APAI POLYMORPHISM FREQUENCIES AND PROSTATE CANCER PATIENTS IN PUNJAB, PAKISTAN

MAZHAR, M. W.[1*] – TAHIR, H.[1] – MAHMOOD, J.[1] – SHAHZADI, M. B.[1] – SAIF, S.[1]

[1] *Department of Biotechnology and Bioinformatics, Government College University, Faisalabad, Pakistan.*

**Corresponding author*
e-mail: waqarmazhar63[at]gmail.com

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Abstract. Prostate cancer is the third most common malignancy in Pakistani males. Vitamin D receptor (VDR) gene has been a subject of extensive pharmacogenetic research recently. Association studies between different types of cancers including prostate cancer (PCa) and VDR gene polymorphism have also great importance. Vitamin D has an anticancer effect, so VDR gene polymorphisms have got much attention. It is proposed that vitamin D deficiency may underlie the major risk factors for prostate cancer, including age, black race and genetic variation in vitamin D-binding protein. Clinical diagnosis of prostate cancer can be done by PSA and biopsy. The clinical diagnosis does not provide a definitive diagnosis of progression of PCa. Most common symptoms of prostate cancer are Nocturia (increased urination at night), difficulty in urination, Hematuria (blood in urine), and Dysuria (frequent and painful urination). It may influence sexual function, for instance, trouble in accomplishing an erection or agonizing discharge. Advance prostate cancer may spread to other organs of the body, causing pain in pelvis or ribs. In Benign prostate hyperplasia, prostate enlarges and cause urinary symptoms. Diagnosis of prostate cancer can be done by needle biopsy or DRE (digital rectal examination). In benign prostatic hypertrophy/hyperplasia (BPH), prostate continues to enlarge over time. Prostate-specific antigen (PSA) is a glycoprotein, produced by epithelial prostate cells and is unique to the prostate gland. We found ApaI CC genotypes to increase the prostate cancer risk while CA and AA show less than 50% association with the disease. ApaI polymorphism has a strong correlation with PCa than TaqI. Different VDR gene polymorphisms seem to have an association with the PCa but not consistent with other ethnic groups
Keywords: *PSA, VDR gene polymorphisms, ApaI polymorphism, PCa*

Introduction

The prostate cancer was first demonstrated by Flemish anatomist Andreas Vesalius in 1538J and reported by Venetian anatomist Niccolo Massa in 1536. A surgeon named Adam, at London Hospital, reported the first case of prostate cancer in 1853 while doing histological examination. He noted this was “a very rare disease” (Sriprasad et al., 2009). The estimation of new cases worldwide was about 1.1 million in 2012, and death rate of about 307,000. Among American Men, Prostate Cancer is second common cancer (Bashir, 2015). Recent studies show that in Pakistani males prostate cancer is a third common malignancy (Murtaza et al., 2016). When compared with other types of cancers, Prostate cancer is somewhat unusual. The reason is because numerous prostate tumors don't spread rapidly to different parts of the body. Some prostate cancer may not cause symptoms or problems for years or more because of their slow growth. For a long time prostate cancer can be managed even when it spread to other parts, allowing men to stay healthy for many years. However, if it cannot be controlled with the treatments, it can cause manifestation or may lead to death. To determine its growth prostate cancer monitor over time.

No signs and symptoms show in the early stages of the disease. But when it shows symptoms it affects urination mostly. Most common symptoms of prostate cancer are Nocturia (increased urination at night), difficulty in urination, Hematuria (blood in urine), Dysuria (frequent and painful urination). It may influence sexual function, for instance, trouble in accomplishing an erection or agonizing discharge. Advance prostate cancer may spread to other organs of the body, causing pain in pelvis or ribs. In Benign prostate hyperplasia, prostate enlarges and cause urinary symptoms. Diagnosis of prostate cancer can be done by needle biopsy or DRE (digital rectal examination) (Catalona et al., 1994). In benign prostatic hypertrophy/hyperplasia (BPH), prostate continues to enlarge over time. Prostate-specific antigen (PSA) is a glycoprotein, produced by epithelial prostate cells and is unique to the prostate gland (Gjertson and Albertsen, 2011). The purpose of PSA is to breakdown the high molecular weight protein into polypeptides, so semen becomes more liquid (Bunting, 2002).

Literature review

Prostate gland

The prostate is a reproductive secondary gland found in men. It is placed behind the shaft of a man's genitals, next to the rectum, and just below the bladder, and is roughly the size of a walnut. Its primary role is to produce seminal fluids that preserve, promote, and aid in the passage of sperm to the penis. The prostatic urethra is the part of the urethra that passes through the prostate. Urination and ejaculation are two functions of the urethra in males. The prostate contractions during ejaculation and discharge the sperm into the prostatic urethra, which leads to the penis. A good prostate weighs around 11 grammes on total, varying from 7 to 16 grammes (Leissner and Tisell, 1979). It is encased in a fibroelastic tissue layer, which causes septa to grow forwards, separating the prostate in lobes (Khan, 2011).

Testosterone is the primary male hormone, which is generated in the testicles. Dihydrotestosterone is a hormone that regulates the prostate. The prostate produces a slightly alkaline fluid that constitutes about 25% of seminal fluid and permits sperm movement and viability. Because the reproductive system is acidic, the alkalinity of the sperm helps to neutralise the environment, allowing the sperm to survive. Prostate-specific antigen (PSA), together with citrate, zinc, spermine, and cholesterol, is a key component of prostatic excretion. Zones and lobes are two alternative classification methods for the prostate. The zonal classification, which divides the prostate into four distinct areas, is more commonly employed in diagnostics. The peripheral zone (PZ) surrounds the urethra and makes up roughly 70% of the prostate. Prostatic malignancies occur in the PZ in over 80% of cases. The central zone (CZ) is a 25 percent of the prostate that encompasses the ejaculatory channels. This area is home to just 2.5 percent of prostatic malignancies, but the tumors that do occur here are more deadly (Cohen et al., 2008).

Prostate cancer incidence and death

Prostate cancer is the world's sixth most frequent cancer, and men's second most prevalent cancer (Hilal et al., 2015). In 2000, there were anticipated to be 513,000 new cases, but in 2012, there were anticipated to be 1.1 million new cases. This shows that prostate cancer has become more common in the last ten years. Globally, it is projected that 1.7 million new cases and 499,000 fatalities would occur by 2030 (Jain et al.,

2014). In 2008, Asian nations had the greatest impact of the illness, with 14 percent of all cases occurring in Tian Jin, China (1.9/100,000 person-years). The greatest rates were seen in North America and Scandinavia, particularly among African-Americans (137 per 100,000 person-years) (Kitagawa et al., 2013). Prostate cancer incidence is directly proportional to age. Nearly 75% of new cancer diagnoses occur in adults over the age of 85. In other words, the incidence of this cancer rises as one's life expectancy rises (Pienta and Esper, 1993). The aetiology of this malignancy, however, remains uncertain (Hsing and Chokkalingam, 2006). Prostate cancer was estimated to have killed about 90,000 men in Europe in 2008, making it the third most prevalent cause of cancer mortality in males behind lung and colorectal cancers (Ferlay et al., 2010).

Prostate cancer risk factors

Though other signs and symptoms are not entirely either one, they can be categorised as intrinsic or extrinsic (e.g., race, aging, oxidative stress). Some factors could be influenced by both endogenous and exogenous factors.

Endogenous risk factors

Endogenous risk factors for prostate cancer include; (1) family history; (2) hormones; (3) race; and (4) aging and oxidative stress.

Exogenous risk factors

Exogenous risk factors for prostate cancer include: (1) diet; (2) environmental agents; and (3) occupation and other factors.

Progression of prostate cancer

As advancement and movement of malignancy are driven by sub-atomic adjustments, the examination of sub-atomic highlights may empower a superior expectation of the conduct of individual tumors. Since tissues are heterogeneous, modifications on the serum level would be particularly fit as demonstrative and prognostic markers in prostate malignancy (Schlomm et al., 2007). Expanding proof from epidemiological and lab contemplates recommends that diet and way of life may have a part in the advancement of prostate malignancy (Kronenwetter et al., 2005). The admission of explicit vegetables, tomato items (lycopene), nutrient E, selenium, nutrient C and soy items has been contrarily connected with prostate malignancy hazard. Also, epidemiological proof and transient investigations show that the occurrence of clinically huge prostate malignancy is a lot of lower in pieces of the reality where individuals eat prevalently low fat, plant-based eating regimen (Catalona et al., 1991).

Genetic epidemiology of prostate cancer

A large portion of the human genome is non-coding DNA; accordingly, most of hereditary changes are innocuous. Just transformations in the exon locales of the genome are dependent upon hurtful changes which may influence protein arrangement (Pearson et al., 1996). Albeit the hereditary qualities of prostate disease is inadequately perceived, we know malignancies quite often emerge from a solitary substantial cell that goes through various hereditary changes which cause an adjustment of quality movement and subsequently aggregate (Wang and Gotoh, 2010). Disease causing

transformations as a rule emerge in qualities associated with the guideline of cell development or passing (Petros et al., 2014). By far most of disease cells have six unique capacities; independence in development signals, heartlessness toward hostile to development signals, avoidance of apoptosis, endless replication capacity, supported angiogenesis and capacity to attack tissue and metastasise (Carpenter, 2007). A transformation explicit for prostate disease is yet to be recognized. Likewise, regular changes in oncogenes and tumor concealment qualities for different diseases are shockingly uncommon in essential prostate malignant growth (Fan et al., 2011).

Genome-wide association studies

Genome-wide affiliation considers (GWAS) has been profoundly fruitful in finding defenselessness loci for prostate malignancy. At present, in excess of twenty GWAS have recognized in excess of fifty normal variations related with powerlessness with PCa (Chen et al., 2013). The first prostate malignancy GWAS in 2005, in excess of 1300 investigations have been added to the Catalog of Published Genome-Wide Association Studies (Juran and Lazaridis, 2011).

Vitamin D receptor gene

Vitamin D is involved in a number of biological processes like bone metabolism, regulation of cell proliferation, differentiation, and immune response modulation, it is a steroid hormone. It has a key role in the growth of normal and malignant human prostate cells maintaining their regulation (Uitterlinden et al., 2004). The vitamin D receptor (VDR) is a protein formed from VDR gene which have the instruction for the formation of VDR receptor protein, which allows the body to respond to vitamin D. VDR exists on chromosome 12q13.11 and are 100 kb in size (Mutti et al., 2011). Recent data revealed that vitamin D receptor gene (VDR) shows high polymorphism rate but their influence on the functioning and signaling of VDR protein is not known (Uitterlinden et al., 2004). It is widely seen that Vitamin D receptors are present in bone, intestine, kidney, and the parathyroid gland. Significantly High levels of VDR indicate the prostate cancer regression. The genomic action of vitamin D is mediated by vitamin D receptor (VDR) which is a ligand-activated transcription factor. Genomic actions of vitamin D are calcium homeostasis regulation, cell growth, differentiation and xenobiotics detoxification process as well as the modulation of adaptive and innate immunity; the latter including activation of monocyte-macrophages (Vanessa et al., 2013).

VDR gene polymorphisms

Vitamin D gained much attention due to its anticancer effect and polymorphism. Vitamin D receptor gene (VDR) polymorphism is linked to different diseases conflictingly (Medeiros et al., 2002). A number of studies revealed a correspondence in between prostate cancer and VDR polymorphisms. However, some studies are also there finding no significant association between them (Guo et al., 2013). A number of series polymorphisms in the vitamin D receptor gene were recently reported to be associated with bone density and risk of osteoporosis. Four most commonly studied VDR polymorphisms are FokI, TaqI, ApaI, and BsmI. All of the polymorphisms are strongly associated with one another except FokI, and the presence of one

polymorphism can be used to detect the presence of other one as all are interlinked and occur together.

ApaI

This polymorphism is found in exon 9 at codon 352 of the VDR gene. The Apa1 A allele has reported to found at a higher frequency in the Asian population which is about 74% (Köstner et al., 2009).

FokI

The FokI polymorphism is present in the exonic region and is correlated to any change in the VDR gene's reading frame. The Fok1 polymorphism is the sole VDR gene polymorphism that is used to generate an altered protein (Chauhan and Sakharkar, 2017).

TaqI

TaqI polymorphisms have no link to protein sequence and found on the 3'-UTR regions. T allele of TaqI indicates the higher chances of occurrence of prostate cancer (Iqbal and Khan, 2017).

BsmI

Bsm1 resides in intron 8 and at the 3′ end of the gene. The Bsm1 restriction enzyme is associated with other polymorphisms like Apa1, Taq1, and the variable-length poly(A) which is disequilibrium link (Mohammadi et al., 2014).

VDR polymorphism studies in Pakistan

Several studies were conducted in Pakistan to check the association of VDR gene and associated diseases within Pakistani population. Results revealed that Pakistani population is at high risk of exposure of VDR gene polymorphism that is a significant risk factor of Rheumatoid arthritis as well as Osteoarthritis onset (Mukhtar et al., 2019). Also, the GG genotype of VDR-Cdx2 polymorphism can enhance the risk of onset of breast cancer in females (Khan and Maqbool, 2015). Some studies said that VDR polymorphisms may be the liable regions for T1D development (Mukhtar et al., 2017).

Materials and Methods

Study design

This study was carried out to address the objectives of this report. Study included different volunteers and patients of prostate cancer. Blood samples were collected from these patients in order to determine the association of VDR polymorphisms ApaI and TaqI with this disease.

Study area and population

The proposed study was carried out by collection of blood samples from MINAR (Multan Institute of Nuclear Medicine and radiology) Multan, Punjab Pakistan from June to July. The patients were from different cities of southern Punjab region. Samples

were collected from patients with a confirmed diagnosis of prostate cancer following the histopathological examination. Informed written consent was signed from all the participants. Most of this study group were married, having children and in the age group of 28-80 years. Naswar, hookah and cigarette were found to be a regular part of their routine. Most commonly occurring symptoms included pain, difficulty in urination and lump.

Blood sampling

Blood (5ml) was drawn; using disposable syringes by venepuncture and was immediately transferred to EDTA coated vacutainers and then stored at 4°C until used for the study.

DNA extraction

DNA isolation was carried out by using WizPrep gDNA Mini kit. The following method was used:

(1) 200µl of whole blood was added into a 1.5ml microcentrifuge tube;
(2) 200µl of GB buffer and 20µl of Proteinase K was added and mixed by vortexing;
(3) The tubes were incubated at 56ºC for 10min. During incubation, the tubes were inverted every 5 minutes;
(4) 200µl of absolute ethanol was added to the sample lysates and mixed by vortexing;
(5) DNA Spin Column was connected to the 2.0ml collection tube;
(6) The mixture was applied to the DNA Spin Column and centrifuged for 1 min at 13,000 rpm;
(7) The DNA Spin Column was transferred to the new collection tube and flow-through was discarded;
(8) 500µl of W1 buffer was added to the DNA Spin Column and centrifuged for 1 min.at 13,000 rpm;
(9) The flow-through was discarded and re-connected with DNA Spin Column;
(10) 500µl of W2 buffer was added in the DNA Spin Column and centrifuged for 1 min. at 13,000 rpm;
(11) The flow-through was discarded; DNA Spin Column was reconnected and centrifuged for 2 min at 13,000 rpm;
(12) The DNA Spin Column was connected to a new 1.5ml tube;
(13) 50~100µl of Elution Buffer was added and incubated at room temperature for 1 min and centrifuged for 1 min at 13,000 rpm;
(14) The DNA Spin Column was discarded and purified DNA was eluted for use;
(15) The purified DNA sample was stored at -20ºC.

Gel electrophoresis for DNA

In order to check the presence of DNA, gel electrophoresis was done by using the following method:

(1) 1% agarose gel was prepared by adding 0.4g agarose to 40ml of the TAE buffer in a flask;

(2) Flask was covered with aluminum foil and heated in a microwave oven to dissolve the agarose and 2µl Ethidium Bromide was added;
(3) The prepared agarose gel was then poured into a gel casting tray carefully, avoiding bubble formation;
(4) The comb was inserted in the gel for the formation of wells and kept for 20 mins at room temperature to let it polymerize;
(5) Then the gel tray was placed into the electrophoresis apparatus;
(6) TAE buffer was added in the apparatus and the comb was removed;
(7) Sample DNA was prepared by adding 2µl bromo-phenol dye with 5µl DNA sample;
(8) DNA with dye was then loaded in the wells;
(9) 1µl of 1kb ladder was also loaded in a well for comparison;
(10) The power supply was switched on and the voltage was kept at 90 volts for 90 minutes;
(11) Then gel was visualized under UV light in gel documentation system and picture of the gel was taken using GDS.

Allele specific PCR

Two different polymorphisms Taq1 and Apa1 were genotyped in patients of prostate cancer. For this purpose 2 different sets of allele specific primers were designed and amplification of particular alleles was carried out by means of PCR to identify polymorphisms. For optimization studies, DNA of a healthy individual was used and different conditions for PCR were adjusted by changing the concentrations of MgCl2, dNTPs, DNA and primers.

ApaI genotyping

Allele-specific primers were designed for PCR. Standard PCR reaction was carried by using genomic DNA. A master mix was prepared for PCR by using the following method (*Table 1* and *Table 2*): (1) all reagents were thawed on ice; (2) reagents were added in the following order: buffer, dNTPs, $MgCl_2$, dH_2O, taq polymerase, template DNA and primers; (3) gently mixed by tapping tube; (4) a negative control reaction was prepared without template DNA; and (5) a positive control reaction was prepared with a template of known size and appropriate primers.

Table 1. *Reaction mixture for ApaI PCR.*

Reagents	Quantity
10x PCR buffer	2 µl
dNTPs	0.8 µl
25mM $MgCl_2$	2 µl
dH_2O	11.7 µl
Taq DNA polymerase	0.5 µl
Forward primer	1 µl
Reverse primer	1 µl
Sample DNA	1 µl

Table 2. *Amplification profile for ApaI PCR.*

Step	Temperature	Duration	No. of cycles
Initial Denaturation	95°C	3min	1
Denaturation	94°C	1min	30
Primer Annealing	64°C	40sec	
Extension (go to denaturung step and repeat 35 cycles)	72°C	1min	
Final Extension	72°C	5min	1
Hold (technique)	4°C	Infinite	Hold

TaqI genotyping

Allele-specific primers were designed for PCR. Standard PCR reaction was carried by using genomic DNA. A master mix was prepared for PCR (*Table 1* and *Table 2*).

Statistical analysis

All the data was entered in Microsoft excel sheet for statistical analysis. Frequency distribution was calculated for each polymorphism and their association with specific age groups was established. The effect size of both genotypes and alleles was calculated by comparison.

Results and Discussion

This study was performed to evaluate the polymorphisms by using the PCR technique. Results showed association between the disease and genotype. The patient population was classified into 2 groups depending on their ages, the first group was below 50 and the second group was above 50.

Frequencies and association of TaqI polymorphism in cases

A strong association between TaqI genotype 'TT' and prostate cancer was observed which is 95%. The 'TC' genotype and 'CC' genotype showed a decrease in risk for the disease as both genotypes have only 5% association. In below 50 age patients, there is 100% association with PCa for 'TT' genotype but for genotype 'TC' and 'CC', there is 0% association so we can say that there is no chance of the disease in these genotypes. In above 50 age patients, the genotype 'TT' show 92% association with PCa and only 8% risk is found to be associated with genotype 'TC' and 'CC' (*Table 3* and *Figure 1*).

Table 3. *TaqI polymorphism frequencies and effect estimation by age in prostate cancer patients.*

TaqI		Cases with frequency (N) and percentage (%)
Genotypes (N=18)	TT	18 (95%)
	TC	1 (5%)
	CC	1 (5%)
Age<50 N=7	TT	7 (100%)
	TC	0 (0%)
	CC	0 (0%)

Age>50 N=12	TT	11 (92%)
	TC	1 (8%)
	CC	1 (8%)

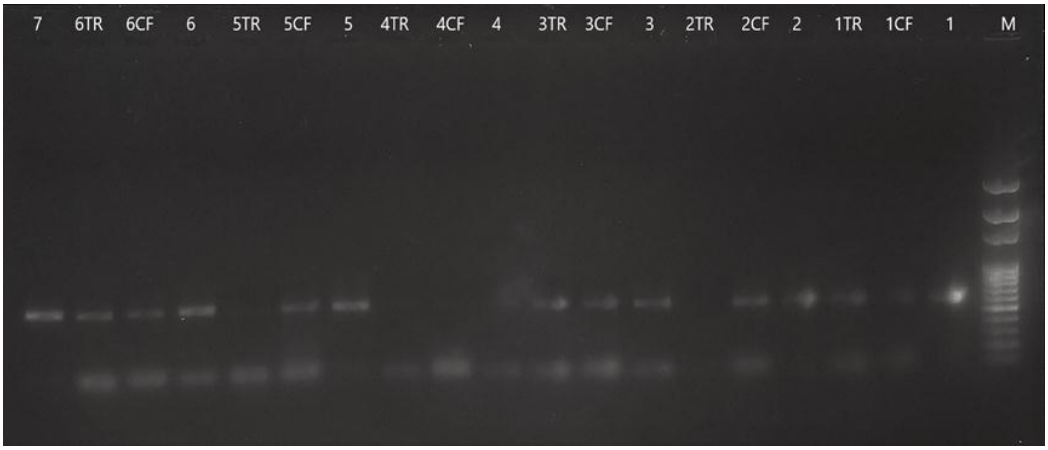

Figure 1. *Agarose gel image of PCR products using allele specific primers of TaqI.*

Frequencies and association of ApaI polymorphism in cases

100% association between ApaI genotype 'CC' and PCa was found, which showed a high risk of disease in this genotype. The 'CA' genotype showed 42% risk with the disease and 'AA' genotype showed a 47% risk for the disease. 'CC' genotype was found to be highly risky and associated with progression of disease in both age groups. The 'CA' genotype was found to have 43% risk in below age and 42% in above 50 age patients. In above 50 age patients the genotype 'AA' was having 29% association with PCa but in above 50 age it showed 58% risk. Both these genotypes 'CA' and 'AA' were found to be less risky than 'CC' genotype (*Table 4* and *Figure 2*).

Table 4. *ApaI polymorphism frequencies and effect estimation by age in prostate cancer patients.*

ApaI		Cases with frequency (N) and percentage (%)
Genotypes N=19	CC	19 (100%)
	CA	8 (42%)
	AA	9 (47%)
Age<50 N=7	CC	7 (100%)
	CA	3 (43%)
	AA	2 (29%)
Age>50 N=12	CC	12 (100%)
	CA	5 (42%)
	AA	7 (58%)

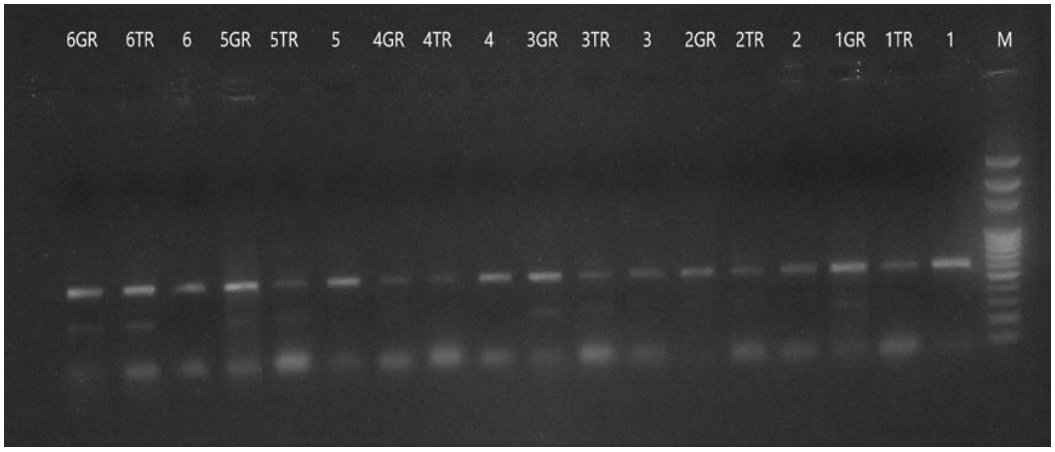

Figure 2. *Agarose gel image of PCR products using allele specific primers of ApaI.*

Prostate cancer is the third most common malignancy in Pakistani males. Vitamin D receptor (VDR) gene has been a subject of extensive pharmacogenetic research recently. Association studies between different types of cancers including prostate cancer (PCa) and VDR gene polymorphism have also great importance. Vitamin D has an anticancer effect, so VDR gene polymorphisms have got much attention (Medeiros et al., 2002). It is proposed that vitamin D deficiency may underlie the major risk factors for prostate cancer, including age, black race and genetic variation in vitamin D-binding protein (SCl'lWARTZ and Hulka, 1990). Clinical diagnosis of prostate cancer can be done by PSA and biopsy. The clinical diagnosis does not provide a definitive diagnosis of progression of PCa.

The frequency of prostatic carcinoma is very low among other neighbouring countries like India, Iran, Turkey, and Bangladesh. This tumour was included among ten commonest tumours in all districts but it was found to be most frequent in Gujrat district (Ahmad et al., 1991). The apparent lower risk for prostate cancer may be an indicator of 'missing cancers' diagnosed on the basis of clinical investigations. The lower life expectancy in Pakistan and also the lack of post-mortems substantially contributes to the low age-standardised incidence rates for prostate in comparison to the developed countries (Bhurgri et al., 2005).

Recent studies have suggested that vitamin D is an important determinant of prostate cancer risk and inherited polymorphisms of the vitamin D receptor (VDR) gene are associated with the risk and progression of prostate cancer. Substantial experimental evidence indicates that the hormonal form of vitamin D promotes the differentiation and inhibits the proliferation, invasiveness, and metastasis of human prostatic cancer cells (John et al., 2005). Genetic variation in the Androgen Receptor (AR) genes was associated with prostate cancer, and the gene appears to preferentially confer risk for advanced disease.

Conclusion

In this study, we have analyzed the role of VDR gene polymorphisms with prostate cancer and found a significant association between them. The results show that there is a significant association between the TT genotype of TaqI with the risk of prostate cancer but the other genotypes do not show that much association. We found ApaI CC genotypes to increase the prostate cancer risk while CA and AA show less than 50%

association with the disease. ApaI polymorphism has a strong correlation with PCa than TaqI. Different VDR gene polymorphisms seem to have an association with the PCa but not consistent with other ethnic groups. The difference in the population may be one of the reasons. Black people have more risk of having prostate cancer than white people. Because of the high melanin content of black skin, it inhibits the formation of previtamin D (Powe et al., 2013). Although small sample size, fewer resources, some unstudied risk factors and lack of time also seem to influence pharmacogenetic findings. The tendency of consanguineous marriages in Pakistani society can also be a risk factor. Age group also a reason, the elderly is D deficient because the aged often receive little exposure to UV light and ability to synthesize Vit D decline with age (Llewellyn et al., 2010). Future studies, with large enough sample size, the separate study of different ethnic groups may be able to find a better correlation between VDR gene polymorphisms and PCa risk.

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Conflict of interest

There are no conflict of intrest involve any parties in this research study.

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