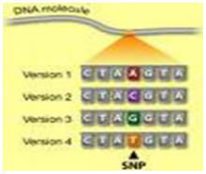
CHAPTER -5

CHAPTER- 5

To understand the genetic association of single nucleotide polymorphism in human prostate genes from benign prostate hyperplasia patients.

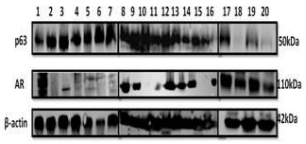
Prostate Tissue Sample(TURP)

Blood

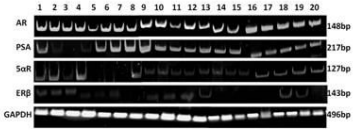


SNP Analysis

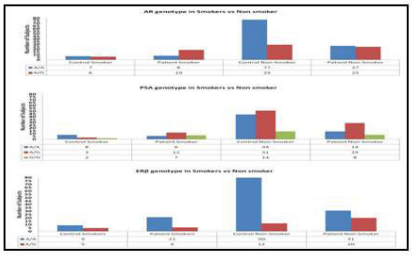
Consent form



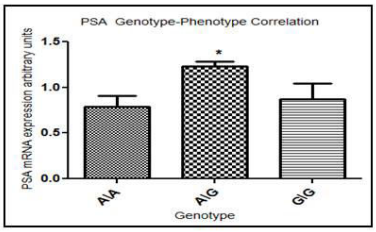
Protein Profiling



Relative gene expression



Gene-Environmental interaction



Genotype- Phenotype correlation

5.1 Introduction

The prostate is a hormonally regulated organ whose growth accelerates at sexual maturity due to androgen actions on the both stromal and epithelial cells. In men over the age of 40-50 years, prostate gland represents a major medical problem in the form of benign prostate hyperplasia (BPH) and prostate cancer (PCa). Steroid hormones are involved in normal prostate growth and carcinogenesis. They maintain the homeostasis of cell survival & 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-mediated cellular proliferation and (b) inflammatory response to local infections (Konwar.R et al. 2008). Androgen and other steroid metabolites along with estrogen have been considered as major risk factors in the pathogenesis of BPH (Geller et al. 1976; Winter and Liehr 1996; Rohrmann et al. 2007). The proper understanding of aetiology and pathogenesis of the prostate enlargement are still unknown. Epidemiological data from several studies indicated that both diseases are becoming increasingly prevalent worldwide (SUZUKI 2009; Orsted and Bojesen 2013).

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

Many attempts have been made during the last decade to obtain a thorough understanding of the BPH pathogenesis. It is a disease where symptomatic treatment is only option for medical professionals and the treatment methods do fail frequently which result in repetitive Trans Urethral Rectal Prostectomy (TURP). To understand Pathophysiology of the disease we established a BPH rodent model and human BPH epitheal cell line described in earlier chapters. Genetic targets such as single nucleotide polymorphisms (SNPs) are considered very promising genetic markers for a better understanding of the genetic basis for various complex diseases like Breast cancer, Lung Cancer etc (Hajiloo et al. 2013). But till now there is no early diagnostic genetic marker for proper identification of BPH pathogenesis. However, it is still unclear whether such genetic variations (single nucleotide polymorphism in prostate specific genes) play pivotal role in progression and pathogenesis of prostate enlargement.

Genetic polymorphisms for pathogenesis of BPH and their association have been reported (Konwar et al. 2008). Several studies have been conducted to evaluate the AR polymorphism in BPH. Giovannucci, 1999 reported an association of short CAG repeat length in BPH patients (Giovannucci et al. 1999). Further studies also supported the hypothesis that the shorter CAG repeats length of AR gene was related to prostate enlargement (Shibata et al. 2001; Roberts et al. 2004). Polymorphisms in AR have also been linked to differences in rates of PSA production (Xu et al. 2002). It was also found that men carrying two copies of the PSA G allele had nearly three folds increased risk of prostate cancer. Moreover, the risk was more elevated among men who also carried a short AR CAG alleles (Xue et al. 2000). report has shown four SNPs (rs298793 T/C, rs1887994 G/T, rs1256040 C/T and rs1256062 A/G) in ERβ. Among them one SNP in promotor region (-13950 T/C) (rs2897983) is associated with BPH and PCA risk (Thellenberg-Karlsson et al. 2006). Another SNP of ERβ gene at position 1730 A/G in 3'UTR region found significantly associated with PCa in African population (Zhao et al. 2004). However, no biological functional studies have been published to support the epidemiological findings, and analyses of gene-environment interaction were rarely performed. 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 or to the locations of functional SNPs.

So in light of that, we aimed to investigate the susceptibility of polymorphism in candidate genes (Androgen receptor, Prostate Specific Antigen & Estrogen Receptor β) with BPH risk in Western part of Indian population *(Table 5.1)*. Moreover, genotype-phenotype correlation and assoiciation of factors like smoking habits, emphasizing on environmental impact has been elucidated in the BPH patients for the disease progression in western part of the India.

Prostatic Gene Name	**Polymorphism & Ref.Seq. no.**
Androgen Receptor (AR)	A/G at position 1754 in Exon-1 **(rs 6152)**
Prostate specific antigen (PSA)	Promotor -158 A/G **(rs 266882)**
Estrogen Receptor-β (ERβ)	1730 A/G 3'UTR in Exon -8 **(rs 4986938)**

Table 5. 1: *Polymorphisms studied in the study*

5.2 Materials and Methods:

5.2.1 Study subjects:

The study was performed during 2011 to 2014 in western part of Indian population with 200 samples out of which 80 were BPH patients who underwent TURP (blood) and 120 were healthy match control (blood). 2ml Blood and approx 1-5g tissue (20 samples) of the patients who underwent TURP (Trans Urethral Resection of Prostate) (age group 40-80 year) from were collected in sterile EDTA coated vial and transport medium, respectively. For control samples a healthy match individual between 40-80 years who did not have any BPH history were chosen for the study. Control blood samples were collected in sterile EDTA coated vial. The study was Approved by Institute Human Ethics Committee, IHEC, The M.S.U. Baroda. Detailed demographic and anthropometric data were collected in structured questionnaire which included age, genetic pre-disposition, clinical complications, smoking habit and socio-economic status. (for consent form see annexure 1). Further patients' prostatic tissue samples were processed for histo-pathological evaluation to assess Benign Prostate Hyperplasia or Prostate cancer by a consultant pathologist.

5.2.2 Chemicals:

All the gene specific primers were synthesized from IDT, India. PCR Master Mix and gel electrophoresis reagents were obtained from Sigma, USA. Reverse Transcriptase -PCR reagents, genomic DNA isolation kit and restriction endonuclease enzymes were procured from Fermentas, Germany. Other required reagents were obtained from local distributors. All the chemicals were extra pure and of molecular biology grade**.**

5.2.3 Isolation of Genomic DNA and PCR:

Around 200µl of blood sample was used for genomic DNA isolation using Fermentas pure extreme DNA purification kit according to the manufacturer's instructions. PCR was performed for AR, PSA & ERβ genes with respective appropriate primers *(Table 5.2 and 5.3)*. 20 ng of genomic DNA was used to amplify products of respective genes in a 50 ul system using 0.30µm of each Primers, 1.5 units of Taq DNA Polymerase, 200 µM dNTP, 1.5mM $MgCl_2$. and 5µl of 10X buffer (500mM KCl and 200mM Tris- HCl).

AR	**Forward**	5' CAG AGG CTA CCT GGT CCT GG 3'
	Reverse	5' CTG CCT TAC ACA ACT CCT TGG C 3'
PSA	**Forward**	5' TTG TAT GAA GAA TCG GG ATC GT 3'
	Reverse	5' TCC CCC AGG AGC CCT ATA AAA 3'
ERβ	**Forward**	5'-GTA GAC TGG CTC TGA GCA AAG AGA GC-3'
	Reverse	5'CCA AGC CTG CCA TCA CCA AAT GAG-3'

Table 5. 2: *Gene specific Primers sequence for SNP study*

PCR condition for AR gene	**PCR condition for PSA gene**	**PCR condition for ERβ gene**
95°C for 3 mins	95°C for 3 mins	95°C for 3 min
94°C for 30 sec	94°C for 30 sec	95°C for 30 sec
60°C for 30 sec	59°C for 30 sec	58°C for 30 sec
72°C for 45 sec	72°C for 45 sec	72°C for 45 sec
72°C for 10 mins	72°C for 10 mins	72°C for 10 mins
30 cycles	30 cycles	30 cycles
Product size: 416bp	Product size: 300bp	Product size: 405bp

Table 5. 3: *PCR conditions for prostate genes*

5.2.4 Confirmation of amplified gene:

PCR Amplified fragment of DNA was then confirmed by running on 2% Agarose gel or 15% DNA-PAGE after staining with Ethidium Bromide(EtBr) along with DNA marker ladder.

5.2.5 Restriction pattern for polymorphism:

Amplified fragment of DNA was incubated with appropriate restriction endonuclease enzyme (*NheI* for PSA, *Stu1* for AR and *Alu1* for ERβ) *(Table 5.4)* subjected to analyze the digestion pattern. In order to find out SNPs which are specific to this population, the digested products were then analyzed by 15% DNA-PAGE after staining with EtBr *(Fig. 5.1).*

AR – *Stu I*	PSA – *Nhe I*	ERβ - *Alu I*
Recognition site: 5'-----AGG↓CCT-----3' 3'-----TCC↑GGA-----5' Incubation at 37 ℃ for 1 hr Restriction product size: **327bp and 89bp**	Recognition site: 5'-----G↓CTAGC-----3' 3'-----CGATC↑G-----5' Incubation at 37 ℃ for 4 hrs Restriction product size: **300bp and 150bp**	Recognition site: 5'----AG ↓ CT-----3' 3'----TC↑ GA----5' Incubation at 37 ℃ for 1 hr Restriction product size**:** **323bp and 82bp**

Table 5. 4: *Restriction endonucleases and their recognition sites.*

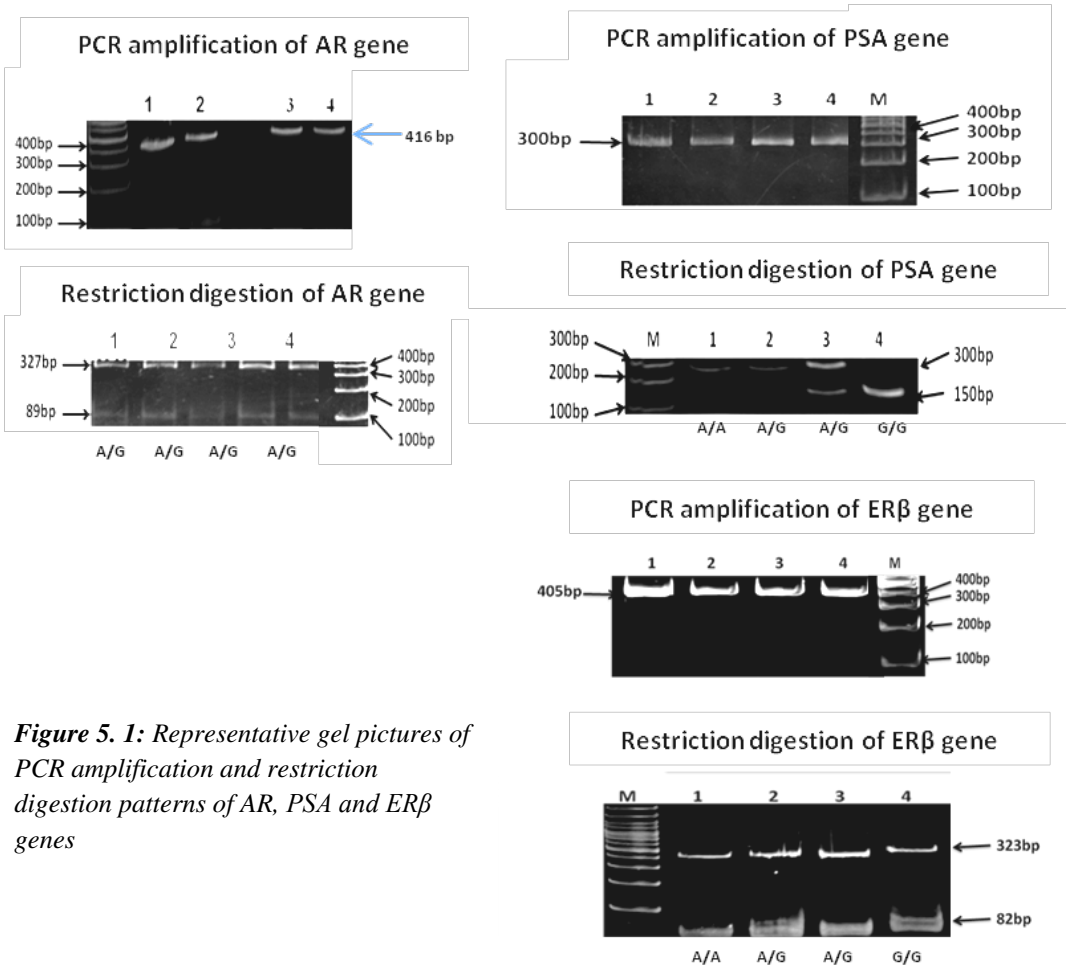


Figure 5. 1: *Representative gel pictures of PCR amplification and restriction digestion patterns of AR, PSA and ERβ genes*

5.2.6 RNA extraction and cDNA synthesis:

Total RNA was isolated from the TURP excised prostate tissue sample (20 patients) and re-suspended in RNA stabilizing solution procured from Amresco laboratories. RNA samples (n=3) were quantified by spectrophotometer at 260/280 nm. Complementary DNA (cDNA) was synthesised by reverse transcriptase (RT) using 1µg RNA (Fermentas First stand cDNA

synthesis kit). After Reverse transcription cDNA samples were amplified by RT-PCR using gene specific primers for AR, ERα, ERβ and 5 α reductase (type -2) genes. GAPDH was used as an endogenous control. Reactions were carried out in an Eppendorf Gradient PCR. The PCR products were electrophoresed on an ethidium bromide stained 2% agarose gel in Tris-acetate-EDTA (TAE) buffer. Gels were photographed by Gel documentation unit from UVITEC Cambridge alliance 4.7 and densitometrical analysis were carried out using Image J software.

5.2.7- Protein Profiling:

Western blotting of excised prostate TURP samples (20 samples) were performed as previously described (Carson and Rittmaster 2003). The tissues were lysed with urea containing lysis buffer (1mM EDTA, 50 mM Tris-HCl pH 7.5, 70 m MNaCl, 1% Triton, 50 mM NaF) supplemented with protease inhibitor cocktail (Fermentas INC.). Total Protein estimation was carried out using Bradford reagent according to manufacturer's suggestions (BIO-RAD). Tissue lysates (40µg) were separated on Polyacrylamide gel using Mini-tetracell electrophoresis system (BIO-RAD) and transferred onto nitrocellulose blotting membrane (Millipore). 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 various prostatic proteins (AR and p63) were added to blots and incubated overnight at 4^{o}C. Anti-rabbit and Anti-mouse IgG conjugated with HRP were used to develop the blots using Ultra-sensitive enhanced chemiluminiscence reagent (Millipore, USA) and blot images were documented using chemi-doc instrument Cambridge UV tech, UK.

5.2.8 Statistical Analysis:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for all the polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-square analysis. The distribution of the genotypes and allele frequencies of all the polymorphisms in patients and control subjects were compared by chi-square test. Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated. *p*- values less than 0.05 were considered as statistically significant. Statistical analyses were performed with Newman-keuls post hoc one way ANOVA and t-test by using Graph Pad Prism software version 5.0.

5.3 Results

The genotype work of all the polymorphisms in 80 patients and 120 control subjects was accomplished by PCR- RFLP analysis using restriction enzymes *Stu I, Nhe I* and *Alu I.* BPH patient's data were analyzed in the terms of allelic frequency and their geographical distribution using Hardy Weinberg equilibrium (HWE). The distribution of all the polymorphisms genotype frequencies were consistent with Hardy Weinberg expectations in control and patient group ($p>0.05$). Genotype- phenotype studies were accomplished by gene expression and SNP's analysis.

5.3.1 Analysis of association between AR gene A/G polymorphism at position 1754 in exon-1 and susceptibility to BPH:

Total 198 subjects were studied for the AR polymorphism *(Table5.5).* This study found A/G polymorphism at position 1754 in exon-1 has a 3 fold increase risk in BPH progression (OR 3.0; 95% CI (1.67-5.46). The A/G genotype frequency of AR was found to be significant in patients compared with age matched healthy controls (55.7 %.).

	Observed genotype count		Expected genotype count		Chi sq. test $\chi 2$	p value for HWE	p value for Association
	A/A	A/G	A/A	A/G			
Control (n=119)	84	35	85.68	88.06	32.0	0.06	0.0002
Patients (n=79)	35	44	41.08	71.1	11.23	0.006	

Table 5. 5*: Association studies for the androgen receptor (AR) gene A/G polymorphism at position 1754 in exon-1in BPH patients and controls.*

5.3.2 Analysis of association between Prostate Specific Antigen (PSA) gene A/G polymorphism at ARE1 of PSA Promotor -158 A/G and susceptibility to BPH:

Total 196 subjects were studied for the PSA polymorphism *(Table 5.6).* This study found A/G polymorphism at ARE1 of PSA Promotor -158 A/G has a 2 fold increase risk in BPH progression(OR 2.0; 95% CI (1.07-3.74). However, a non significant association of the A/G genotype frequency of PSA was observed.

	Observed genotype count			Expected genotype count			Chi sq. test χ^2	p value for HWE	p value for association
	A/A	A/G	G/G	A/A	A/G	G/G			
Control (n=120)	50	54	16	49.45	55.44	15.6	0.056	0.81	0.08
Patients (n=76)	20	41	15	21.36	38	16.8	0.54	0.46	

Table 5. 6: *Association studies for the Prostate Specific Antigen (PSA) gene A/G polymorphism at ARE1 of PSA Promotor -158 A/G in BPH patients and controls.*

5.3.3 Analysis of association between Estrogen Receptor-β (ER- β) gene A/G polymorphism at position 1730 A/G 3'UTR in Exon -8 and susceptibility to BPH:

Total 194 subjects were studied for the ER- β polymorphism *(Table 5.7).* This study found A/G polymorphism at position 1730 A/G 3'UTR in Exon-8 has a 6.5 fold increase risk in BPH progression(OR 6.5; 95% CI (3.27-12.74). The A/G genotype frequency of the ER- β was found to be significant in patients compared with age matched healthy controls (52.6 %.).

	Observed genotype count		Expected genotype count		Chi sq. test χ^2	P value for HWE	p value for Association
	A/A	G/G	A/A	G/G			
Control (n=116)	99	17	84.68	2.61	104.38	0.394	0.0001
Patients (n=78)	37	41	17.55	21.84	38.37	0.001	

Table 5. 7*: Association studies for the Estrogen Receptor β (ERβ) gene A/G polymorphism at position 1730 A/G 3'UTR in Exon -8 in BPH patients and controls*

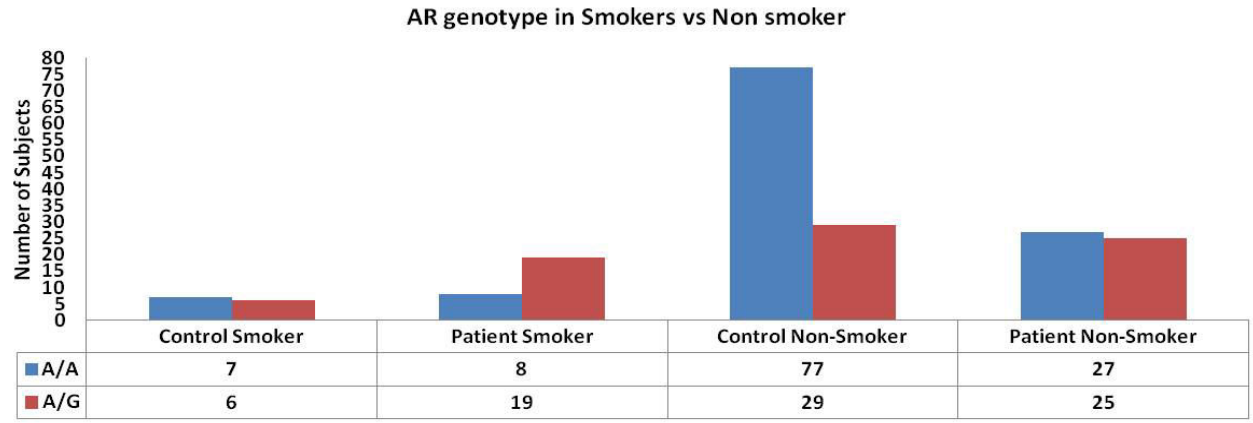
Gene	No. of Controls (%)	No. of Patients (%)	Odd Ratio (95% CI)
AR genotype A/A(418bp) A/G(329/89bp) Total	A/A 84 (70.6) A/G 35 (29.4) **119**(100)	A/A 35(44.3) A/G 44(55.7) **79**(100)	OR 3.0 ; (95% CI 1.67-5.46) p Value=0 .0002 ***
PSA genotype A/A(300bp) A/G(150/300bp) G/G(150bp) Total	A/A 50(41.7) A/G 54(45.0) G/G 16(13.3) **120**(100)	A/A 20(26.3) A/G 41(54.0) G/G 15(19.7) **76**(100)	OR 2.0; (95% CI 1.07-3.74)
ERβ Genotype A/A(405bp) A/G (323/82bp) Total	A/A 99(85.3) A/G 17(14.7) **116**(100)	A/A 37(47.4) A/G 41(52.6) **78**(100)	OR 6.5; (95% CI 3.27-12.74) p value= 0.0001 ***

Table 5. 8: *Distribution of AR, PSA and ERβ Genotypes among cases and controls*

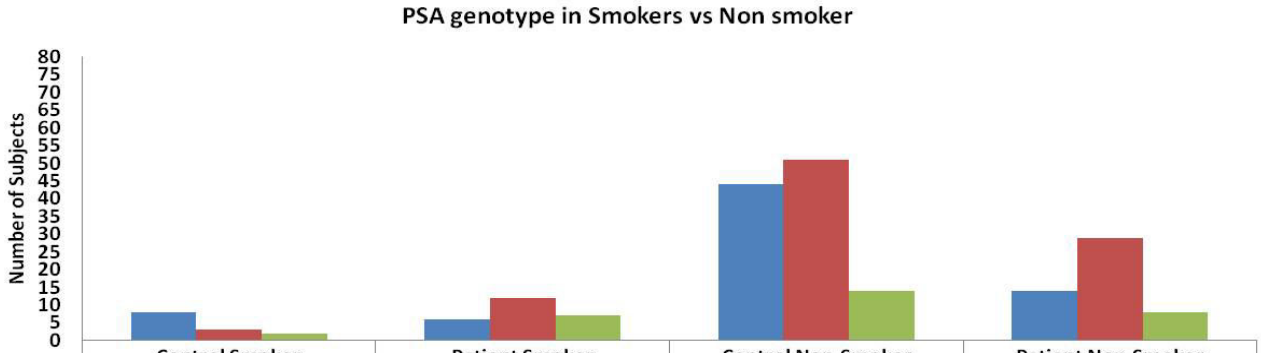
5.4.4 Genotype correlation with smokers and non smokers:

Smoking behaviour is influenced by both genetic and environmental factor. Moreover, smoking initiation and its persistence have heritability at least 50 percent (walter 2006). There are several potential mechanisms where by smoking may increase the risk of PCa and hyperplasia. Data was further investigated for the correlation between association of SNPs of prostate candidate genes, i.e. AR, PSA and ERβ, with smoking habits among the patients and control in gene specific manner.

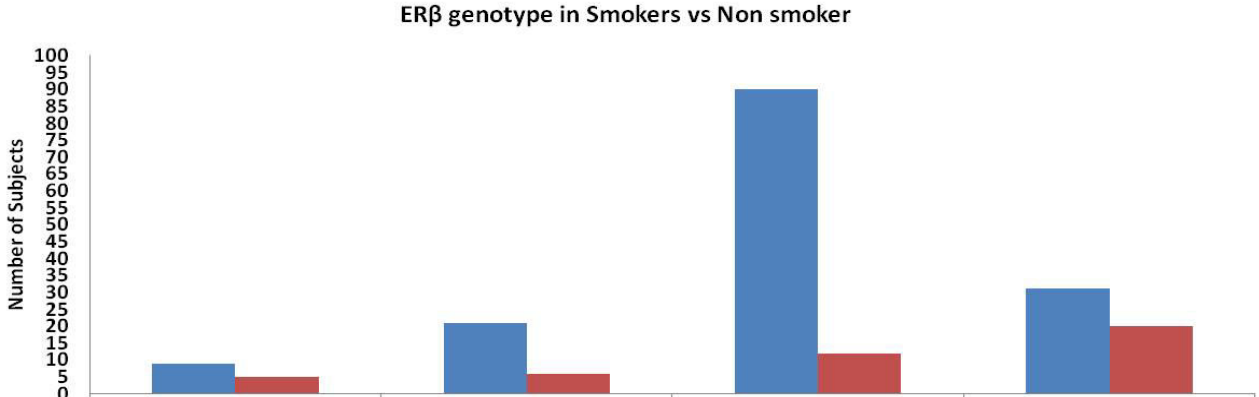
In our results, we found A/G genotype of AR gene more in smoker patients as compared to smoker control subjects, whereas A/A genotype had been observed more in control non-smoker population. Similarly, A/G genotype of PSA gene found to be more in smoker patients compared with smoker control population and the frequency of A/G was found more with then Non-smoker individuals. A/A genotype of PSA gene was observed more in control population. In ERβ, no difference was observed in both genotypes with respect to control population *(Fig. 5.2).*



	Control Smoker	Patient Smoker	Control Non-Smoker	Patient Non-Smoker
A/A	7	8	77	27
A/G	6	19	29	25



	Control Smoker	Patient Smoker	Control Non-Smoker	Patient Non-Smoker
A/A	8	6	44	14
A/G	3	12	51	29
G/G	2	7	14	8



	Control Smokers	Patient Smokers	Control Non-Smoker	Patient Non-Smoker
A/A	9	21	90	31
A/G	5	6	12	20

Figure 5. 2: *Genotype correlation of smokers v/s non-smokers in control and patients (A) AR genotype in Smokers vs Non smoker, (B) PSA genotype in Smokers vs Non smoker), (C) ERβ genotype in Smokers vs Non smoker.*

5.4.5 Genotype- Phenotype correlation:

We also investigated the correlation between the SNPs in the three prostatic genes and their phenotypes by mRNA expression of the three genes and protein profile of AR and p63 from prostate tissue samples collected during TURP. Total twenty subjects were assessed for the study. The expression level (arbitrary units) for each genotype of the three genes (AR, PSA and ERβ) were calculated using ImageJ software and was normalized with GAPDH expression *(Fig 5.3)*. We found significant increase of AR mRNA expression in patients A/G gentype of with mRNA expression of PSA was found to be significantly increased in A/G genotype compared to A/A and G/G genotypes among the patients, whereas these genotypes did not display any significant difference in ERβ genotype *(Fig. 5.4).*

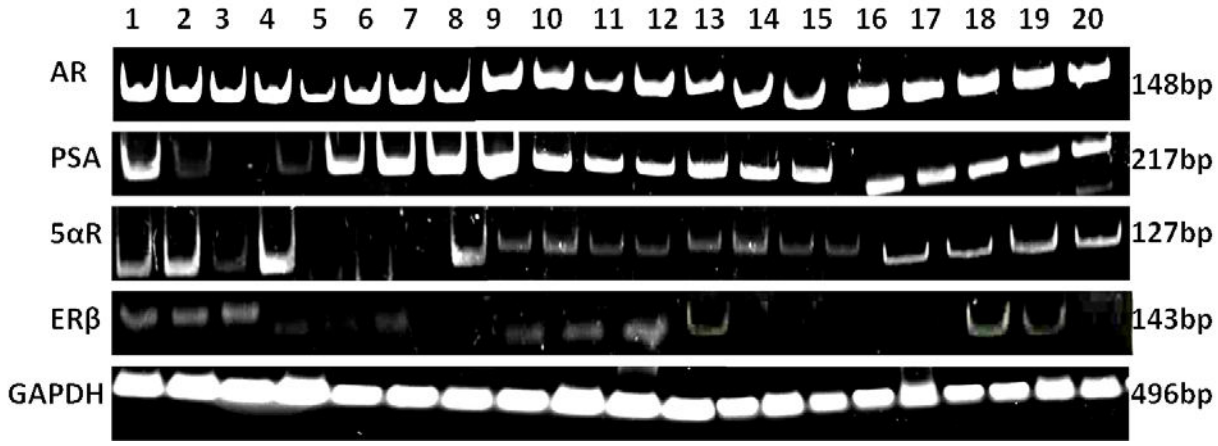


Figure 5. 3: *Human TURP sample gene expression of AR, PSA, 5α Reductase and ERβ. Internal control: GAPDH.*

Furthermore, we also tried to correlate protein profile of AR and p63 with genotype. Our result showed significant increase of AR protein expression in A/G genotype of AR gene, which was further, supported our mRNA expression data and indicating its role in BPH progression *(Fig 5.6)*. p63 protein profile showed significant increase in A/G genotype compared to A/A in patients *(Fig 5.5*). However, no significant difference could be observed in 5α Reductase with both the genotypes.

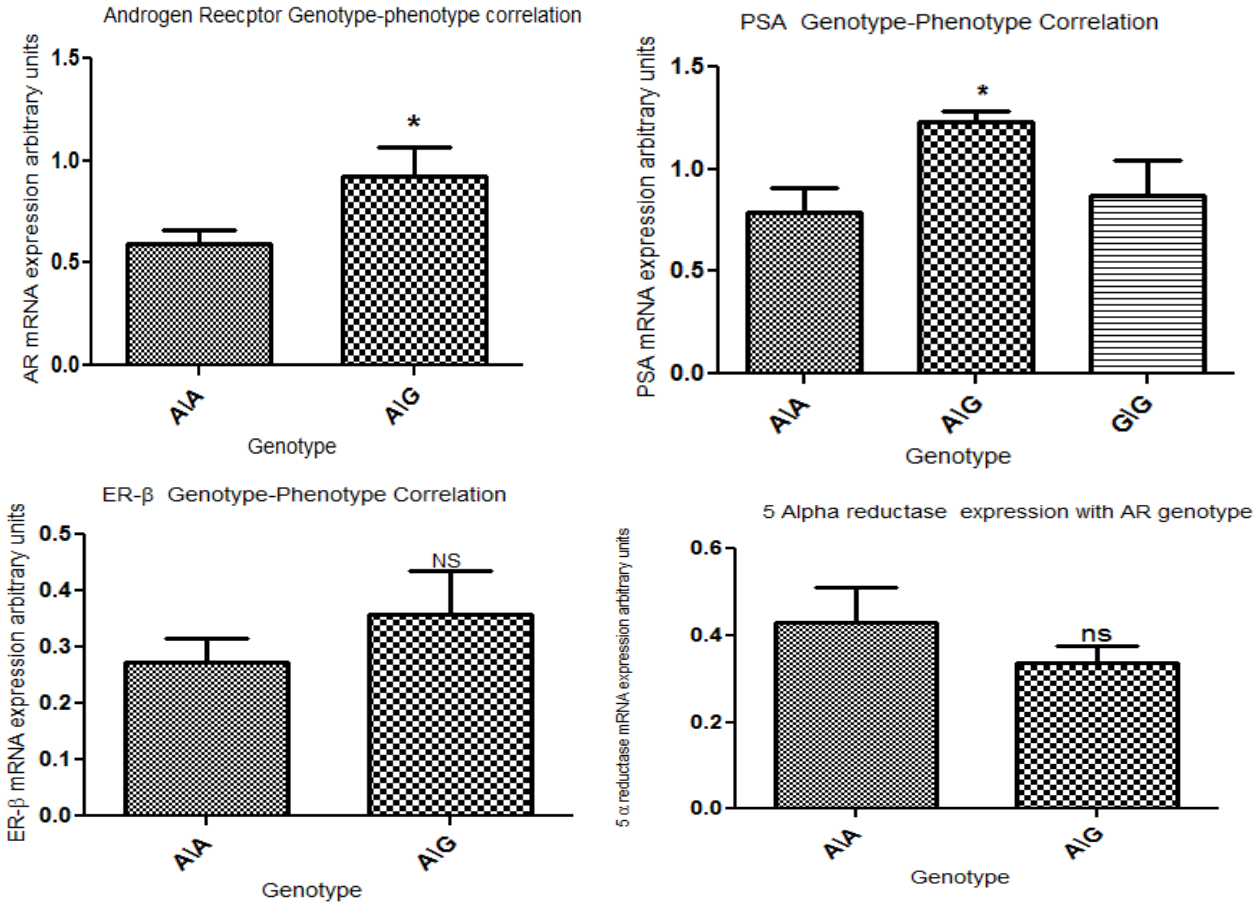


Figure 5. 4: *Genotype Phenotype correlation of AR (*p<0.05, A/G vs A/A), PSA(*p<0.05, A/G vs A/A), ERβ and 5α Reductase genes. (ns; Non-significant)*

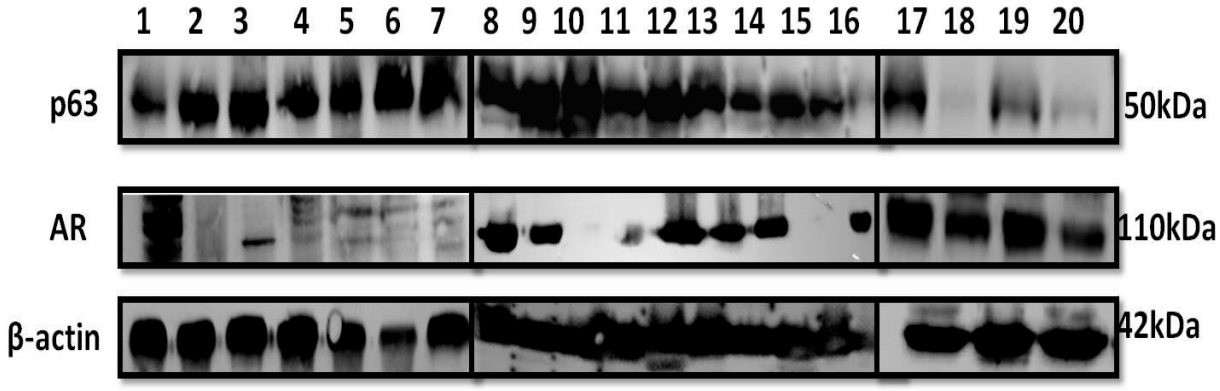


Figure 5. 5: *Human TURP sample protein profile of AR and p63 genes. Internal control: β Actin.*

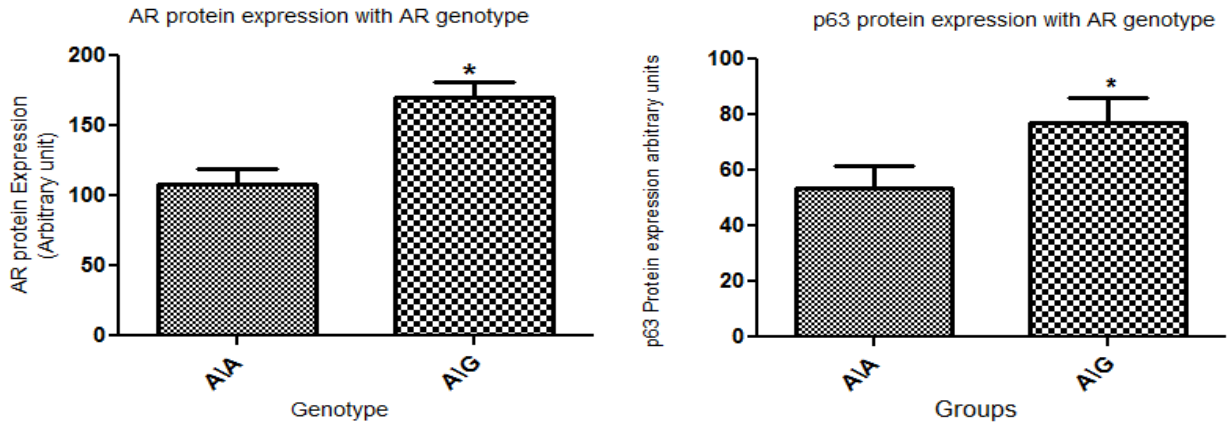


Figure 5. 6: *Correlation of AR and p63 protein expression with AR genotype. (*p<0.05, A/G vs A/A)*

5.5 Discussion

The geographical distribution of prostate cancer is not consistent worldwide, with a higher incidence in some regions such as USA, and lower incidence in Asian countries. Asian population has a high susceptibility for BPH (Pandya et al. 2013). There is a striking difference in Prostate cancer risk between different racial and ethnic groups, with African American men reported 40 to 60 fold higher incidence rates than those reported for Asian men (Ruijter et al. 1999). Till date, there is no genetic marker(s) identified for early diagnosis of BPH in humans.

To address the underlying genetic cause of benign prostate burden in western part of Indian population, we obtained blood samples from a cohort, and looked for polymorphisms of prostatic genes, Androgen receptor (AR), Prostate Specific Antigen (PSA), Estrogen Receptor β(ERβ). A strict exclusion and inclusion criteria were implemented for subject selection. The polymorphism in the AR gene is in exon 1 which forms the NH_2-terminal domain of the protein. This domain (exon 1) harbors the major transcription activation functions and it also binds to the COOH-terminal LBD (N/C interaction). The hormone dependent interaction of the NH_2-terminal domain with the ligand binding domain can play a role in stabilization of the androgen receptor dimer complex by slowing the rate of ligand dissociation and decreasing receptor degradation (Doesburg et al. 1997; Centenera et al. 2008). If the SNP under study affects any of these interactions, it will result in increased cell proliferation, as the growth of the prostate gland is dependent on circulating androgens and intracellular steroid signalling pathways mediated through the androgen receptor (AR) (Chen et al. 2002; Konwar.R et al. 2008). AR also regulates

the genes like 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) for glutamic acid. The E213 G/A SNP (rs6152) SNP has been studied in different population worldwide. A very 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 5.8)*. We found significant association of AR polymorphism (rs6152) with 3 fold increased risk of BPH progression in western Indian population.

The PSA gene, a member (hK3) of the human kallikrein (hK) gene family, is located at the 19q13.41 chromosome 19 (Klobeck et al. 1989; Lilja 2003). It encodes 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* gene promoter. The most proximal of the three AREs in the *PSA* promoter—ARE1, which is lying 170 BP upstream of the transcription start site and has two allelic variants: AGAACAnnnAGT**A**CT and AGAACAnnnAGT**G**CT. These allelic variants are known to have a transcriptional control role for AR-regulated expression (Xu et al. 2002). So, it is possible that the AR binds these two alleles with differing affinities, producing quantitative differences in *PSA* mRNA expression. Association studies of the A-158G polymorphism in ARE1 of PSA promoter have demonstrated that men with an A/G genotype had a 2.4-fold increased risk (95% CIs 51.23–4.81) of developing prostate cancer (Lai et al. 2007). Therefore, ARE mutations interfering with the PSA regulation lead to an increase in PSA secretion. An increase in PSA secretion will eventually cause prostate cells to develop into tumor tissue (Xu et al. 1998; Lange et al. 1999). PSA also plays a role in normal prostate growth and possibly in prostate carcinogenesis (Peehl 1995). 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 is also known to cause AR over expression, thus, leading to enhanced AR activity and increased cell growth (Culig et al. 1994). Our results are concordance with above cited literatures.

Xue *et. al.,* has reported that A/G genotype of AR with A/G genotype of PSA may lead to rapid and early disease progression (Xue et al. 2000). However, a recent report from D. Alptekin et al. 2012 has reported no statistically significant difference between controls, BPH and adenocarcinoma for the PSA A/G polymorphism in Turkish patients (Alptekin et al. 2012). Similarly in our study no statistically significant association has been observed. In the A/G genotype frequency of PSA was 54 % in patients study(OR 2.0, 95% CI 1.07-3.74);*(Table 5.8).*

ERβ has an anti-proliferative role, which maintain the homeostasis of the prostate gland (Weihua et al. 2001). Bardin *et. al.,* 2004 suggested that there is reduced expression of ERβ on the epithelial cell layer of the prostate gland, leading to more proliferation of prostate cells causing prostate enlargement (Bardin et al. 2004). A very recent study by Safarinejad MR et al 2012 on Iranian population demonstrated significant risk of prostate cancer with 1730 A/G 3'UTR in Exon -8 (rs 4986938) SNP in ERβ gene. The polymorphisms of ER in the 3 'un-translated regions (3'-UTR) are known to affect mRNA stability (Safarinejad et al. 2012). It was reported that this polymorphism presumably had no functional implication, but its allele might be in linkage disequilibrium with relevant mutations in the gene. The specific control sequence of the ERβ mRNA degradation pathway was found to be located in the 3' UTR region (Kenealy et al. 2000).

This mutation has also studied by D. Surekha et al 2009 with reduced ERβ gene expression in south Indian cohort leads to an increased risk for breast cancer development (Surekha et al. 2009).

Thus, if this SNP in ERβ, decreases the stability of ERβ mRNA, it would decrease the anti-proliferation signals and will thus lead to increased cell growth in prostate gland. We have also found the A/G genotype frequency of ERβ to be 52.6 % (OR 6.5, 95% CI 3.27-12.74) *(Table 5.8)* and significant association of disease pathogenesis had been observed with A/G genotype.

Smoking behaviour is influenced by both genetic and environmental factors. Moreover, smoking initiation and its persistence have at least 50 percent heritability. Our comparative study between SNP and smoking habit further strengthen the fact. We observed higher percent of associated genotype with smoking habit of the patients.

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 the gene and protein expressions.

In order to correlate the genotype with the phenotype, we compared SNP data with mRNA expression of the respective genes. Interestingly, we found significant increase in protein and gene expression of AR with A/G genotype, increase PSA gene expression in A/G genotype with no significant ERβ expression in A/G genotype of ERβ. It is also reported in studies where SNP in ERβ, decreases the stability of ERβ mRNA hence, disturbance in proliferation mechanisms. Reports also demonstrated that in benign condition AR protein expression was increased compared to prostate cancer (Gaston et al. 2003; Koochekpour et al. 2014). Similar profile of AR protein had been observed in our study. It is known that ER β has anti proliferative effect in the prostate gland development. In genotype-phenotype correlation study we found no significant changes in ERβ group.

P63 is a prostate stem cell marker and used as a distinctive marker between PCa and BPH. A very 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 (Yu and Jiang 2011) but the exact mechanism is still not known. Increased protein expression of p63 had been observed with A/G genotype of AR in patients compared to A/A, indicating hyperplasia condition and role of AR in pathogenesis of BPH. This is further supported by our study in earlier chapter where p63 expression was observed in BPH cell line.

5α Reductase type II, is an enzyme responsible for conversion of Testosterone into DHT. However, no significant difference in 5α Reductase type mRNA was observed with AR SNP genotype. similarly reports from Luo and colleagues found no significant difference in the 5α Reductase mRNA level between BPH and normal samples (Luo et al. 2003). Additionally, it remains controversial how the 5α Reductase level change with PCa development and progression. Wako *et al.* recently found no significant change in both 5α Reductase-I and II level between localized prostate cancer samples and normal prostate tissue (Wako et al. 2008)

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

In conclusion, the association of BPH pathogenesis and severity can be due to the alteration in multiple candidate prostate genes which leads to the pathology of the prostate gland at transcriptional and translational level. Further this study can be used for future therapies for BPH pathogenesis and to design personalised medicine.

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