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Research Article

Genetic Association Study of Two Variants within *RFX6* and *SLC22A3* for Their Potential Role in Prostate Cancer Predisposition

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Abstract

Prostate cancer is one of the leading causes of cancer-related deaths among men worldwide. Research has shown that genetic variations can increase an individual's risk of developing this disease. In this study, we investigated the potential role of two genetic variants, one within *RFX6* and another within *SLC22A3*, in predisposition to prostate cancer. A genetic association study was conducted, involving a case-control design with 112 prostate cancer cases and 95 individuals affected by benign prostatic hyperplasia who served as controls and had no history of cancer. The genotypes of the two variants, rs339331 in *RFX6* and rs9364554 in *SLC22A3*, were determined using tetra-primer ARMS-PCR. In this study, a multi-stage strategy was employed to analyze the data obtained from genotyping and to assess the association of these two variants with prostate cancer risk. For statistical analysis, Chi-squared, Fisher's exact test and logistic regression were performed to evaluate the association of variants with prostate cancer and Gleason score. The results suggest that the rs9364554 variant in *SLC22A3* is not associated with prostate cancer risk under either additive or multiplicative genetic models, while the variant in *RFX6* is significantly associated with prostate cancer susceptibility. The TT genotype of rs339331 indicated a possible protective effect against prostate adenocarcinoma (CI 95% = 0/009 - 0/725, P-value = 0/009, OR = 0/083). In conclusion, our study provides evidence that the genetic variant rs339331 within *RFX6* is significantly associated with prostate cancer susceptibility and may have a potential protective effect against prostate adenocarcinoma. It should be mentioned that more research is needed to investigate the possible protective effect of the TT genotype of rs9364554 against prostate cancer risk in other populations and various ethnic groups and particularly larger sample sizes are required to confirm this association.

***Keywords*:** Prostate Cancer, *SLC22A3*, *RFX6*, Variant, Benign prostatic hyperplasia (BPH)

Introduction

Prostate cancer is the second most commonly diagnosed malignancy among men worldwide after lung cancer. Many studies have shown that prostate cancer is even more common among men in developed countries (Rawla, 2019). According to Farhood et al., the incidence of prostate cancer varies by region. In Iran, as well as some other Asian countries, the incidence rate is lower compared to Western countries. For example, Ardabil, a city in Iran, showed an incidence rate of 3.5 per 100,000 men, and in Fars Province, it is among the ten most common cancers (Farhood et al., 2018). Investigations have reflected that there is a positive relation between the incidence and mortality of prostate cancer with increasing age and the median age at diagnosis is 66 years old (Boyle et al., 2019; Pakzad et al., 2015). Prostate cancer is divided into two categories, depending on the cell of origin. Adenocarcinoma, the most common type of prostate cancer, arises from epithelial cells and stromal sarcoma is a rare type of prostate cancer that arises from stromal cells (Yang et al., 2018).

The adult prostate gland consists of three types of epithelial cells, including luminal, basal, and neuroendocrine cells. Prostate cancers are mainly composed of cells with a luminal phenotype, and it was historically thought that they are the origin of prostate cancer. However, studies in mice models have shown that both basal and luminal cells can

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serve as cells of origin for prostate cancer (Park et al., 2016). Furthermore, tumors of prostate cancer commonly appear multifocal, meaning there are multiple tumors in the prostate gland (Andreoiu and Cheng, 2010). At the early stage of prostate cancer, cancer may be asymptomatic (Shore, 2014) and generally inactive, so affected individuals may not require treatment or may need minimal treatment. However, men in this period commonly experience difficulty with urination, increased frequency, and enuresis. It is worth noting that these symptoms can be caused by benign prostatic hyperplasia (BPH). This condition is a noncancerous enlargement of the prostate gland which causes urinary tract problems (Langan, 2019) (Allemailem et al., 2021). As prostate cancer progresses, patients may experience symptoms such as urinary retention and back pain because the axial bone is the most common site of metastatic bone (Wong et al., 2019).

Prostate cancer metastasis occurs when cancer cells leave the prostate gland and travel to other parts of the body via the blood or lymphatic system. The most common metastatic sites for prostate cancer are the bones, liver, lung and lymph nodes (Gandaglia et al., 2014). The molecular basis of metastasis involves the interaction between cancer cells and the extracellular matrix (ECM), a network of proteins and other molecules that surrounds the cells. Adhesion proteins such as integrin play an important role in this process by allowing cancer cells to attach to the ECM and invade tissue (Wang et al., 2022). Metastasis is a multi-step process that involves multiple molecular pathways, including the regulation of cell adhesion. Adhesive proteins play an important role in metastasis by facilitating the detachment of cancer cells from the primary site, their invasion into surrounding tissues, their survival in the bloodstream or lymphatic system, and their establishment in a new tissue or organ (Sulekha Suresh and Guruvayoorappan, 2023). Studies have shown that patients with metastatic prostate cancer have a low survival rate of five years (Jin et al., 2020; Sulekha Suresh and Guruvayoorappan, 2023). Due to the high prevalence and low survival rate among patients with metastatic prostate cancer, there is an urgent need to enhance screening and diagnostic techniques. Nowadays, the major screening strategies are based on evaluating serum Prostate-specific antigen (PSA) levels, clinical manifestations, and physical examinations (Ilic et al., 2018).

Investigations have shown that genetic variants can influence the development, progression, and response to treatment of prostate cancer. Approximately 60% of prostate cancer patients have a genetic predisposition, and more than 100 putative single nucleotide polymorphisms (SNPs) have been shown to be associated with a greater risk of prostate cancer development (Allemailem et al., 2021). SNPs are common genetic variations that occur when a single base pair in the DNA sequence is altered. Experiments have shown that these variations can have a profound effect on the enzymatic activity of PSA and thereby influence the development and progression of prostate cancer (Allemailem et al. 2021). Finding variants in the genes that are involved in prostate cancer will help identify individuals who may be at higher risk for developing the disease or progressing to more advanced stages and may aid in the development of more precise screening plans (Villers and Grosclaude 2008).

The aim of this study was to assess the relationship between the genetic variants rs9364554 and rs339331, prostate adenocarcinoma, and clinical data in comparison to benign prostatic hyperplasia by applying a multi-stage approach on the Iranian population. The SNPs investigated in this study are rs339331 (on *RFX6*) and rs9364554 (on *SLC22A3*). These SNPs are located at the 6q locus, which is considered a susceptibility locus for many cancers. *SLC22A3* is seen as a member of the solute carrier family 22 and it performs as a transporter of cation in different organs consisting of prostate tissues. Studies have shown that lower expression levels of this gene were reported among various cancers, particularly in high Gleason-grade prostate cancer. Multiple SNPs of *SLC22A3* have been found in association with alterations of *SLC22A3* mRNA and it also showed association with risk level of prostate cancer, colorectal cancer, coronary artery disease and other human disease (Chen et al., 2013; Grisanzio et al., 2012). On the other hand, the rs339331 regulates the function of *RFX6* by recruitment Androgen receptor (AR), Forkhead box A1 (*FOXA1*) and Homeobox B13 (*HOXB13*) transcription factors and affects cell migration and proliferation (Daram et al., 2020; Li et al., 2019).

Materials and Methods

Patients

This study was carried out on a sample of 205 people. The participants included 112 men with prostate adenocarcinoma in the patient group

(experimental group) and 93 men with benign prostatic hyperplasia in the control group, all referred to Shahid Hasheminejad Hospital in Tehran, Iran. The researchers used a random selection strategy to select the participants. The diagnosis was based on PSA levels, digital rectal examination (DRE), prostate biopsy, and physical confirmation.

Table 1. Primer sequences for genotyping *RFX6* rs339331 and *SLC22A3* rs9364554 variants, and the related amplicon size.

Gene	SNP	Primer	Primer sequence	Amplicon Size (bp)
RFX6	rs339331	Forward outer	TCAGAGTAACCTAGAGGATAAGCATCAGGT	420
		Reverse outer	CAGTTTCTTCTGGAGCCAACAAAATAAC	
		Forward inner	TGCATGAACTCTCTCTCCCCAGTGTT	189 (T allele)
		Reverse inner	CCTCCTAGTCACTAAAGATAAACCTCCTG	286 (C allele)
SLC22A3	rs9364554	Forward outer	AGAACTGGGTTTTGTTGGTTCTATTGTC	481
		Reverse outer	CAAAAGAGCAGAGATTATTCACTGGATG	
		Forward inner	CCACTATGATTAGTCCATCCTTGTCAATT	215 (T allele)
		Reverse inner	ACACAGCTCAAATGTGTTCACTCACAG	322 (C allele)

Therefore, only patients with prostate adenocarcinoma and BPH, without a history of cancer, were selected for this study. In addition, patients' clinical data were evaluated, including serum PSA level, Gleason score, and perineural infiltration (PI) of peripheral blood DNA. The Ethics Committee of the Islamic Azad University - North Tehran Branch approved this study (Approval ID: IR.IAU.TNB.REC.1400.085)

DNA extraction

The FavorPrep™ Blood Genomic DNA Extraction Mini Kit (Favorgen, Taiwan) was used to extract genomic DNA according to the instructions. The quantity of each DNA sample was confirmed by 1.5% agarose gel electrophoresis and the quality was confirmed using a NanoDrop™ spectrophotometer.

SNP genotyping

To determine the SNP genotyping of each sample, the researchers employed the Tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR) assay. The primer design was performed using the Primer1 database and validated through the Primer-BLAST-NCBI database. Four primers were used for genotyping in a single reaction with two non-specific outer primers and two allele-specific inner primers.

The T-ARMS-PCR was performed on a DNA Engine thermal cycler (Eppendorf, Germany) using a reaction volume of 25 µL. The reaction mixture contained 1.5 µL (10 pmol) of each primer, 12.5 µL of Taq DNA Polymerase 2X Master Mix Red (Amplicon, Odense, Denmark), 2 µL of genomic DNA, and 4.5 µL of PCR-grade water. The amplification program started with a pre-denaturation step at 95°C for 10 min, followed by denaturation at 95°C for 30 seconds, annealing at 54°C (rs9364554) and 55°C (rs339331) for 30 seconds, and extension at 72°C for 1 min, for a total of 35 cycles. The program was terminated with a final extension of 5 minutes at 72°C. The PCR products were analyzed by electrophoresis on a 2% agarose gel containing Gel green stain (Ana Cell, Iran) in 0.5X tris/borate/EDTA (TBE) for the separation of the amplified fragments.

Statistical analysis

To evaluate the genotype frequency, allele frequency, Hardy-Weinberg equilibrium (HWE), and genetic models, chi-square and Fisher tests were utilized with a p-value threshold of <0.3 in the first step and <0.05 in the second step. In addition, to examine the association between the SNP and prostate cancer risk, as well as the Gleason score, additive and multiplicative models were applied to calculate the odds ratio (OR) with a 95% confidence interval (95% CI) using regression and chi-square tests. Statistical significance was defined as a p-value of <0.016 in the evaluation of rs339331 in the second step, with a Bonferroni correction used to address multiple-testing issues. Statistical analysis was conducted using SPSS software version 25.

Result

At first, TETRA-ARMS-PCR was performed on 58 patient samples, including 28 cancer samples and 30 BPH samples. The cancer samples were selected based on positive perineural invasion with a Gleason score greater than 7, while BPH samples were selected based on a PSA level of less than 4. The age of the patients in both the case and control groups ranged from 50 to 84 and 47 to 78 years, respectively. The mean age of the case group was 71.77±9.22, while the mean age of the control group was 62.66±7.848.

Statistical analysis (Stage I)

Firstly, a complete set of SNPs was investigated with a liberal P-value threshold of <0.3 to identify any meaningful variants. The frequency of genotypes for these polymorphisms in each of the case and control groups was reported independently in Table 2. The results showed no significant difference in the distribution of the rs9364554 genotype (p=0.741) between the case and control groups. Using the Chi-square test, the prostate cancer (p=0.864) and BPH (p=0.818) groups were found to be in Hardy-Weinberg equilibrium. Therefore, additive and multiplicative genetic models were applied to assess the association between this variant and the incidence of cancer. In this study, the CC genotype at rs9364554 was considered as wild type genotype and accepted as the reference genotype. Based on Table 3 and using logistic regression and Fisher's exact test, rs9364554 was not found to be associated with prostate cancer in either the additive or multiplicative genetic models. Also, rs9364554 was not significant in any of the analyses, so it was removed, and in the next step only the rs339331 polymorphism was assessed

Patients' Characteristics (Stage I)

At this stage, genotyping of the rs339331 polymorphism was performed in the remaining individuals (145 men). The demographic and clinical information of these patients is shown in Table 4.

Table 2. The genotype frequency of polymorphisms calculated for both groups at stage I

dbSNP	Genotype	Case N (%)	Control N (%)	*P-value*
rs9364554	CC	16 (57.1)	20 (66.7)	0.741
	CT	10 (35.7)	8 (26.7)	
	TT	2 (7.1)	2 (6.7)	
rs339331	CC	1 (3.6)	4 (13.3)	0.141
	CT	27 (96.4)	24 (80)	
	TT	0 (0)	2 (6.7)	

Table 3. Multiplicative and additive genetic models

dbSNP	Multiplicative model		Additive model		
rs9364554	C vs T		CC vs TT	CC vs CT	CC
	OR	1.33	1.25	1.56	1 (reference)
	95%CI	0.55-3.99	0.15-9.87	0.5-4.87	
	P-value	0.519	1	0.441	

Table 4: Clinical and demographic information of the participants

		Prostate cancer n	BPH n
PSA (ng/ml)	≤4	10	19
	4.1–10	45	54
	> 10	57	20
Gleason score	< 7	20	-
	7	55	-
	≥8	37	-
Perineural invasion	+	80	-
	-	32	-
Age	<60	17(16.5)	26(26)
	60-90	37(35.9)	41(41)
	>70	49(47.6)	33(33)

Statistical analysis (Stage II)

As the analysis in the first stage was only for the rs339331 polymorphism, all participants (205 males) were involved in the second stage to assess significance. First, the Hardy-Weinberg balance between the two groups was evaluated using the chi-square test. The distribution of rs339331 genotypes in the adenocarcinoma (p=0) and BPH (p=0.014) groups was outside the Hardy-Weinberg equilibrium. The genotype frequencies of these polymorphisms are shown in Table 5.

Based on the additive genetic model, the homozygote TT was found to have a protective effect against prostate cancer when considering CC as a reference genotype. There was a significant difference observed between the genotype frequency TT + CC in the case group compared to the control group (OR [95% CI] = 0.083 [0.009 - 0.725], P=0.009). In Table 6, the genotype and allelic frequency, OR [95%CI] (logistic regression), and P-value (Fisher's exact test) of the risk allele compared to the wild allele for three categories of Gleason score were presented. According to the statistical results shown in Table 6, no significant difference was observed between the frequencies of allele C vs T in all three categories of Gleason score at rs339331.

Discussion

Prostate cancer is one of the most common cancers in men worldwide, and many people die from it each year. Although it is more prevalent among older males, it can also affect younger men (Bleyer et al., 2020). Prostate cancer is a significant public health concern due to its high global incidence and mortality rate. In addition to its high incidence and mortality rate, prostate cancer also poses a challenge for clinicians due to the diverse range of disease presentations and varying clinical outcomes. Thus, developing effective screening strategies and treatments for prostate cancer is crucial in reducing its impact on public health. Although environmental factors such as lifestyle have been associated with prostate cancer risk, genetic variation has also been identified as a significant risk factor. Genome-wide association studies (GWASs) have identified numerous prostate cancer susceptibility loci and implicated many pathways involved in prostate tumorigenesis (Jiang et al., 2022).

Table 5. Genotype frequency of rs339331 and the additive genetic model used in the analysis of the data

dbSNP		**Case n (%)**	**Control n (%)**	***P*-Value**	**Additive model**			
rs339331	Genotype					CC vs TT	CC vs CT	CC
	CC	13 (11.6)	14 (15.1)	0.001	OR	0.083	1.59	1 (reference)
	CT	98 (87.5)	66 (71)		95%CI	0.009-0.72	0.7-3.62	
	TT	1 (0.9)	13 (14)		*P-value*	0.009	0.257	

Table 6. The genotype and allelic frequency, odds ratio (OR) [95% confidence interval (CI)], and P-value of the risk allele compared to the wild allele calculated for three categories of Gleason score

rs339331		**CC n (%)**	**CT n (%)**	**TT n (%)**	**C**	**T**	**OR (CI95%)**	***P*-Value**
Control		13(11.6)	98(87.5)	1(0.9)	94 (50.5)	92 (49.5)	1 (reference)	-
Gleason score	< 7	5 (25)	14 (70)	1 (5)	24 (60)	16 (40)	0.68 (0.34-1.36)	0.277
	7	6 (10.9)	49 (89.1)	0	61 (55.5)	49 (44.5)	0.82 (0.51-1.31)	0.413
	≥8	2 (5.4)	35 (94.6)	0	39 (52.7)	35 (47.3)	0.91 (0.53-1.57)	0.753

Table 7. Genotype distribution of rs339331 and p-value in relation to the mentioned clinical feature

rs339331		**CC**	**CT**	**TT**	***P-value***
Perineural invasion	+	6	25	1	0.085
	-	7	73	0	

Here we analyzed two variants within *SLC22A3* (rs9364554) and *RFX6* (rs339331). The gene *SLC22A3* is present in a wide range of tissues, such as the liver, prostate, skeletal muscle, brain, and heart. Currently, researchers are investigating the function of this gene in relation to prostate cancer (Eeles et al. 2008). Various SNPs in this gene have been investigated for association with colorectal cancer risk, coronary artery disease and prostate cancer (Grisanzio et al. 2012; Haiman et al. 2011; Huang et al. 2014; Nies et al. 2009; True et al. 2006; Waters et al. 2009). Our results showed no significant association between rs9364554 and the risk of prostate cancer in the Iranian population. Our findings are consistent with previous studies on Japanese and African populations (Lindström et al. 2012). For *RFX6*, previous studies showed altered expression level of *RFX6* contributes to cancer progression. Huang et al. found that rs339331 can affect *RFX6* expression via interaction with *HOXB13*. Up to now, multiple studies showed this variant has a significant association with prostate cancer among Iranian, African, European, and Chinese populations (Huang et al., 2014).

The current study's results showed that there was a significant difference in rs339331 *RFX6* genes between the control and patient groups. Our findings also support previous studies. The TT genotype indicated a protective effect against prostate adenocarcinoma (CI 95% = 0.009-0.725, P-value = 0.009, OR = 0.083). It should be mentioned that more research is needed to investigate the possible protective effect of the TT genotype of rs9364554 against prostate cancer risk in other populations and various ethnic groups and particularly larger sample sizes are required to confirm this association.

Conflict of interest
None.

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