	Forward Reverse	5′-CAG AGG CTA CCT GGT CCT GG-3′ 5′-CTG CCT TAC ACA ACT CCT TGG C-3′	***AR – Stu I*** **Recognition site:** 5′-AGG↓CCT-3′ and3′-TCC↑GGA-5′ **Restriction product size:** 327bp and 89bp
Prostate specific antigen (PSA) (*klk*) Promotor– 158 A/G (rs 266882)	Forward Reverse	5′-TTG TAT GAA GAA TCG GG ATC GT-3′ 5′-TCC CCC AGG AGC CCT ATA AAA-3′	**PSA *(klk)* – *Nhe I*** **Recognition site:** 5′-G↓CTAGC-3′ and 3′-CGATC↑G-5′ **Restriction product size:**300bp and 150bp
Estrogen Receptor-β *(ERβ)* 1730 A/G 3′UTR in Exon-8 (rs 4986938)	Forward Reverse	5′-GTA GAC TGG CTC TGA GCA AAG AGA GC-3′ 5′-CCA AGC CTG CCA TCA CCA AAT GAG-3′	***ERβ - Alu I*** **Recognition site:** 5′-AG ↓ CT-3′ and3′-TC↑ GA-5′ **Restriction product size:** 323bp and 82bp

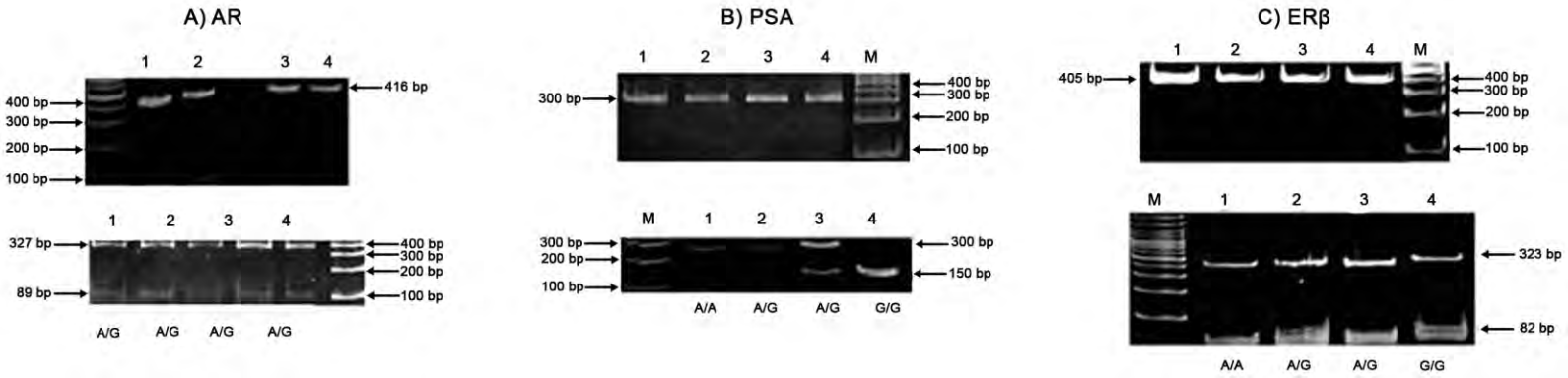

Figure 1: Agarose gel electrophoresis of PCR productspre- and post-restriction enzyme digestion. A) AR; B) PSA; and C) ER β genes.

OH, USA). RNA samples (n = 3) were quantified by spectrophotometer at 260/280 nm. Complementary DNA (cDNA) was synthesized by reverse transcriptase (RT) using 1 µg RNA (Fermentas First stand cDNA synthesis kit). After Reverse transcription cDNA samples were amplified by RT-PCR using gene specific primers for AR, ER-α, ER-β and 5α reductase (type-2) genes. GAPDH was used as an endogenous control. Reactions were carried out in a Gradient PCR (Eppendorf, NY, USA). The PCR products were electrophoresed in agarose gel (2%) in Tris-acetate-EDTA (TAE) buffer, and stained with EtBr. Gels were photographed by Gel documentation unit (UVITEC Cambridge Alliance 4.7, Cambridge, UK) and densitometrical analysis was carried out using Image J software.

Protein Profiling

Protein profiling for Androgen Receptor and p63 by western blotting of TURP biopsy samples were performed as previously described (Carson and Rittmaster, 2003). The tissues were lysed with urea containing lysis buffer (1 mM EDTA, 50 mM Tris-HCl pH 7.5, 70 mM NaCl, 1% Triton, 50 mM NaF) supplemented with protease inhibitor cocktail (Fermentas INC.,GMBH, Germany). Total Protein estimation was carried out using Bradford reagent according to manufacturer's suggestions (BIO-RAD laboratories, CA, USA). Tissue lysates (40 µg) were separated on Polyacrylamide gel using Mini-tetracell electrophoresis system (BIO-RAD laboratories, CA, USA) and transferred onto nitrocellulose blotting membrane (Merck Millipore, MA, USA). Blots were then incubated with blocking milk buffer (5% fat free skimmed milk with 0.1% Tween-20 in Phosphate Buffer Saline (PBS)). Dilutions of primary antibodies against prostate proteins AR and p63 were added to blots and incubated overnight at 4ºC. Anti-rabbit and anti-mouse IgG conjugated with HRP were used to develop the blots using Ultra-sensitive enhanced chemi-luminiscence reagent (Merck Millipore, MA, USA) and blot images were documented using chemi-doc instrument (UVITEC Cambridge Alliance 4.7, Cambridge, UK).

Statistical Analysis

The Hardy-Weinberg equilibrium (HWE) was used to evaluate the polymorphisms in patients and control subjects by comparing the observed and expected

Table 2. Percentage frequencies of AR, PSA and ERβ genotypes.

AR Genotypes % frequency (n)				
	Control (n = 119)	Cases(n = 79)	*P* value	OR (95% CI)
A/A	70.6 (84)	44.3 (35)		
A/G	29.4 (35)	55.7 (44)	0.0002	3.0 (1.67-5.46)
PSA Genotypes % frequency (n)				
	Control (n = 120)	Cases (n = 76)		
A/A	41.7 (50)	26.3 (20)		
A/G	45.0 (54)	53.9 (41)	0.08	2.0 (1.07-3.74)
G/G	13.3 (16)	19.8 (15)		
ERβ Genotypes % frequency (n)				
	Control (n = 116)	Cases (n = 78)		
A/A	99 (85.3)	37 (47.4)		
A/G	17 (16.7)	41 (52.6)	0.0001	6.5 (3.27-12.74)

frequencies of the genotypes using chi-square analysis. The distribution of the genotypes and allele frequencies of the polymorphisms in patients and control subjects were compared by chi-square test. Odds ratio (OR) with 95% confidence interval (95% CI) for disease susceptibility was calculated. *P-value* less than 0.05 were considered as statistically significant. Gene and protein studies (Genotype–Phenotype) statistical analyses were performed with Newman-keuls post hoc one way ANOVA and t-test by using GraphPad Prism software version 5.0.

RESULTS

The genotype of the polymorphisms in patients and control subjects used PCR-RFLP protocol and digestion with restriction enzymes *Stu I, Nhe I* and *Alu I*. BPH patient's data were analyzed in the terms of allelic. The distribution of the genotype frequencies followed the Hardy Weinberg equilibrium in control and patient groups ($p > 0.05$). Genotype–phenotype studies were accomplished by gene and protein expression and SNP's analysis.

Association of AR, PSA and ERβ SNPs with Risk to BPH

The frequencies of the mentioned SNPS in AR, PSA and ER followed the Hardy Weinberg Equilibrium in the representative population from the state of Gujarat in Western India. The percentage frequency distribution in the controls and BPH samples indicated an association of increased risk with the heterozygous SNPs in AR [($p = 0.0002$; OR 3 (CI 1.67 – 5.46)]; and ERβ [$p =$

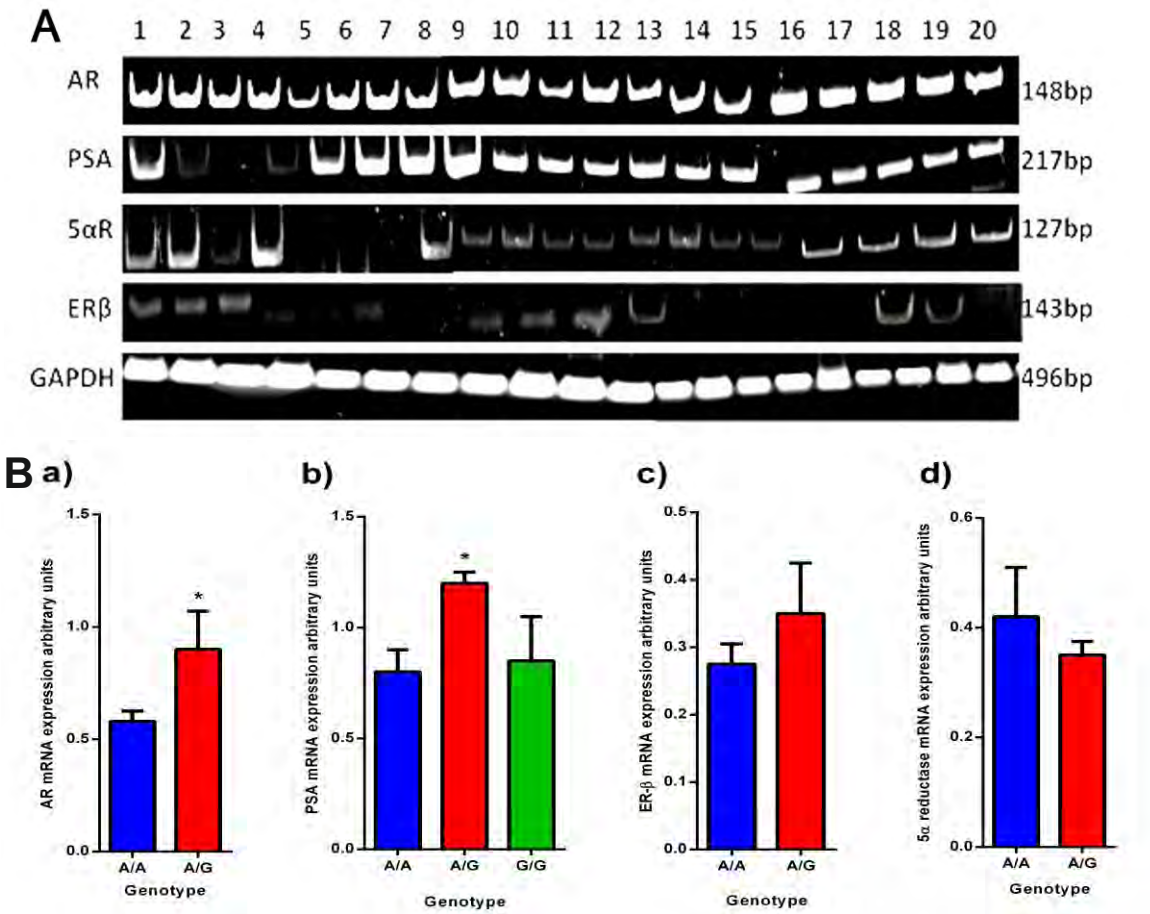

Figure 2: A) Pictorial representation of gene expression pattern of AR, PSA, 5α reductase, ERβ and GAPDH (internal control) in TURP biopsy samples. (lane 1–20 represents individual patient's sample); B) Genotype–phenotype correlation of a) AR (*p < 0.05, A/G vs A/A), b) PSA (*p < 0.05, A/G vs A/A), c) ERβ and d) 5α Reductase genes.

0.0001; OR 6.5 (CI 3.27 – 12.74)] (Table 2). Whereas the PSA SNP frequencies in the control and BPH patients does not show a significant difference [p = 0.08; OR 1.35 (0.75 – 2.4)].

Genotype–phenotype Correlation

We investigated correlation between the three polymorphisms in the prostate cancer associated genes and the phenotypes by mRNA expression of AR, PSA (KLK), ER-β, 5Alpha Recductase genes, and protein profile of AR and p63 from prostate tissue samples collected during TURP surgery. Twenty subjects were assessed for the study. The expression level (arbitrary units) for each genotype of the three genes (AR, PSA(KLK) and ER-β) were calculated using ImageJ software and GAPDH expression for normalization (Fig. 2A).We observed significant increase of AR mRNA expression in patients with A/G genotype (*p < 0.05, A/G vs A/A)

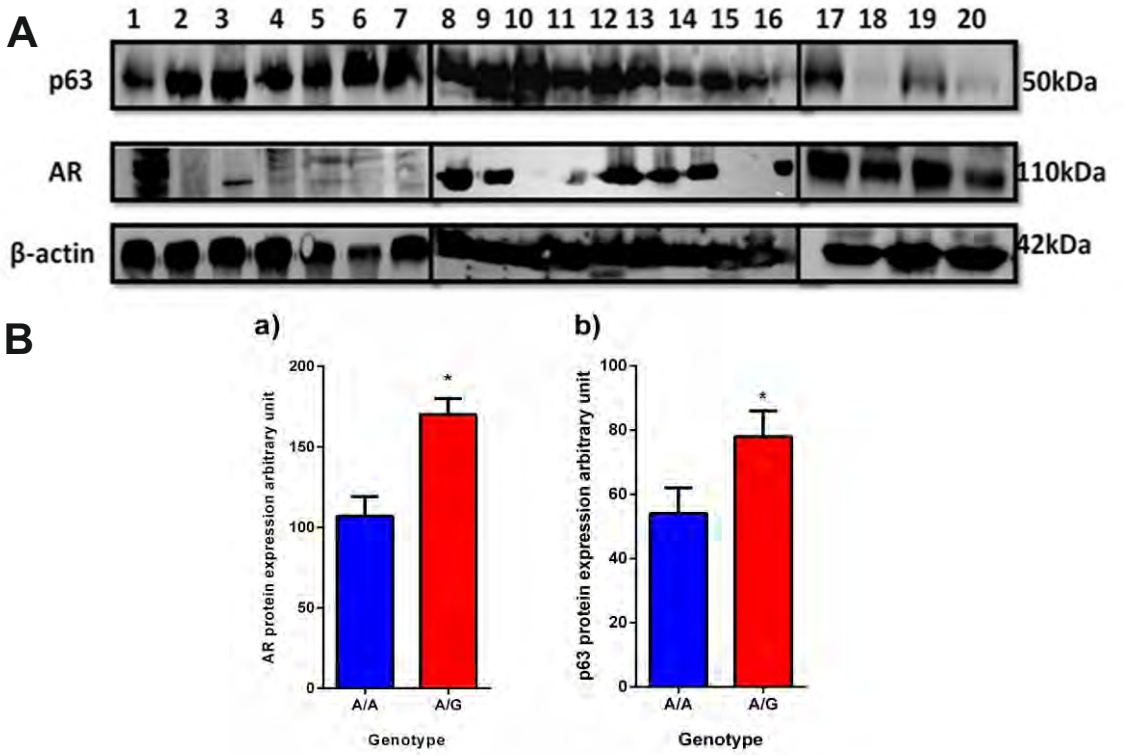

Figure 3: A) Pictorial representation of Protein profile of p63, AR and β-Actin (internal control) in TURP biopsy samples (lane 1–20 represents individual patient's sample); B) Correlation of a) AR and b) p63 protein expression with AR genotype (*p < 0.05 A/G vs A/A)

similarly, mRNA expression of PSA(KLK) was found to be significantly increased in A/G genotype (*p < 0.05, A/G vs A/A) compared to A/A and G/G genotypes among the patients; whereas, ERβ genotypes did not display significant difference in phenotype (Fig. 2B).

Furthermore, protein profile of AR and p63 were correlated with AR genotype. Our result showed significant increase of AR protein expression in A/G genotype of AR gene, supporting mRNA expression data and indicating a role in BPH progression (Fig. 3B). p63 protein profilealso showed significant increase in AR A/G genotype compared to A/A in the patients (Fig. 3A). However, no significant difference could be observed in 5α Reductase with both the genotypes.

DISCUSSION

The geographical distribution of prostate cancer is not consistent worldwide, with a higher incidence in USA, and lower incidence in Asian countries, despite the Asian population showing a high susceptibility for BPH (Pandya *et al*., 2013), and between different racial and ethnic groups. African Americans are

reported with 40–60 fold higher incidence rates than those reported for Asian men (Ruijter *et al*., 1999).

To address the underlying genetic cause of benign prostate burden in western part of Indian population, we obtained blood samples from a cohort, and analysed for polymorphisms of prostatic genes, AR, PSA (KLK), and ERβ. A strict exclusion and inclusion criteria (AUASI) were implemented for subject selection. The polymorphism in the AR gene is in exon 1, which is the NH_2-terminal domain of the protein and harbors major transcription activation functions also binding to the COOH-terminal LBD (N/C interaction) of the gene. The hormone dependent interaction of the NH_2-terminal domain with the ligand binding domain plays a role in stabilization of the androgen receptor dimer complex by slowing the rate of ligand dissociation and decreasing receptor degradation (Centenera *et al*., 2008; Doesburg *et al*., 1997). The SNPs under study may affect these interactions, resulting in increased cell proliferation, as the growth of the prostate gland is dependent on circulating androgens and intracellular steroid signaling pathways mediated through the androgen receptor (AR) (Chen *et al*., 2002; Konwar *et al*., 2008). AR also regulates additional genes including cyclin dependent kinases (CDK), CDK2 and CDK4, and CDK inhibitor p16, p63 and thus regulates cell cycle (Lu *et al*., 1999; Yu and Jiang, 2011).

The AR-*Stu I* restriction site, commonly known as E213 G/A SNP (rs6152) is a synonymous change (GAG > GAA) coding glutamic acid. The E213 G/A SNP (rs6152)SNP has been studied indifferent population worldwide. A recent study by Shahriar Koochekpour *et al*. (2014) in African American population showed significant association of rs6152 in black African American population.In our study the A/G genotype frequency of AR was found to be 55.7% (OR 3.0 (95% CI 1.67 – 5.46) (Table 2).

The PSA (KLK) gene, a member of the human kallikrein (hKLK) gene family, is located at the 19q13.41 chromosome 19 (Lilja, 2003), encoding a glycoprotein containing 240 amino acids, also known as serine protease (33 kDa). Since PSA secretion is regulated by androgens via AR-dependent pathways, an increase in the amount of androgen in turn induces PSA secretion from the prostate epithelium (Medeiros *et al*., 2002; Rao *et al*., 2003). The AR also

interacts with the three AREs in the PSA (KLK) gene promoter. The most proximal of the three AREs in the PSA (KLK) promoter — ARE1, located 170 bp upstream of the transcription start site has two allelic variants: AGAACAnnnAGT**A**CT and AGAACA-nnnAGT**G**CT. These allelic variants play a transcriptional control role for AR-regulated expression (Xu *et al*., 2002). The AR may bind two alleles with different affinities, producing quantitative differences in PSA (KLK) mRNA expression. Association studies of the A-158G polymorphism in ARE1 of PSA(KLK) promoter have demonstrated that males with A/G genotype showed a 2.4-fold increased risk (95% CIs 51.23 – 4.81) of developing prostate cancer (Lai *et al*., 2007). Thus, ARE mutations interfering with the PSA (KLK) regulation will lead to an increase in PSA secretion and subsequent prostate cells transformation to malignant phenotype (Xu *et al*., 1998). PSA has been identified as the protease for the major IGF-binding protein, IGFBP-3. Cleavage of IGFBP-3 by PSA increases IGF-I and IGF-II bioavailability thus increasing cell growth (Cohen *et al*., 1994). IGF-I also causes AR over expression, thus, leading to enhanced AR activity and increased cell growth (Culig *et al*., 1994). Our results are in concordance with above cited literature.

Xue *et al.* (2000) have reported that coinheritance of ARA/G genotype and PSA(KLK) A/G genotype may lead to rapid and early disease progression. However, Alptekin *et al*. (2012) reported no statistically significant difference between controls, BPH and adeno-carcinoma association with PSA A/G polymorphism in Turkish patient. ERβ has an anti-proliferative role, maintaining homeostasis of the prostate gland (Weihua *et al*., 2001). Bardin *et al*. (2004) suggested reduced expression of ERβ on the epithelial cells of the prostate gland, leading to proliferation of prostate cells and prostate enlargement. Safarinejad *et al*. (2012) demonstrated significant risk of prostate cancer with 1730 A/G 3′UTR in Exon-8(rs 4986938) SNP in ERβ gene in Iranian population.The polymorphisms of ER in the 3′-untranslated regions (3′-UTR) affects mRNA stability. Although the polymorphism showed no functional implication, the allele might be in linkage disequilibrium with relevant mutations in the gene. The specific control sequence of the ER-β mRNA degradation pathway is located in the 3′-

UTR region (Kenealy *et al*., 2000). Thus, if the SNP in ERβ decreases the stability of ERβ mRNA, it result in decreasing anti-proliferation signals and thus lead to increased cell growth in prostate gland. We observed A/G genotype frequency of ER-β as 52.6% (OR 6.5, 95% CI 3.27 – 12.74) (Table 2) with significant association in disease pathogenesis. The mutation has been studied in South Indian cohort and the results demonstrated reduced ER-β gene expression with an increased risk for breast cancer (Surekha *et al*., 2009).

Further reports suggest that mRNAs containing different bases at SNP sites may vary in their interactions with cellular components involved in mRNA synthesis, maturation, transport, translation or degradation. It has been documented that a number of single base-pair substitutions alter or create essential sequence elements for splicing, processing, or translation of human mRNA (Shen *et al*., 1999). Such elements may affect the expression of the mRNA, thus affecting gene and protein expressions. To correlate genotype with phenotype, we compared SNP data with mRNA expression in the respective genes. Interestingly, we observed significant increase in protein and gene expression of AR with A/G genotype, increase PSA (KLK) gene expression in A/G genotype and no significant change in ERβ expression in A/G genotype of ERβ.Several reports have demonstrated that in benign condition, AR protein expression was increased compared to prostate cancer (Gaston *et al*., 2003; Koochekpour *et al*., 2014). A similar profile of AR protein was observed in our study. Although ER-β has anti-proliferative effect in the prostate gland, the genotype–phenotype correlation reports no significant changes in ER-β group.

P63 is a prostate stem cell marker and used as a distinctive marker between PCa and BPH. A recent study by Yu *et al.* (2011) demonstrated significantly lower expression of p63 in ARKO (Androgen Receptor Knockout) mice, indicating AR dependant expression of p63. Increased protein expression of p63 was observed with A/G genotype of AR in patients compared to A/A genotype, indicatinga role of AR in BPH pathogenesis. This is further supported by our earlier study where p63 expression was observed in BPH derived cell line (Prajapati *et al*., 2014).

5α Reductase type II, is an enzyme responsible for conversion of

Testosterone into DHT. However, no significant difference in 5α Reductase type II mRNA was observed with AR polymorphism in the present study. Earlier report from Luo and colleagues have also observed no significant difference in the 5α Reductase mRNA level between BPH and normal samples (Luo *et al*., 2003). Additionally, changes in 5α Reductase level with PCa development and progression remains controversial. Wako *et al*. (2008) observed no significant change in both 5α Reductase I and II level between prostate cancer and normal prostate tissue.

The SNPs investigated in the present study for AR and ER-β genes were found to be significantly associated with BPH pathogenesis as compared to control cohort. Additionally, the genotype–phenotype study provided evidence that gene–environment interactions play an important role in the etiology of BPH. However, a large cohort study from different parts of India is mandatory to verify the association of SNPs and the environmental factors that may modify the relationship between genetic polymorphisms and disease pathogenesis.

CONFLICT OF INTEREST

The authors claim no conflict of interest.

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AUTHORSHIP CONTRIBUTION

AP and SG[1] designed the research. SG[1] supervised data collection. AP, GC, PP and SK involved in sample collection and carried out all of the experiments and analyses. Both AP and SG[1] are taking responsibility for the statistical methods employed and interpretation of the findings. SG[2] helped in procurement ofsamples and provided clinical advice regarding the analysis of histopathological interpretation. AP and SG[1] wrote paper.

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