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Original Article

Genetics Section

Association of Androgen Receptor (AR) CAG Repeats and Cytochrome P450 3A5*3 (CYP3A5*3) Gene Polymorphisms in South Indian Men with Prostate Cancer

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ABSTRACT

Introduction: Prostate Cancer (PCA) is the second most common cancer diagnosed in men worldwide. Prostate Specific Antigen (PSA) is the readily used biomarker for PCA screening, but lack of specificity limits its usefulness. Hence, role of Androgen receptor (AR) CAG repeat polymorphism and Cytochrome P450 3A5*3 gene polymorphism involved in the metabolism of testosterone, growth and differentiation of prostate gland were evaluated to assess their association with pathogenesis of prostate lesions.

Aim: To determine CAG repeats in AR gene and CYP3A5*3 gene polymorphism in South Indian men with PCA and Benign Prostate Hyperplasia (BPH).

Materials and Methods: Genomic DNA was isolated from 312 (100 men with PCA, 109 BPH patients and 103 controls). Formalin fixed paraffin embedded tissue/peripheral blood samples using salting out method were used. Polymerase Chain Reaction (PCR) procedure was carried out using specific primers for AR and CYP3A5*3 genes. Bands were visualised by electrophoresis on 2% ethidium bromide (EtBr) stained agarose gel. Odds ratios were calculated using MedCalc^{R} version 18.2.1 and Chi-square analysis was carried out to determine the association among groups.

Results: AR gene with <22 CAG repeats and 'GG' genotype of CYP3A5*3 gene polymorphism were identified in 61 (61%), PCA patients, 11 (10%) BPH and 13 (13%) control individuals. A statistically significant association was observed between AR short repeats and CYP3A5*3 'GG' genotype with PCA (p-value <0.05).

Conclusion: The results suggest that the best age for PCA screening is 48 years or above for early detection and prevention of this malignancy. A <22 CAG repeats in AR gene and 'GG' genotype of CYP3A5*3 (rs#776746) gene can be together considered as specific molecular marker for identifying men at a risk of developing PCA.

Keywords: Benign prostate hyperplasia, Genetic markers, Polymorphism, Prostate specific antigen

INTRODUCTION

PCA is the second most frequently diagnosed cancer among men worldwide and its incidence is higher in Western as compared to Asian population [1]. According to GLOBOCAN 2018, it is estimated that the incidence of PCA cases is almost 1.3 million with a mortality rate of 3,59,000 per 1,00,000 men in 2018 and it is the fifth leading cause of cancer related death in men [2]. Age and ethnicity play a key role in the incidence and mortality of PCA [3,4]. Over the past decade, there has been a significant decrease in the incidence of PCA in the developed countries due to enhanced awareness, broad implementation of prostate screening and the development of comprehensive cancer screening strategies [3]. However, in developing countries like India, due to lack of such screening strategies, the actual prevalence has yet to be established.

Enlargement of the prostate gland also known as BPH is a common pathology affecting elderly men above 50 years of age that often progress to a characteristic pattern of growth to develop into PCA [5,6]. Apart from age, the major risk factors for prostate pathologies are ethnicity, family history and genetics [6]. It was known that inherited factors contribute about 5-9% to the aetiology of PCA [7]. Alterations in DNA sequence underline the development of PCA. Ample studies suggests that CAG repeat polymorphisms in AR gene and cytochrome P4503A5*3 (CYP3A5*3) gene polymorphism are involved in the metabolism of androgens such as testosterone, and influence the risk of PCA thus making them novel candidate genes for PCA risk assessment [4,8,9].

AR gene located on chromosome Xq11-12 encodes a four domains protein known to be involved in androgen signaling that regulates male secondary sexual characteristics [10]. AR binds to androgens, mainly testosterone and 5-α-dihydrotestosterone and plays a fundamental role in the growth, differentiation and maintenance of normal and malignant growth of the prostate [11,12]. AR gene has a triplet CAG repeat in its exon 1, which regulates the expression of AR. AR gene with shorter CAG repeats have higher transcription activity, and while high number of CAG repeats leads to decreased testosterone activity [10].

CYP3A5*3 gene located at 7q21-22 belongs to a multi-gene family of cytochrome P450. It is most abundantly expressed in liver, small intestine and to a lesser extent in normal prostate cells implying a more intense and localised influence on the prostate gland [13]. CYP3A5*3 is involved in drug metabolism, and oxidation and inactivation of testosterone to a less biologically active form like 2β, 6β, or 15β -hydroxytestosterone, which are readily eliminated from the body. CYP3A5*3 sequence variant 6986A>G affects the enzyme activity and increases the bioavailability of testosterone hence it serves as an important aetiological factor in development of PCA [13-15].

Based on these findings, the present study was conducted to evaluate CAG repeats in AR gene and CYP3A5 gene 6986A>G variation in men with PCA and BPH to determine their association with cancer and benign prostate pathologies as compared to controls.

MATERIALS AND METHODS

This case-control study was conducted at the Department of Genetics and Molecular Medicine, Kamineni Hospitals, Hyderabad, Telangana, India, from January 2014 to December 2016. Ethical approval was obtained from the Institutional Ethics Committee of Kamineni Hospitals (Registration Number: ECR/58/Inst/AP/2013) Hyderabad, India.

The sample size was calculated based on the formula n=z*sigma/5 by taking seven studies into consideration. The estimated sample size was 87 hence the study was carried out on 100 PCA patients and 109 BPH cases with 95% CI. Apparently healthy 103 men without any prostate problems visiting the Department for the Master Health Checkup (MHC) were recruited as controls. An informed verbal consent was obtained from the participants of the study.

PCA and BPH samples were collected from clinically identified men, who were radiologically diagnosed by Trans Rectal Ultrasonography (TRUS) or Trans Urethral Resection of Prostate (TURP) and confirmed histopathologically by salting out method collected from men above 40 years of age with a normal ultrasound of abdomen, PSA levels <4ng/mL and without any prostate related problems.

Isolation of Genomic DNA

Genomic DNA was extracted from 312 formalin fixed paraffin embedded tissue/peripheral blood samples by method, previously standardised by our group. The amount of DNA was quantified using NanoDrop™ 2000/2000cc, (Thermo Scientific™, model number: ND-2000) and stored at -20°C deep freezer until further analysis [16,17].

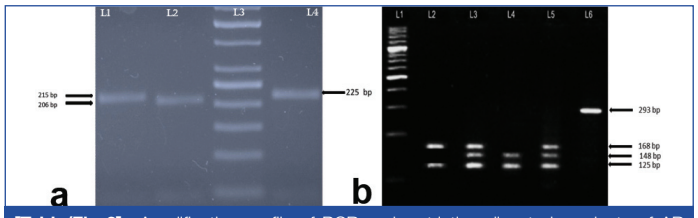
AR Gene CAG Repeats and CYP3A5*3 Gene 6986A>G Polymorphism Genotyping

A three-step PCR procedure was carried out in a gradient PCR thermal cycler, (Applied Biosystems 9902 Veriti) using AR and CYP3A5*3 specific-gene primers purchased from BioArtis Life Sciences Private Limited, Hyderabad, India [Table/Fig-1] [18,19].

Gene	Orientation	Primers (5'-3')	Reference
AR gene	Forward	TGCGCGAAGTGATCCAGAAC	[18]
	Reverse	CTTGGGGAGAACCATCCTCA	
CYP3A5*3	Forward	CATCAGTTAGTAGACAGATGA	[19]
	Reverse	GGTCCAAACAGGGAAGAAATA	

[Table/Fig-1]: Forward and reverse primers for AR and CYP3A5*3 gene [18,19].

Restriction Fragment Length Polymorphism (RFLP), of CYP3A5*3 gene polymorphism was carried out using SsPI restriction enzyme (Thermo ScientificTM catalog number ER0771) [19]. Amplified PCR and restriction digestion products were separated on 2% agarose gel by electrophoresis [17]. Gel was photographed using UVITEC gel documentation system [Table/Fig-2].



[Table/Fig-2]: Amplification profile of PCR and restriction digested products of AR and CYP3A5*3 gene polymorphisms. a) AR gene CAG repeats. Lane 1: Band size 215 bp Allele 23 (A23) has 23 CAG repeats, Lane 2: 206 bp Allele 22 (A22) has 22 CAG repeats, Lane 3: 50bp DNA ladder, and Lane 4: 225 bp Allele 24 (A24) has 24 CAG repeats. b) CYP3A5*3 (rs#776746) gene polymorphism. Lane 1: 100bp DNA ladder, Lane 2: Homozygous GG genotype (168/125 bp), Lane 3 & Lane 5: Heterozygous AG genotype (168/148/125) bp), Lane 4: Homozygous AA genotype (148/125 bp) and Lane 6: Undigested PCR product size 293 bp.

STATISTICAL ANALYSIS

Odds Ratios (OR) and Chi-square analysis tests were used to determine association between groups with 95% Confidence Intervals (CIs) using MedCalc® statistical software version 18.2.1 [17].

RESULTS

Baseline Characteristics of the Participants

The risk factor for onset of prostate pathologies and cancer increases with age. In the present study age range of participants was 40 to 87 years. The mean age of PCA patients was 69.46±9.25 years, while that of individuals with BPH was of 66.99±8.30 years Right, 72 (72%) PCA and 71 (65%) BPH cases were aged ≥65 years [Table/Fig-3].

Clinically, PSA level ≤4 ng/mL is considered as normal by the National Institutes of Health, USA. In the present study, PSA levels ranged from 0.09 ng/mL to one of the cases showing 1230 ng/mL. The mean PSA of controls was 50.28±51.51 ng/mL, 20.48±19.06 ng/mL and 3.86±13.36 ng/mL in PCA, BPH and control groups, respectively. Majority of the PCA 94 (94%) and BPH 88 (81%) cases had PSA levels >4 ng/mL as opposed to 12 (12%) control individuals [Table/Fig-3].

Clinical parameters	Groups		
	PCA (n=100)	BPH (n=109)	Controls (n=103)
Age			
<65 years	28 (28%)	38 (35%)	87 (84%)
≥65 years	72 (72%)	71 (65%)	16 (16%)
PSA			
≤4 ng/mL	6 (6%)	21 (19%)	91 (88%)
>4 ng/mL	94 (94%)	88 (81%)	12 (12%)
PV			
≤25cc	18 (18%)	14 (13%)	100 (97%)
>25cc	82 (82%)	95 (87%)	3 (3%)
GS	(n=81)	NA	NA
<7	13 (16%)		
≥7	68 (84%)		

[Table/Fig-3]: Categorization of PCA, BPH and controls individuals on the basis of clinical parameters.
PCA: Prostate cancer; BPH: Benign prostate hyperplasia; GS: Gleason score; PCA: Prostate cancer; PSA: Prostate specific antigen; PV: Prostate volume; NA: Not applicable

Enlargement of prostate gland diagnosed by ultrasound is another marker for Prostate Cancer. In adult men, Prostate Volume (PV) depicts prostate size and PV ≤25cc is considered normal. In the present study, PV levels ranged from 10cc to 210cc, with mean PV of 45.33±27.50cc, 50.81±25.76cc and 18.02±3.51cc in PCA, BPH and control groups, respectively. Prostate enlargement was observed in 82 (82%) PCA and 95 (87%) BPH cases, while 100 (97%) control individuals had normal prostate size [Table/Fig-3].

Histopathological grading of the PCA was based on the Gleason Score (GS) that ranges from 5 to 10 and helps in assessing aggressiveness of the cancer [20]. Based on the International society of urological pathology (ISUP), GS is categorised as: (i) <7; and (ii) ≥7 and in the present study, 68 (84%) of the PCA cases had a GS of ≥7 which indicates aggressive state of disease [Table/Fig-3].

AR gene CAG Repeats

AR gene amplified PCR products had a band size ranging in length from 85bp (9 CAG repeats) to 300bp (32 CAG repeats) in cases and controls. A <22 CAG repeats corresponding to increased transcriptional activity was observed in 94 (94%) PCA cases, in contrast to only 20 (18%) BPH cases and 18 (17%) control individuals. The <22 CAG repeats of AR gene were found to be significantly associated with PCA (OR-73.9815, 95%CI- 28.0617 to 195.0436) (p-value <0.0001) [Table/Fig-4].

CYP3A5*3 Gene 6986A>G Polymorphism (rs#776746)

The frequency of 'G' allele from control population was established as 0.54. In PCA cases, the frequency of 'G' allele was observed to be 0.77 and 0.66 in BPH cases [Table/Fig-5]. The risk ratio for the individuals with GG genotype was found to be seven fold higher when compared to AG and AA genotype in our study population. Dominant model of inheritance explains the 'G' allele association with PCA.

Groups	<22 CAG repeats	≥22 CAG repeats	Categories, Odds ratio (OR), 95%CI	p-value
PCA (n=100)	94 (94%)	6 (6%)	PCA vs. controls OR- 73.9815, (95% CI- 28.0617 to 195.0436)	<0.0001*
BPH (n=109)	20 (18%)	89 (82%)	BPH vs. controls OR- 1.0612, (95%CI- 0.5255 to 2.1428)	0.8524
Controls (n=103)	18 (17%)	85 (83%)	PCA vs. BPH OR- 69.7167, (95%CI- 26.7668 to 181.5836)	<0.0001*

[Table/Fig-4]: AR gene CAG repeats analysis in PCA, BPH and control groups.
OR (Odds ratios) *p-value <0.05 statistically significant. PCA: Prostate cancer; BPH: Benign prostate hyperplasia

Groups	Allele frequency		Allelic frequency 'G' vs. 'A' allele		p-value
	G	A	Categories	Odds ratio, 95%CI	
PCA (n=100)	155 (0.77)	45 (0.23)	i) PCA vs. controls	OR- 2.9479, 95%CI- 1.9170 to 4.5333	<0.0001*
BPH (n=109)	145 (0.66)	73 (0.34)	ii) BPH vs. controls	OR- 1.7000, 95%CI- 1.1480 to 2.5175	0.0081
Control (n=103)	111 (0.54)	95 (0.46)	iii) PCA vs. BPH	OR- 1.7341, 95% CI- 1.1224 to 2.6793	0.0131
Groups	**Genotype frequency**			**Dominant model**	**Odds ratio, 95% CI**	**p-value**
	AA	**AG**	**GG**	**GG+AG vs. AA**		
PCA (n=100)	8 (8%)	29 (29%)	63 (63%)	i) PCA vs. controls	OR- 7.9180, 95%CI- 3.4789 to 18.0217	<0.0001*
BPH (n=109)	27 (24.77%)	19 (17.43%)	63 (57.8%)	ii) BPH vs. controls	OR- 2.0911, 95%CI- 1.1636 to 3.7578	0.0136
Control (n=103)	42 (40.77%)	11 (10.68%)	50 (48.55%)	iii) PCA vs. BPH	OR- 3.7866, 95%CI- 1.6294 to 8.7998	0.0020

[Table/Fig-5]: Genotype frequency of CYP3A5 gene in PCA, BPH and control groups.
OR (Odds ratios) *p-value<0.05 statistically significant. PCA: Prostate cancer; BPH: Benign prostate hyperplasia

AR, CYP3A5*3	<22, GG	<22, AG	<22, AA	>22, GG	>22, AG	>22, AA
PCA (n=100)	61 (61%)	27 (27%)	6 (6%)	4 (4%)	1 (1%)	1 (1%)
BPH (n=109)	11 (10%)	5 (4%)	4 (4%)	52 (48%)	14 (13%)	23 (21%)
Control (n=103)	13 (13%)	0 (0%)	5 (5%)	37 (36%)	11 (10%)	37 (36%)

Short CAG repeats (<22) in AR gene and with 'GG' genotype in CYP3A5 gene in groups	Comparison of proportions in categories- 95%CI	p-value
PCA (n=100) <22, GG (n= 61) (61%)	i) PCA vs. controls- 50.105, 95%CI- 35.4494 to 58.3715	<0.0001*
BPH (n=109) <22, GG (n=11) (10%)	ii) BPH vs. controls- 0.468, 95% CI- N5.7604 to 11.9643	0.4941
Control (n=103) <22, GG (n=13) (13%)	iii) PCA vs. BPH- 57.095, 95%CI- 37.8113 to 60.0906	<0.0001*

[Table/Fig-6]: Association of <22 CAG repeats in AR gene and 'GG' genotype of CYP3A5 gene in PCA cases.
Chi-square analysis *p-value<0.05 statistically significant. PCA: Prostate Cancer; BPH: Benign Prostate Hyperplasia

Assessment of Short CAG Repeats (<22) in AR Gene with 'GG' Genotype in CYP3A5 Gene Polymorphisms in Cases and Controls

In the present study, 61 (61%) PCA cases, 11 (10%) BPH cases and 13 (13%) control individuals had <22 CAG repeats and 'GG' genotype. Chi-square analysis showed that AR gene with <22 CAG repeat polymorphism and the GG genotype of CYP3A5*3 was found to be significantly associated with PCA (X^2=50.105, 95%CI-35.4494 to 58.3715) (p-value<0.0001) [Table/Fig-6].

DISCUSSION

PCA is a heterogeneous cancer in men that progresses gradually with age. Several candidate genes are associated with PCA risk, and in the present study genes linked with androgen metabolism leading to PCA cell growth and progression were evaluated. The mean age of men at the time of diagnosis was 69.46±9.25 years, which was similar to Finland, Philadelphia and Australian population where the mean age was 68.8 years, 62.9±8 years and 63 years, respectively [2,21,22]. Based on these values, age at diagnosis was considered as >65 years. Since the estimated age of screening is calculated as mean age ±2xSD, the men aged 48 years or above are recommended to screen for PCA. National cancer institute (NIH) recommends PCA screening in men >50 years of age and with elevated PSA levels [23,24].

In our study, both PCA and BPH patients had PSA levels >4 ng/mL indicating that PSA is not a specific marker for prostate malignancy. According to NIH, there is no specific normal or abnormal level of PSA, but a clinical value of ≤4 ng/mL is taken as normal and that of >4 ng/mL requires a follow-up biopsy [25]. PSA is the only marker currently available and routinely used in screening and diagnosis of prostate Pathologies, however, it lacks specificity, and cannot differentiate between prostatitis, benign and malignant tumours which leads to over diagnosis and overtreatment [26,27]. Therefore, a better and more specific molecular genetic marker for the assessment of PCA risk in men is the need of the hour.

CAG repeats in AR and CYP3A5*3 gene polymorphisms involved in testosterone metabolism were evaluated for PCA risk assessment. A meta-analysis study in Asian population, reported that short number of (<22) CAG repeats in AR gene and CYP3A5 6986A>G variation along with conventional risk factors are associated with increased risk of PCA [28]. Numerous studies report similar findings in Macedonian, Mexican, Indian and Taiwanese men [29-32].

A meta-analysis of worldwide population based sequencing on CYP3A5*3 revealed a Minor Allele Frequency (MAF) of 100% in South Asians, 98.6% in East Asians, 99.5% in Europeans, 41.2% in Africans and 96.7% in admixed Americans [33]. According to 1000G and GnoMAD database MAF of CYP3A5*3 the frequency of allele 'G' is 62.1% and 73.47%, respectively. The 'GG' genotype of CYP3A5*3 gene was documented to play a central role in increasing the bioavailability of testosterone; thereby increasing the PCA cell growth and progression [15,34].

Previous literature reports a significant association of 'GG' genotype of CYP3A5*3 polymorphism with PCA and BPH in dominant mode indicating a role of testosterone metabolism in prostate pathologies. In the present study, CYP3A5 gene polymorphism was found to be associated with increased risk of developing PCA and these findings were in agreement with various other studies [13,15,34-37].

Primarily used Androgen Deprivation Therapy (ADT) for treatment modality for PCA show a positive initial response but inevitably relapse to an androgen-independent phenotype, and ultimately fails to suppress the presence of androgens at the tissue level. Therefore, genotypic analysis of CAG repeats of AR and GG genotype of CYP3A5 gene polymorphisms can also help in development of personalised therapy in treatment and management of the disease [14,38,39].

LIMITATION

Sample size was relatively small and study population was restricted to patients visiting the hospital. Further cohort studies on different ethnic groups and larger sample size are required for better understanding of the disease.

CONCLUSION

AR gene with <22 CAG repeats and GG genotype of CYP3A5*3 gene polymorphisms together can be used as a specific molecular marker for screening, diagnosis and management of PCA. Men over the age of 48 years, with short (<22) CAG repeats in AR gene and 'GG' genotype in CYP3A5*3 gene polymorphism are at an increased risk of developing PCA and require regular surveillance for risk assessment.

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