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Vitamin D Receptor Gene Polymorphisms in Indian Children with Idiopathic Nephrotic Syndrome

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ABSTRACT Idiopathic nephrotic syndrome (INS) is the most common glomerular disorder of childhood. In the present study we have investigated the prevalence of VDR gene polymorphisms in INS patients and healthy controls in North Indian population to assess the role of VDR genes in INS as these patients are at high risk to develop metabolic bone disease. Genotyping of four polymorphic sites (FokI, ApaI, TaqI and BsmI) in the Vitamin D receptor (VDR) gene of 108 unrelated nephrotic patients and 569 healthy controls were performed by PCR-based method. The genotype frequencies were compared among INS and controls. There was significant difference at three polymorphic sites except at TaqI. When the two high risk genotype ff of FokI and BB of BsmI of VDR were combined we found that the risk was increased to ~3.5 folds. Our results revealed the VDR gene polymorphism may have a significant role.

INTRODUCTION

Idiopathic nephrotic syndrome (INS) is the most common glomerular disorder in children and is characterized by massive proteinuria, and hypoalbuminemia resulting into edema and hypercholesterolemia. It has an overall incidence of 2 per 1,00,000 and prevalence of 16 per 1,00,000. The occurrence of nephrotic syndrome is estimated to be almost 6 times higher in Asian children. The vitamin D3 receptor (VDR) gene maps to the locus 12q12-q14 (Haussler et al. 1998). It comprises of 11 exons and spans approximately 75 kb. The noncoding 5-prime end of the VDR gene includes exons 1A, 1B, and 1C. Translated product is encoded by exons 2 to 9. Exons 2 and 3 are involved in DNA binding, and exons 7, 8, and 9 are involved in binding to vitamin D. The vitamin D3 receptor (VDR) is an intracellular hormone receptor, which specifically binds to the active form of vitamin D (1,25-dihydroxyvitamin D_3 or calcitriol). It interacts with target-cell nuclei and produces a variety of biologic effects. To specify, VDR is a ligand-dependent transcription factor belonging to the nuclear receptor super-family. After the binding of $1,25(OH)_2D_3$ or other VDR ligands, VDR forms a heterodimer with the retinoid X receptor (RXR) and associates with vitamin D–response elements (VDREs) on target genes. It can then either positively or negatively affects the expression of its target genes (Haussler et al. 1998).

The pathogenesis of INS still remains controversial. Recent studies have revealed that disturbance in Th1 and Th2 cytokines is involved in the pathogenesis of INS (Topaloglu et al. 1994; Shimoyama et al. 2004). Th1 cytokines such as interleukin-2 and IFN-γ preferentially induce cell-mediated immunity, while Th2 type cytokines such as IL-4 and IL-10 primarily supports antibody production (Mosmann et al. 1989).

It has been reported that VDR and 1α, 25-dihydroxy vitamin D ($1,25(OH)_2D_3$) play role in the Th1/Th2 balance through the transcriptional inhibition of cytokine genes. $1,25(OH)_2D_3$ inhibited IFN-γ secretion by Th1 cells in dose dependent manner (Lemire et al. 1995). $1,25(OH)_2D_3$ enters the cell and binds with nuclear receptor termed as vitamin D3 receptor (VDR) (Klein et al. 1993), On binding with VDR it forms a heterodimer with retinoid X receptor (RXR) (Shaffer et al. 2004). This heterodimer complex

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interacts with VDRE (vitamin D responsive elements) which are present in the promoter region on target genes and are involved in regulating their own transcription (Beato et al. 1989). Thus acting as immuno-modulator. Another study found that repression of IL-2 gene transcription by $1,25(OH)_2D_3$ occurs via VDR-dependent inhibition of NFATp/AP-1 complex formation (Alroy et al. 1995). Several frequent polymorphisms found in the VDR gene were reported to be associated with many skeletal as well as non-skeletal diseases (Peacock et al. 2002).

As VDR acts as a potent immuno-modulator and INS pathogenesis has also been associated with biochemical derangements resulting into metabolic bone disease. We have therefore examined VDR gene in 108 NS children to test whether different alleles are associated with the pathogenesis of the disease.

MATERIALS AND METHODS

A prospective analysis was done in 108 children (68.5% boys, 31.5% girls) with idiopathic nephrotic syndrome, who were admitted to the Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Lucknow, India during the period from Jan-2006 to Aug-2008. All patients were diagnosed according to the International Study of Kidney Disease in Children (ISKDC) criteria as defined in our earlier study (Gulati et al. 1994, 2003) and had developed nephrotic syndrome before the age of 18 years. Renal biopsies were carried out in all steroid resistant cases. Children with poor compliance and not on regular follow-ups were excluded from the study.

All children were subjected to a detailed history and physical examination for clinical evidence of disturbances in calcium metabolism In addition the following biochemical tests were done to confirm the diagnosis of nephrotic syndrome – serum creatinine, total protein, albumin, cholesterol, triglycerides and urinary routine and microscopy and urine protein and creatinine ratio in a spot sample.

Control subjects were 569 (70.2%boys, 29.8%girls) individuals from the same population with no history of renal diseases. The controls were age, sex, and ethnically matched. An informed written consent was obtained from the patient and if the children were less than 15 years of age written consent was obtained from parents or the guardian. The study was approved by the Ethical committee of SGPGIMS and Department of Biotechnology, Government of India.

Blood Collection and Genotyping of VDR Gene

Blood samples for measuring serum biochemical and lipid profiles were obtained in the morning after fasting of 8 hours. 3 ml of venous blood sample was collected in EDTA vials for the extraction of genomic DNA. DNA was extracted from blood by using a commercial kit (Qiagen). Various VDR markers used for the present study were TaqI, ApaI, FokI, and BsmI. The genotyping was done according to Vupputuri et al. (2006).

Statistical Analysis

Statistical analysis was performed by using SPSS statistical software (version 15). Student's t test was used to assess the significance of difference in mean values of different biochemical markers among the patient and control group. For each variable, the values are expressed as mean±SD. Data was evaluated by One-Way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison test. Alleles and genotypic frequencies for VDR alleles were calculated by using gene counting method. Comparison of the categorical data i.e. different VDR genotypes among controls and patients was done by Fisher's exact and χ^2 test. Odd's ratios were calculated with a 95% confidence interval limit from 2×2 contingency table. "P" value <0.05 was considered significant. Hardy-Weinberg equilibrium was determined by Pearson's c^2 goodness-of-fit tests.

RESULTS

Various biochemical parameters were studied in both patient and control samples were shown in table 1. The mean value of serum Albumin, Proteinuria, BUN and ALKP among nephrotic patients was found to be 2.6±0.8, 2.9±1.0, 16.9±8.3 and 241.7±101.8 respectively. We observed that there was significant difference in biochemical parameters between the nephrotic patients and controls (Table 1).

VDR's ApaI restriction Site Polymorphism

The allele frequency for the A and a alleles of ApaI were 79(36.6%) and 137(63.4%) in the

Table1: Clinical characteristics of nephrotic patients and controls

Parameters (SI)	*Patients=108* (*Mean±SD)*	*Controls= 569* (*Mean±SD)*	*P-Value*
S Alb (mg/dL)	2.6± 0.8	3.9± 0.2	<0.0001*
S Prot	6.5± 1.1	6.9± 0.3	0.001*
S. Calc (mmol/L)	8.4± 0.9	9.6± 0.4	<0.0001*
Hb	11.4± 1.4	13.5± 1.1	0.001*
S. URIC (mg/dl)	5.1± 0.9	4.9 ± 1.0	0.0457*
S. BUN (mg/dl)	16.9± 8.3	9.8± 2.7	<0.0001*
S. ALKP (U/L)	241.7± 101.8	87.8± 16.7	<0.0001*
S. Na (mmol/L)	135.9± 8.4	138.4± 2.6	0.0005*
S. K (mmol/L)	4.6± 0.7	3.7± 0.24	<0.0001*
S. PHOS (mg/dl)	4.4± 1.0	3.7± 0.3	<0.0001*
S. Creatinine (mg/dl)	0.81± 0.7	0.81± 0.2	0.9947
Proteinuria	2.9± 1.0	0.33± 0.6	<0.0001*
Gender,%(male/female)	74 (68.5%)/34(31.5)	399 (70.2)/ 170(29.8)	——

*Significant value (p<0.05)

patient group and 666 (58.6%) and 472 (41.4%) in the control group. In children with nephrotic syndrome frequencies of the AA, Aa, and aa genotypes were 18 (16.7%), 43 (39.8%), and 47 (43.5%) respectively, whereas in controls, they were 171 (30.0%), 324 (57.0%), and 74 (13.0%). There was significant difference between allele frequency (p=<0.0001; OR=2.447, 95%CI=1.811-3.306), and genotype frequency (p=<0.0001; OR=5.154, 95%CI=3.279-8.101) in the INS and control group (Table 2).

VDR's TaqI Restriction Site Polymorphism

The allele frequency for the T and t of TaqI were 130(60.2%) and 86(39.8%) in the patient group and 762(67.0%) and 376(33.0%) in the control group. There was no significant difference in genotype frequency (p=0.4431; OR=1.25, 95%CI=0.7048-2.216) as well as in the allele frequency (p=0.0602; OR=1.341; 95%CI= 0.994-1.808) between the INS and control group (Table 2).

Table 2: Distribution of VDR among patients and healthy control group

Variants	*Patients (n =108)* *No. (%)*	*Control (n=569)* *No. (%)*	*OR (95% CI)*	*P- value*
APA1				
AA	18 (16.7%)	171 (30.0%)	2.148 (1.256-3.675)	0.0064*
Aa	43 (39.8%)	324 (57.0%)		
aa	47 (43.5%)	74 (13.0%)		
Allele A	79 (36.6%)	666 (58.6%)	2.447 (1.811-3.306)	<0.0001*
Allele a	137 (63.4%)	472 (41.4%)		
TAQ1				
T T	39(36.1%)	267 (47.0%)	0.6393 (0.4176-0.9787)	0.0495*
T t	52(48.1%)	228 (40.0%)		
t t	17 (15.8%)	74 (13.0%)		
Allele T	130 (60.2%)	762 (67%)	0.7459 (0.5530-1.006)	0.0648
Allele t	86 (39.8%)	376 (33%)		
FOK1				
FF	43 (39.8%)	270 (47.4%)	0.7326 (0.4818-1.114)	0.1757
Ff	53 (49.15%)	284 (50.0%)		
f f	12 (11.1%)	15 (2.6%)		
Allele F	139 (64.4%)	824 (72.4%)	0.6879 (0.5059-0.9354)	0.0207*
Allele f	77 (35.6%)	314 (27.6%)		
BSM1				
bb	69 (63.8%)	434 (76.3%)	8.309 (2.303-29.971)	0.0007*
Bb	33 (30.6%)	131 (23.0%)		
BB	6 (5.6%)	4 (0.7%)		
Allele b	171(79.2%)	999 (87.8%)	1.891 (1.302-2.747)	0.001*
Allele B	45 (20.8%)	139 (12.2%)		

*Significant value (p<0.05)

VDR's FokI Restriction Site Polymorphism

In children with nephrotic syndrome, homozygous FF was 39.8%, heterozygous Ff was 49.15%, and homozygous ff was 11.11% respectively, whereas in controls, they were 270(47.4%), 284(50.0%), and 15(2.6%). Incidence of F and f alleles of FokI were 139(64.4%) and 77(35.6%) in the childhood nephrotic syndrome group and 824(72.4%) and 314(27.6%) in the control group. There was significant difference in genotype frequency (p=0.0003; OR=4.617; 95%CI=2.096-10.168) and allele frequency (p=0.0216, OR=1.454; 95% CI= 1.069-1.977) between the INS and control group (Table 2).

VDR's BsmI Restriction Site Polymorphism

In children with nephrotic syndrome frequencies of the BB, Bb, and bb genotypes were 5.6%, 30.6%, and 63.8% respectively, while in controls, they were 0.7%, 23.0%, and 76.3%. The frequency for the B and b alleles of BsmI were 45 (20.8%) and 171(79.2%) in INS patient and 139(12.2%) and 999(87.8%) in the control group. There was significant difference between

Table 3: Combined analysis of VDR genotypes among INS patients and controls

Genotype	*Patients (108)*	*Controls (569)*	*P- value*	*OR (95 % CI)*
	No. (%)	*No. (%)*		
Double: TaqI and ApaI				
T1 & A1	7 (6.48%)	165 (29.0%)	<0.0001*	5.893 (2.682-12.95)
T1 & A0	32 (29.64%)	176 (31.0%)	0.821	0.9402 (0.5996-1.474)
T0 & A1	11 (10.18%)	80 (14.0%)	0.3556	0.6932 (0.3558-1.351)
T0 & A0	58 (53.70%)	148 (26.0%)	<0.0001*	3.3 (2.163-5.033)
Double: TaqI and FokI				
T1 & F1	12 (11.11%)	182 (32.0%)	<0.0001*	0.2658 (0.1422-0.4969)
T1 & F0	27 (25.0%)	159 (28.0%)	0.5590	0.8595 (0.5358-1.379)
T0 & F1	31 (28.70%)	103 (18.1%)	0.0171*	1.821 (1.140-2.910)
T0 & F0	38 (35.19%)	125 (21.9%)	0.0046*	1.928 (1.239-3.000)
Double: TaqI and BsmI				
T1 & B1	25 (23.15%)	284 (50.0%)	<0.0001*	0.3023 (0.1877-0.4868)
T1 & B0	14 (12.96%)	57 (10.0%)	0.3908	1.338 (0.7162-2.499)
T0 & B1	44 (40.75%)	154 (27.0%)	0.0055*	1.853 (1.210-2.837)
T0 & B0	25 (23.14%)	74 (13.0%)	0.0108*	2.015 (1.210-3.354)
Double: Apa1 And FokI				
A1 & F1	8 (7.41%)	114 (20.0%)	0.0010*	0.3193 (0.1510-0.6753)
AI & F0	10 (9.26%)	131 (23.0%)	0.0007*	0.3412 (0.1729-0.6731)
A0 & F1	35 (32.41%)	171 (30.1%)	0.6488	1.116 (0.7179-1.735)
A0 & F0	55 (50.92%)	153 (26.9%)	<0.0001*	2.822 (1.853-4.296)
Double: ApaI and BsmI				
A1 & B1	11 (10.18%)	176 (30.9%)	<0.0001*	0.2532 (0.1324-0.4844)
A1 & B0	7 (6.48%)	68 (11.9%)	0.1305	0.5106 (0.2278-1.144)
A0 & B1	58 (53.70%)	262 (46.1%)	0.1716	1.359 (0.8996-2.054)
A0 & B0	32 (29.64%)	63 (11.1%)	<0.0001*	3.382 (2.074-5.516)
Double: FokI and BsmI				
F1 & B1	29 (26.86 %)	199 (35.0%)	0.1198	0.6825 (0.4312-1.080)
F1 & B0	14 (12.96%)	86 (15.1%)	0.6580	0.8365 (0.4560-1.534)
F0 & B1	40 (37.03%)	239 (42.0%)	0.3937	0.8122 (0.5312-1.242)
F0 & B0	25 (23.15%)	45 (7.9%)	<0.0001*	3.507 (2.042-6.025)
Triple: TaqI, ApaI, and FokI				
T1, A1 & F1	2 (1.86%)	85 (15.0%)	<0.0001*	0.1074 (0.02602-0.4436)
T0, A0 & F0	22 (20.37%)	80 (14.1%)	0.1060	1.564 (0.9253-2.642)
T1, A0 & F0	10 (9.26%)	97 (17.0%)	0.0439*	0.4965 (0.2499-0.9865)
T0, A0 & F1	6 (5.55%)	28 (4.9%)	0.8095	1.137 (0.4589-2.815)
T0, A1 & F0	5 (4.63%)	51 (8.9%)	0.1806	0.4931 (0.1921-1.266)
T0, A1 & F1	33 (30.55%)	74 (13.0%)	<0.0001*	2.943 (1.827-4.742)
T1, A0 & F1	25 (23.15%)	74 (13.0%)	0.0108*	2.015 (1.210-3.354)
T1, A1 & F0	5 (4.63%)	80 (14.1%)	0.0043*	0.2967 (0.1173-0.7508)
All together: TaqI, ApaI, FokI and BsmI				
T1, A1, F1 & B1	1 (0.93%)	11 (2.1%)	0.7018	0.4741 (0.06054-3.712)
T0, A0, F0 & B0	12 (11.11%)	57 (10.0%)	0.7291	1.123 (0.5805-2.172)

*Significant value (p<0.05), 0=mutant + heterozygous, 1=wild type genotype

genotype frequency (p=0.0018, OR=8.309; 95%CI=2.303-29.971) and allele frequency (p=0.0011, OR=1.891; 95%CI=1.302-2.747) in the INS and control group (Table 2).

To see the cumulative effect, we analyzed our data by pooling two, three and four genotypes of the VDR gene among patients and controls in different combinations as shown in table 3.

Our result revealed that the risk of the disease was 3.5 fold increased when we combined ff (mutant) genotype of FokI and BB (mutant) genotype of BsmI (p=<0.0001; OR=3.507), the risk of disease increased to 1.5 fold when we combined tt (mutant) of TaqI, aa (mutant) of ApaI and ff (mutant) of FokI (p=0.1060; OR=1.564) whereas no significant difference was observed when all the four genotypes were combined (Table 3).

DISCUSSION

Present study is the first report from North India regarding the role of different genetic variants of VDR gene polymorphism like TaqI, ApaI, FokI and BsmI gene in causation of Idiopathic Nephrotic Syndrome. There are some studies on different malignancies on Indian population (Selvaraj et al. 2008; Mittal et al. 2007; Mitra et al. 2006) but no study is available to evaluate the role VDR gene polymorphism in INS from Indian subcontinent. In the present study we have investigated 108 Idiopathic Nephrotic Syndrome patients and 569 healthy controls. These were genotyped for the VDR gene polymorphisms. Our results demonstrated that ApaI, TaqI and BsmI polymorphism may be one of the genetic risk factors for INS among north Indian population We observed that the allele frequency distribution of ApaI (a), TaqI (t), FokI (f) and BsmI (b) was in the order of 63.4%, 39.8%, 35.6%, and 20.8% respectively in the patient group. There was significant difference in allele frequency between the patient and control group in case of a allele of ApaI, f at FokI restriction site and b at BsmI restriction site. No significant difference in case of t at TaqI restriction site. However, when the two high risk genotype ff of FokI and BB of BsmI of VDR were combined, we found that the risk was raised to ~3.5 folds.

Recently VDR gene BsmI polymorphism bb genotype was found to be positively associated with the development of nephrotic syndrome (Ozaki et al. 2000). In the present study we observed that aa, ff, and BB genotypes of ApaI, FokI, and BsmI restriction sites alone or in combination are positively associated with nephrotic syndrome, suggesting that these genotypes may be associated with some factors which participates in the causation and progression of the nephrotic syndrome. Differentiation of VDR genotypes may trigger breakdown of the cytokine relationship directly or indirectly and may be associated with the pathogenesis of the NS, or it may lead to alter the VDR structure ultimately leading to altered receptor function, and lastly it may also increase or decrease the expression of VDR protein thereby causing disease. The data presented in this study is the first report from India regarding the role of genetic variants of VDR gene in nephrotic syndrome.

The FokI polymorphism leads to the production of a VDR protein that differs in length by three amino acids. Individuals with F allele of FokI initiates translation at the second ATG site thereby lacking the three NH-2 terminal amino acid of the full length VDR protein. In contrast, individual with the f allele initiate translation at the first ATG site and synthesize the full length VDR protein (Morrison et al. 1994). Though the BsmI and ApaI locus is intronic, a number of mechanisms have been invoked to explain how these polymorphisms might influence the expression of VDR. One of these explanations includes the disruption of a splice site for VDR mRNA transcription, which may result in truncated or alternatively spliced protein product (Nesic et al. 1993). Another explanation involves the changes in mRNA stability speculating that these intron might influence the level of mRNA product (Farrow et al. 1994; Nesic et al. 1993). The TaqI polymorphism results in a silent mutation in exon 9, with ATT and ATC both coding for isoleucine (Farrow et al. 1994).

Many tissues in the body, like heart, kidney, activated T and B lymphocytes etc have the nuclear receptor for 1, 25 dihydroxyvitamin D_3 (VDR). Thus, it is not surprising that 1,25 dihydroxyvitamin D_3 has multitude of biological effects which are non-calcemic in nature (Holick et al. 2004), and include effects on immunity, muscle and vasculature, and growth and differentiation of many cell types (Van Schooten et al. 1998). The effect of 1,25 dihydroxyvitamin D_3 is mediated through vitamin D receptor. VDR forms heterodimer complex with Retinoid X Receptor (RXR), this

heterodimer than interacts with VDRE (vitamin D responsive elements) which are present in the promoter region on target genes and are involved in regulating their own transcription (Beato et al. 1989). Extensive studies have revealed an association between VDR polymorphism and some complex diseases like osteoporosis (Peacock et al. 2002; Saijo et al. 1991), type 1 (McDermott et al. 1997) or type 2 diabetes (Oh and Barrett-Connor 2002), coronary artery disease (Van Schooten et al. 1998), and sytemic lupus erythematosus (Ozaki et al. 2000).

In addition to the non-availability of various parameters and information, the result of the present study may have also been influenced by the study design and composition of the sample population. Regarding the study design, it may be possible that being a single centre study, the samples are over representative of a particular genotype secondly it has been widely accepted that the Indian society is fragmented into numerous sub-groups identified by the name of the caste and hence there is a high possibility that the social structuring and stringent marital practices have also resulted into genetic struc-turing. Allelic heterogeneity, manifested by different alleles can have the same effect in various populations as this might arise from gene-environment interactions. Thus the same allele might function differently in altered environ-ments. We suggest that multi-centric studies involving a much higher number of subjects and including controls from different socio-cultural strata will lead to validate the strong association found in present study. Since is generally agreed that there are ethnic difference in VDR allele frequency (Sainz et al. 1997). The association between the genotypes and the nephrotic syndrome must still be examined in the other populations.

Conclusively, VDR gene polymorphisms appear to be an important genetic determinant in causation and progression of Idiopathic Nephrotic Syndrome. Considering important predisposition risk factor for INS, we observed that aa of ApaI, BB of BsmI, and ff of FokI, was strongly associated with nephrotic syndrome among Indians. No association was found for TaqI on INS patients. Further studies in this regard will open a number of options like timing, type, and doses of anti-steroid therapy. Incorporation of such approaches will allow an advance anticipation of the clinical outcome.

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