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LACK OF ASSOCIATION BETWEEN VITAMIN D RECEPTOR *FOK*1 POLYMORPHISM AND URINARY TRACT INFECTION AMONG KARACHI POPULATION

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ABSTRACT

Present investigation was the first of its type carried out on Pakistani population with respect to vitamin D receptor (VDR) gene polymorphism in Urinary tract infection (UTI) patients. Vitamin D, a secosteroid belongs to the nuclear receptor family. In urinary tract it produces its action by binding with vitamin D receptor in bladder epithelial cells inducing the expression of cathelicidin, a potent antimicrobial peptide. Polymorphism in VDR gene may results in lowering the activity of vitamin D that leads to the susceptibility of UTI. Here, we evaluated VDR *Fok*1 gene polymorphism with its correlation to the risk of UTI in Karachites. Genomic DNA through proteinase-K method was extracted using mouth epithelial cells from 31 infected and 62 controls of both genders. VDR *Fok*1 gene polymorphism was evaluated though PCR-RFLP. A non-significant (p=0.699) association of VDR *Fok*1 gene polymorphism among cases and control was observed, however risk may be observed for Ff genotype with an odds-ratio of 1.255 (95%CI=0.476-3.310). Moreover, in controls the gene was in Hardy Weinberg Equilibrium (p=0.859).

Key words: *Fok*1, UTI, VDR, polymorphism

INTRODUCTION

Urinary tract infection (UTI) is one of the most frequent infectious diseases seen in clinical practice. It can be classified into pyelonephritis, cystitis and urethritis caused by the bacterial colonization among which *E. coli* is the chief agent (Khan *et al.,* 2013, Tambekar *et al.,* 2006). Because of anatomical differences UTI is most common in women (63%) as compared to men (36%) (Tambekar *et al.,* 2006). It is observed that UTI patients have no definite abnormality related to infection. Therefore attention has been focused on genetic factors to evaluate the possible cause of infection.

Vitamin D a secosteroid is regarded as an immune modulator (Bikle, 2008). Its deficiency is known to lower the host defense towards infections (Youssef *et al.,* 2012). Vitamin D mediates its action upon binding to its receptor, vitamin D receptor (VDR) (Feldman *et al.,* 2013) widely distributed in body. The gene for human VDR is located on chromosome 12p13.1 approximately contains 100kb. Since VDR expresses throughout the body cells, therefore, vitamin D has many functions in body and its deficiency may lead to a disease state.

Vitamin D is known to have antimicrobial actions (Youssef *et al.,* 2012). It has been observed that it binds to the receptor in uroepithilial cells of bladder and expresses cathelicidin antimicrobial peptide and beta defensin 2 gene that mediate innate immune response of body and increases antimicrobial activity against pathogen (Roby and Nardo, 2013). Polymorphisms in VDR increase the incidence of infections (Aslan *et al.,* 2012). Around 200 gene polymorphisms have been identified among which *Fok*1, *Bsm*1, *Apa*1, *and Taq*1 are most common. *Fok*1 (Thymine/Cytosine) polymorphism is start codon polymorphism which results in the formation of two potential initiation codons which may develop variants. In ff variant, translation begins at first ATG site resulting in a full length VDR protein of 427 amino acids while FF variant is obtained when translation begins at second ATG site resulting in VDR protein with 3 less amino acids however in Ff variant both type of proteins are obtained (Swapna *et al.,* 2011). It has been reported that this polymorphism with genotype FF, Ff and ff have varying degrees of influence on vitamin D signal transmission (Swapna *et al.,* 2011; Neyestani *et al.,* 2013) with the FF being most active and ff being least active combination. A study of Turkish children has also determined a significant correlation between *Fok*1 polymorphism and UTI (Aslan *et al.,* 2012). Studies on Indian population have documented a significant correlation of VDR *Fok*1 polymorphism in different disorders (Mohapatra *et al.,* 2013, Sanwalka *et al.,* 2013, Shafia *et al.,* 2013) however present investigation is the first of its kind on Pakistani population studying the correlation of VDR *Fok*1 polymorphism in UTI patients. Since small alteration in VDR

gene may affect sensitivity and susceptibility of receptors which becomes an important reason of morbidity in UTI patients (Aslan *et al.,* 2012).

MATERIALS AND METHODS

Study design

This investigation is a retrospective, case control study performed in subjects clinically diagnosed with UTI.

Sample size

A total of 93 subjects (31 infected and 62 healthy) with an age range of 12-30 years without prior history of UTI were recruited for the present investigation. A pre-tested questionnaire based on demographic and medical information was filled by the subjects.

Inclusion criteria

Patients having upper and lower UTI with fever, costovertebral pain, significant bacteriuria, dysurea, frequency, urgency and suprapubic pain were included in this study.

Exclusion criteria

Patients with kidney failure, pregnancy, renal calculi, hypertension, diabetes, and disorders of hormone imbalances were excluded from the study.

DNA extraction

Genomic DNA was extracted using mouth epithelial cells being the most convenient, easy and effective way to collect DNA in amplifiable amount. DNA was extracted using proteinase K method. In brief, the cells were washed 3 times with lytic solution followed by the digestion in the presence of proteinase K (100 mg) and SDS (10%) at 56°C for 30 min. At the end of incubation proteins were removed using 6M NaCl and DNA precipitated from supernatant using isopropanol. Later, DNA was washed with 70% ethanol, resuspended in DNAase RNAase free water and stored at -80°C till further use.

PCR and RFLP

The VDR gene encoding the *Fok*1 polymorphism was amplified using specific primers forward 5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3' and reverse 5'-TGGAAACACCTTGCTTCTTCTCCCTC-3' (Harris *et al.,* 1997; Morrison *et al.,* 1994). PCR was carried out using Kapa-Taq PCR master mix (Kapa Biosystems, USA) as per manufacturer's protocol. Amplification took place under following conditions: initial denaturation at 95°C for 2min followed by 30 cycles of 95°C for 30s, 58°C for 30s and 68°C for 30sec and one cycle of final extension at 68°C for 7min in an automated thermal cycler (Veriti, Applied Biosystem, USA). The PCR products were then electrophoresed on 1.5% agarose gel and visualized using Vision works LS software Ver 7.1 on ChemiDoc-It^2 (UVP, UK).

Positive samples were subjected to RFLP analysis in a reaction volume of 20µl (5µl of PCR product and 10units of *Fok*1restriction enzyme) at 37°C for 1hr followed by gel electrophoresis (2% agarose gel, 0.5µg/ml ethidium bromide).

Statistical analysis

In order to estimate the association and risk Pearson's chi square, odds ratio were calculated using SPSS 20.0 ver at statistical significance of $p<0.05$. Moreover, Hardy Weinberg equilibrium was calculated to evaluate the genetic distribution in controls.

RESULTS

In our study 50 subjects with UTI and 100 healthy subjects were recruited, however, 31 UTI subjects and 62 controls agreed to provide samples for present investigation. Samples were evaluated for the association of VDR-*Fok*1 polymorphism using PCR-RFLP method. The mean age of patients was 24$\pm$4.6 years, while that of the healthy control subjects was 22$\pm$3.1 years. Among cases the distribution of upper and lower UTI was 25.71% and 74.28% respectively (data not shown). *E.coli* was the main causative agent (80%) for UTI, while a small number of patients had Pseudomonas aeruginosa (15%) or Acineto bacter baumannii (5%) infection.

DNA from 93 subjects was extracted using proteinase-K method and purity determined through ratio of absorbance at 260/280nm. The samples were also electrophoresed on 1% agarose to determine the integrity of extracted DNA. A PCR product of 265bp was obtained upon amplification of promoter region of VDR gene which has *Fok*1 polymorpishm. RFLP analysis was carried out on amplified products. An undigested product of 265bp was obtained presenting FF genotype however the digested products with two combinations of 196, 69 or 265, 196, 69bp were obtained which specifies ff and Ff genotype respectively.

The VDR *Fok*1 genotypic distribution of controls was calculated according to Hardy-Weinberg equilibrium, with p- value of 0.859 (Table 1).

Table 1. VDR *Fok*1 Genotype distribution in the Hardy-Weinberg equilibrium (HWE).

	Genotype	FF	Ff	ff	HWE	p-value
Control	Expected	46.2	14.7	1.2	0.03	0.859
	Observed	46	15	1		

Pearson χ^2 was performed to check the genotypic distribution in cases and controls which showed that the distribution of *Fok*1 genotype is not significantly (P>0.05) associated with UTI in population of Karachi as shown in Table 2. However, a tendency towards UTI exists in Karachiites with Ff genotype having an odd ratio of 1.255 (95%CI=0.476-3.310).

Table 2. Association of VDR *Fok*1 polymorphism and UTI risk.

Genotype	Cases n (%)	Controls n (%)	Pearson's $\chi 2$	p-value	Odd Ratio (95% CI)*	p-value
FF	22 (71)	46 (74)	0.717	0.699	1	
Ff	9 (29)	15 (24)			1.255 (0.476-3.310)	0.624
ff	0	1 (1.6)			NC	1
Total	31	62				
Allelic Frequency						
F	53 (85.4)	107 (86.3)	0.022	0.881	1	
f	9 (14.5)	17 (13.6)			1.069 (0.447-2.557)	0.881

*OR (95%CI) were calculated via binary regression analysis; NC: Not calculated

Ethnicity may also play a role in gene distribution of VDR. The genotypic distribution of FF and Ff genotype was dominant in Urdu speaking and Pathan with UTI whereas these genotypes were also dominant among Urdu speaking and Punjabi control subjects (Table 3).

Table 3. Ethnic distribution of VDR *Fok*1 genotype in cases and control.

Ethnic Groups	**Control n (%)**			**Cases n (%)**		
	FF	**Ff**	**ff**	**FF**	**Ff**	**ff**
Urdu speaking	14 (30.4)	6 (40)	1 (100)	5 (22.7)	4 (44.4)	0 (NC)
Pathan	5 (10.9)	0 (NC)	0 (NC)	5 (22.7)	3 (33.3)	0 (NC)
Panjabi	13 (28.3)	2 (13.3)	0 (NC)	4 (18.2)	1 (11.1)	0 (NC)
Others	14 (30.4)	7 (46.7)	0 (NC)	8 (36.4)	1 (11.1)	0 (NC)
Total	46	15	1	22	9	0

NC: Not calculated

ORs and 95%CI of each ethnic group was also calculated and the results indicated that Pathan, Urdu speaking and Punjabi are at high risk of having Ff genotype with an OR of $1.62X10^9$, 1.867 and 1.625 respectively as shown in Table 4.

Table 4. Association of VDR *Fok*1 polymorphism and UTI risk among ethnic groups.

Ethnic Groups		**FF**	**Ff**	**ff**
Urdu Speaking	OR*	1	1.867	NC
	(%CI)		(0.367-9.487)	
	P-value		0.452	
Pathan	OR*	1	1.62E+09	NC
	(%CI)		NC	
	P-value		0.999	
Punjabi	OR*	1	1.625	NC
	(%CI)		(0.115-22.985)	
	P-value		0.719	
Other	OR*	1	0.25	NC
	(%CI)		(0.026-2.416)	
	P-value		0.231	

*OR (95%CI) were calculated via binary regression analysis; NC: Not calculated

DISCUSSION

UTI is amongst the most common infectious disease in Pakistani population which mostly occurs as a result of Colibacillosis (*E.coli*). Present investigation was focused to analyze vitamin D receptor (VDR) gene polymorphism (*Fok*1*)* in patient with UTI. Many investigations have been carried out to study the correlation between VDR gene polymorphism and various diseases in Asian countries (Mohapatra *et al.,* 2013; Nishijima *et al.*, 2002), but no data on VDR *Fok*1polymorphism is available on Pakistani population to date.

Deficiency of vitamin D has taken the shape of an epidemic among healthy adults and children worldwide (Wacker and Holick, 2013). It has been documented that its deficiency is among the factors causing the development of infections (Schwalfenberg, 2007). Our results have shown that UTI patients with increased melanin pigmentation on skin and have longer duration of sun exposure are less prone to the effects of VDR polymorphism since melanin has a direct relation with sun light absorption by skin while an inverse relationship with vitamin D synthesis in skin Clemens *et al.*, 1982).

Ethnicity seems to plays an important role in the gene polymorphism distribution of vitamin D receptor. A non-significant association has been found between *Fok*1, *Taq*1 and *Bsm*1 VDR polymorphism with diabetes in North Indian population (Bid *et al.,* 2009). A significant FF genotype distribution in healthy individuals has been observed in Finland, Australia and Black Pennsylvania and only Ff genotype was significantly associated with Black Pennsylvania's whereas in all population (Finland, Australia, Japan, England, USA, India, Taiwan) ff genotype was significantly different except Black Pennsylvania (Bid *et al.,* 2005).Our study has shown that Urdu Speaking and Pathan had higher distribution of FF genotype whereas Urdu speaking and Punjabi had higher distribution of FF genotype in comparison to other ethnic groups in Pakistan.

The result of present study revealed that 71% of UTI patients have FF genotype whereas 29% had Ff genotype distribution. A non-significant association was observed with VDR *Fok*1 to UTI patients in Pakistani population which in contrary to the findings of Aslan *et al.*, (2012) who had reported a significant association of *Fok*1 VDR gene polymorphism and UTI in Turkish children. Vitamin D upon binding to its receptor (VDR) modulates immune response through multiple pathways such as; activation of macrophages and NK cells for enhanced phagocytic

activity (Schwalfenberg, 2011), reduce cytokines actions and activates Toll-like receptor. These changes cause an upregulation of VDR expression leading to an over expression of potent antimicrobial peptides i.e. Cathelicidin and β-defensin2 (Liu *et al.,* 2006) in uroepithelial cells of bladder which helps to prevent infection (Fig. 1). Cathelicidin is a vitamin D dependent gene and its varying levels may influence the risk to bacterial infection in urinary tract (Zasloff, 2007).

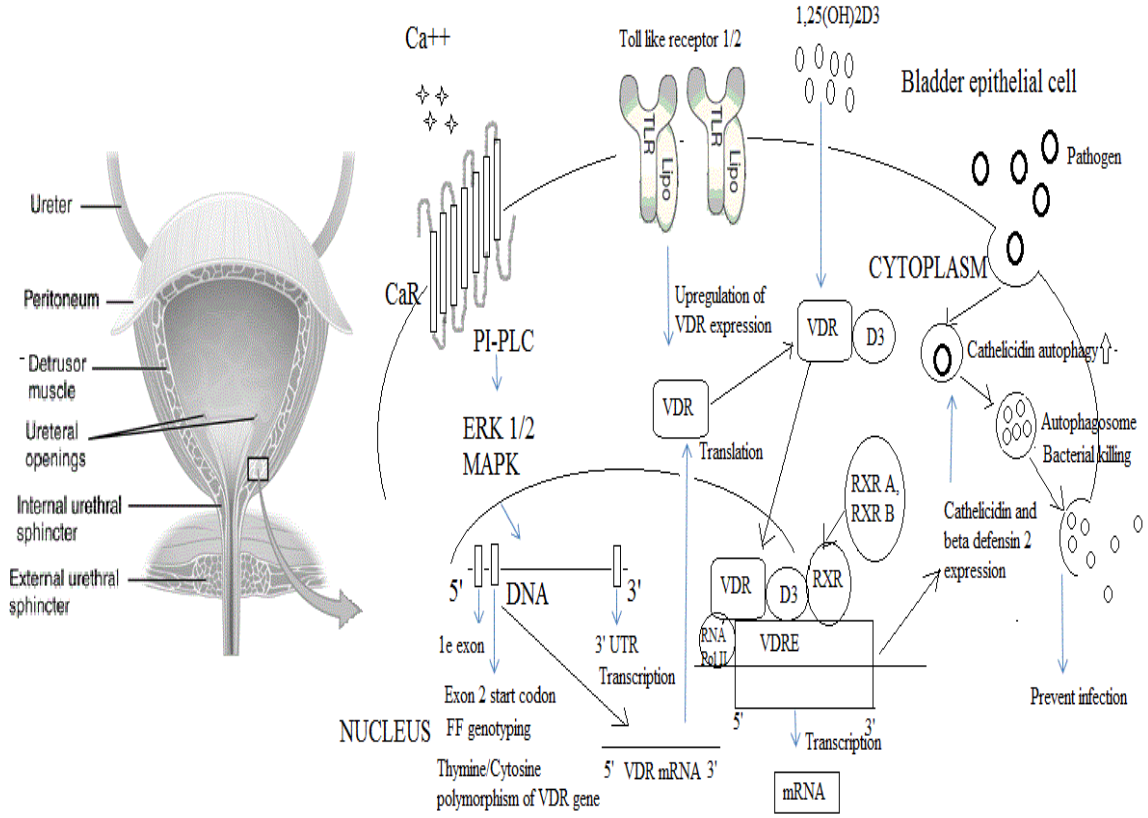

Fig. 1. Signaling pathway indicating the relationship between Fok 1 VDR and UTI.
See text for further details. CaR = Calcium receptors, PI-PLC = Phosphotidyl inositol—phospholipase C, ERK ½ = Extracellular signal regulated kinases 1 and 2, MAPK = Mitogen activated protein kinases, RXR A and B = Retinoid X receptor A and B, VDRE = vitamin D receptor response element.

Our investigation gave an inconsistent distribution of *Fok1* VDR polymorphism in UTI patients. The present studies attempted to answer the possible role of vitamin D receptor in the etiology of UTI as the outcome of present investigation could possibly help to improve the treatment strategies against UTI. Since the existence of a single or multiple VDR polymorphisms may hinder the efficient binding of vitamin D with its receptor and do not prevent UTI. The major limitation of the study was its small sample size, therefore, a more precise evaluation should be carried out including different polymorphism such as *Taq*1, *Apa*1 and *Bsm*1 to establish the correlation of these polymorphisms in UTI among Pakistani population.

Conclusion

A non-significant association of VDR *Fok*1 polymorphism among cases and control was observed, however risk may be observed for Ff genotype.

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