ORIGINAL ARTICLE

POLYMORPHISMS IN THE VITAMIN D RECEPTOR AND THE ANDROGEN RECEPTOR GENE ASSOCIATED WITH THE RISK OF UROLITHIASIS

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ABSTRACT

Transcriptional activity of the vitamin D receptor (VDR) gene is regulated by androgen receptor (AR) gene and both are associated with renal stone formation. We examined gene polymorphisms of VDR (PCR-RFLP) and AR (GeneScan analysis) in 125 stone formers and 150 controls from north India. Genotype Ff of Fok-I and Tt of Taq-I demonstrated significantly higher risk ($P < 0.001$, $OR = 3.559$ and $P = 0.019$, 1.830 respectively). Variant f allele exhibited 1.7-folds higher risk. Ff of Fok-I and Aa of Apa-I gene polymorphism showed higher risk in males only. Mean CAG repeat was significantly higher in hypercalciuric patients as compared to normocalciuric (mean = 21.62 ± 3.384 vs. 20.11 ± 3.182 ; $P = 0.034$). Combined effects 1.8-folds higher risk in patients with Tt genotype of Taq-I and short CAG repeat. Thus, association of FokI and TaqI VDR gene polymorphisms suggest VDR as an important genetic marker for urolithiasis. Further, patients with combination of Tt of Taq-I and short CAG repeat were at higher risk for stone formation.

KEY WORDS

Androgen receptor, Polymorphism, Urolithiasis, Vitamin D receptor.

INTRODUCTION

Calcium oxalate stone disease is the most common metabolic abnormality associated with calcium urolithiasis. The cause of calcium oxalate stone is heterogeneous, involving both genetic and environmental factors. It is a multifactorial disorder resulting from the interaction between environmental influences and hormonal and genetic factors. Rendina et al in their study reported; genetic factors to be important determinants for kidney stone (1). A recent report demonstrated MGP gene polymorphism to be associated with kidney stones formation (2). Besides genetic and environmental factor, hormones also play a very significant role in stone formation

(3). In another study, the total urinary testosterone concentration was found lower among stone patients than among normal healthy subjects, suggesting that androgen may play a role in the pathogenesis of urolithiasis (4).

The VDR and AR genes are both members of nuclear receptor superfamily regulating the genes for hormone-response (5). The transcriptional activity of the VDR gene is regulated in part by AR gene (6). Both these nuclear receptors are ligand-dependent transcriptional regulatory proteins (7). The VDR gene located on chromosome 12, is 5.6 kb in size. Several polymorphisms have been identified and their functional significance and potential effects on disease susceptibility have been investigated (8, 9). Vitamin D produces its effect via the vitamin D receptor (VDR). Any genetic defect in VDR may change the calcium metabolism and increase the risk of stone formation. Allelic variations in the VDR gene may, at least partially, reflect the genetic component of urinary stone disease (10, 11, 12). The AR, a member of the steroid receptor superfamily, is involved in various biological processes such as sexual differentiation, maturation and spermatogenesis (13). The gene is located in the chromosome Xq11-q12 region

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and has a polymorphic cytosine, adenine and guanine (CAG) microsatellite on exon-1, which codes for variable-length glutamine repeats in the N-terminal domain of the AR protein (14). A shorter allele has been shown to increase transactivation activity (15). The CAG repeat length for the AR is related to different diseases like prostate diseases, including prostate carcinoma and BPH (16) and also urolithiasis (17).

We thus chose these two steroid hormone receptors as candidate genes for kidney stone susceptibility. It may be possible that the AR may interact and influence VDR for the risk of stone formation. Genetic association studies are likely to shed light in our current understanding of their roles in the risk of urolithiasis. In the present study, we have evaluated the possible genetic association of two important steroid hormone receptors such as VDR and AR polymorphisms in the risk of urolithiasis in case-control study in Northern India.

MATERIALS AND METHODS

A total of 125 patients (mean age 40.0 ± 11.5 years), treated at the Urology clinic of our tertiary care hospital, Lucknow, India, between the period of November 2004 and January 2006 were enrolled in the study. The control group consisted of 150 unrelated healthy volunteers (mean age 41.5 ± 10.5 years), with no history of stone disease or of renal calcification. The diagnosis of urinary tract calculus was documented by a plain X-ray film, intravenous pyelography and renal ultrasound. Routine tests were done in form of urinary microscopic hematuria and renal ultrasonography in order to exclude any individual with renal calcification. Hypercalciuria was determined by 24-hr collected urine examination. Patients with urinary tract infection were excluded from the analysis. Healthy controls for the study were drawn from general population taking care of matching age, gender and ethnicity. The study was performed with the approval of the hospital ethical committee and informed consent was obtained from the subjects participating in the study.

DNA extraction: Five ml of blood was collected in EDTA vials from cases and controls. DNA was extracted from blood lymphocytes using 'salting out' method (18).

Genotyping of VDR Gene by PCR-RFLP: Table 1 shows the details of the VDR gene screening. The primers of the VDR gene (Fok-I, Taq-I and Apa-I) were used as reported earlier (19, 20, 21).

PCR Assay for AR Polymorphism by Gene Scan: A

fragment of Exon 1 of AR gene was Genotyped using the following primers (22).

F – 5'CAG AAT CTG TTC CAG AGC GTG C 3'

R – 5'AAG GTT GCT GTT CCT CAT CCA G 3'

The forward primer was synthesized (ABI 392 oligosynthesizer, Perkin Elmer, Foster City, Calif) with 5'FAM (Carboxy-fluorescein) label (Perkin Elmer) in order to analyze the PCR product in the automated DNA sequencer (ABI 377). Each reaction sample contained 10ng genomic DNA, 1X PCR buffer containing 1.5 mM $MgCl_2$, 10pM of each primer, 200μM Deoxynucleotide triphosphates and 2U of AmpliTaq Gold (Perkin Elmer). Thermal cycling parameters consist of thirty PCR cycles of 1min at 94°C, 1 min at 60°C and 1 min at 72°C. The cycle programme was preceded by incubation at 94°C for 2min and was followed by a final extension at 72°C for 5min.

Gene Scan and Genotyping: For genescan 3.0 μl of the PCR product was mixed with 0.2 μl of ROX500TM and 6.8 μl of formamide. Upon, denaturation for 5 minute at 95°C and cooling for 5 minute on ice, the samples were run on 3730DNA analyzer (Applied Biosystems, USA). PCR and Genescan were repeated for all the samples to confirm the number of repeats. The raw data were further analyzed using GeneMapper software to obtain the allele (repeat) size. This work has been done at CCMB Hyderabad.

Statistical Analysis: Analysis of data was performed using the computer software SPSS (ver 11.5, Chicago Japan) for windows. Allele frequencies, genotype frequencies and carriage rates of patients and controls were compared using a 2x2 contingency table using Fisher's exact test. Mean, median, mode and standard deviation were calculated as descriptive in each group. Mean CAG repeats in patients and controls were compared by 't' test. The number of CAG repeats was examined as continuous. Differences were considered statistically significant for $P < 0.05$. Odds ratios (OR) with 95% CI were calculated for disease susceptibility or severity in carriers of specific alleles. Haplotype frequencies and linkage disequilibrium were estimated using the expectation-maximization (EM) algorithm implemented in the SNPAnalyzer.

RESULTS

Frequency of VDR (Fok-I, Taq-I and Apa-I) gene polymorphism: The distribution of genotypes of VDR gene (Fok-I, Taq-I and Apa-I) in control and renal stone patients are shown in Table 2. Significant difference in the genotypic frequencies between patients and controls were observed for

Table 1: Main characteristics of VDR gene polymorphisms and techniques used for screening of Vitamin-D receptor (Fok-I, Taq-I & Apa-I) gene polymorphism

	VDR (Fok-I)	VDR (Apa-I)	VDR (Taq-I)
Type of Polymorphism	(C/T)	G/A	T/C
Site of Polymorphism	Exon-2	Intron 8	Codon 352 in exon 9
PCR Primers	F 5' – AGC TGG CCC TGG CAC TGA CTC TGC TCT – 3' R 5' – ATG GAA ACA CCT TGC TTC TCC CTC – 3'	F5'CAACCAAGACTAC AAGTACCGCGTCAGTGA-3' R 5' – AAC CAG CGG GAA GAG GTC AAG GG – 3'	F 5'–CAG AGC ATG GAC AGG GAG CAA–3' R 5'–GCA ACT CCT CAT GGC TGA GGT CTC–3'
Digestion	Fok-I	Apa-I	Taq-I
Allele size, bp	FF/ 265 bp ff/ 196 & 69 bp Ff/ 265 bp, 196 & 69 bp	BB/ 1700 bp and 300 bp Bb/ 2000, 1700, 300 bp bb/ 2000 bp	TT/495 bp and 245 bp, tt/ 290 bp, 245 bp and 205 bp, Tt/ 495 bp, 290 bp, 245 bp and 205 bp
PCR Conditions			
Denaturation	94°C for 30 s	94°C for 30 s	94°C for 30 s
Anneling	58°C for 30 s	63°C for 30 s	63°C for 30 s
Extension	72°C for 1 minutes	72°C for 2 minutes	72°C for 1 minutes
No of Cycles	35	35	36

VDR Fok-I and Taq-I. The genotype Ff of Fok-I and Tt of Taq-I showed significantly higher risk for stone formation ($P<0.001$, $OR=3.559$; $95\%CI=2.06-6.14$ and $P=0.019$, 1.830 ; $95\%CI=1.10-3.03$ respectively). The frequency of low producing genotype FF in Fok-I and Taq-I were higher in controls as compared to patients (46.0% vs 20.0% and 56.0%

vs. 44.8% respectively) and frequency of heterozygous Ff and Tt genotype carrying variant allele were 50.7% and 33.3% in controls and 78.4% and 48.8% in patients respectively. No significant association was seen for Apa-I and the frequencies were comparable. Allele frequencies are shown in Table 3. The variant f allele showed significantly 1.7-folds higher risk for stone formation ($P=0.003$, $OR=1.71$; $95\%CI=1.21-2.44$).

Fig 1: Distribution of CAG Galleles in stone patients and healthy controls

CAG repeat length distribution: Figure 1 shows the distribution of CAG repeats in patients and controls. The number of CAG repeats ranged from 12-38 in Stone patients and controls. The un-paired student's 't'-test demonstrated that mean number of CAG repeats in Stone patients (21.13 ± 3.54) was not significantly different from controls (21.84 ± 4.65) as evident from t-test ($P=0.161$). Median CAG repeat was 21 in both patients as well as controls. CAG repeats were categorized in two groups (more than and less than/equal to mean CAG repeat) and risk was calculated using binary logistic regression analysis but no significant association was established ($P=0.489$), Table 4.

Association of stone formation according to gender: We determined the VDR genotypic frequencies and CAG repeat distribution in male and female stone formers separately (Table 5 and 6). We observed significant difference in Ff of Fok-I and Aa of Apa-I gene polymorphism in males only showing

Table 2: Distribution of VDR gene (Fok-I, Taq-I & Apa-I) polymorphism among the healthy control subjects and the calcium oxalate stone patients

Gene (95%CI)	Genotype	Controls (%) n=150	Cases (%) n=125	P-value	Odds ratio
VDR Fok-I	FF	69 (46.0)	25 (20.0)	-	1.0 (Ref)
	Ff	76 (50.7)	98 (78.4)	<0.001	3.559 (2.06-6.14)
	ff	5 (3.3)	2 (1.6)	0.909	1.104 (0.20-6.05)
VDR Taq-I	TT	84 (56.0)	56 (44.8)	-	1.0 (Ref)
	Tt	50 (33.3)	61 (48.8)	0.019	1.830 (1.10-3.03)
	tt	16 (10.7)	8 (6.4)	0.537	0.750 (0.30-1.87)
VDR Apa-I	AA	57 (38.0)	43 (34.4)	-	1.0 (Ref)
	Aa	71 (47.3)	70 (56.0)	0.309	1.307 (0.78-2.18)
	aa	22 (14.7)	12 (9.6)	0.431	0.723 (0.32-1.62)

higher risk while no association was seen in female group. Though high risk was seen in males with short CAG repeat however, no significant frequency distribution of CAG repeats in either male ($P=0.133$) or female ($P=0.724$) patients and controls were observed.

Distribution according to Hypercalciuric and Normocalciuric: No significant difference in genotype distribution between the groups of normocalciuria and hypercalciuria patients for all the three VDR polymorphism was perceived, Table 7. Mean CAG repeat was lower in normocalciuric (20.11 ± 3.182) as compared to hypercalciuric (21.62 ± 3.384) patients ($P=0.034$).

Association of stone formation with combined effect of CAG repeats length and VDR genotypes: Evaluation was done for CAG repeat length polymorphism in combination with genotypes of all the VDR genes studied in patients and controls. The subjects were divided into two groups: >21 repeat

length and ≤ 21 repeat length and their interaction with genotypes was evaluated with risk of stone formation using binary logistic regression analysis by taking patients and controls as dependent variable and repeat length and genotypes as categorical, Table 8. The combined effect demonstrated 1.8-folds higher risk for Tt genotype of Taq-I and ≤ 21 repeat ($P=0.019$, OR=1.830). No combined association was seen for Fok-I and Apa-I genotypes, Table 8.

DISCUSSION

Urolithiasis is a multifactorial pathology resulting from the interaction between environmental influences, hormonal and genetic factors (23). It is still an enigmatic problem for biologist with very vague understanding of its pathogenesis and mode of early detection due to lack of well-established genetic marker. With the limited understanding of disease and susceptibility genes, functional polymorphism in genes involving, steroidogenesis, steroid action and metabolizing

Table 3: Comparison of allelic frequencies of VDR (Fok-I, Taq-I & Apa-I) gene observed in healthy control subjects and the calcium oxalate stone patients

Gene, allele	Patients (%)	Controls (%)	P/OR
VDR (Fok-I)			
F	148 (59.2)	214 (44.0)	0.003; 1.71 (1.21-2.44)
f	102 (40.8)	86 (56.0)	
VDR (Taq-I)			
T	173 (69.2)	218 (72.7)	0.396; 1.183 (0.81-1.71)
t	77 (30.8)	82 (27.3)	
VDR (Apa-I)			
A	156 (62.4)	185 (61.67)	0.930; 0.969 (0.68-1.37)
a	94 (37.6)	115 (38.33)	

Table 4: CAG repeats length distribution stone patients as compared to the controls

No. of CAG repeats	Stone patients	Controls	Odds ratios	95% CI	P-value
Median					
>21	49 (39.2%)	65 (43.3%)	1.00		
≤ 21	76 (60.8%)	85 (56.7%)	1.186	0.73-1.92	0.489

enzyme, could be major target of further studies in urolithiasis.

The VDR gene influence hormonal signal and regulates calcium homeostasis by affecting bone resorption and increasing calcium absorption. The allelic variations in the 3'-UTR region may affect the mRNA stability or change vitamin-D activity by affecting the regulation of translation and may cause a tendency for stone formation (12). In the present study we observed a significant association of VDR Fok-I and Taq-

I genotypes. The heterozygous genotype Ff of Fok-I and Tt of Taq-I carrying variant allele showed significantly higher risk for stone formation. The frequency of low producing genotype FF in Fok-I and TT Taq-I was higher in controls as compared to patients. Allele frequencies demonstrated that variant f allele showed significantly 1.7-folds higher risk for stone formation.

The ff and Ff forms of the VDR gene are associated with a decreased VDR efficiency (24). The high prevalence of Ff

Table 5: Distribution of VDR (Fok-I, Taq-I & Apa-I) gene polymorphism among the healthy control subjects and the calcium oxalate stone patients in male and female gender

VDR (Fok-I) Genotypes			
Male	FF (%)	Ff (%)	ff (%)
Controls (n=120)	60 (50.0)	58 (48.3)	2 (1.7)
Patients (n=103)	19 (18.43)	83 (80.6)	1 (0.97)
P, OR at 95% CI	1.0 (Ref)	<0.001, 4.519 (2.44-8.36)	0.715, 1.579 (0.13-18.39)
Female	FF (%)	Ff (%)	ff (%)
Controls (n=30)	9 (20)	18 (46.7)	3 (33.3)
Patients (n=22)	3 (13.6)	15 (68.2)	4 (18.2)
P, OR at 95% CI	1.0 (Ref)	0.224, 2.500 (0.57-10.93)	0.171, 4.000 (0.54-29.17)
VDR (Taq-I) Genotypes			
Male	TT (%)	Tt (%)	tt (%)
Controls (n=120)	64 (53.3)	43 (35.8)	13 (10.8)
Patients (n=103)	45 (43.7)	52 (50.5)	6 (5.8)
P, OR at 95% CI	1.0 (Ref)	0.056, 1.720 (0.98-2.99)	0.427, 0.656 (0.23-1.85)
Female	TT (%)	Tt (%)	tt (%)
Controls (n=30)	20 (66.7)	7 (23.3)	3 (10.0)
Patients (n=22)	11 (50.0)	9 (40.9)	2 (9.1)
P, OR at 95% CI	1.0 (Ref)	0.177, 2.338 (0.68-8.01)	0.845, 1.212 (0.17-8.38)
VDR (Apa-I) Genotypes			
Male	AA (%)	Aa (%)	aa (%)
Controls (n=120)	62 (51.7)	44 (36.7)	14 (11.6)
Patients (n=103)	42 (40.8)	55 (53.4)	6 (5.8)
P, OR at 95% CI	1.0 (Ref)	0.031, 1.845 (1.05-3.22)	0.385, 0.633 (0.22-1.77)
Female	AA (%)	Aa (%)	aa (%)
Controls (n=30)	17 (56.7)	10 (33.3)	3 (10.0)
Patients (n=22)	10 (45.5)	9 (40.9)	3 (13.6)
P, OR at 95% CI	1.0 (Ref)	0.484, 1.530 (0.46-5.04)	0.559, 1.700 (0.28-10.08)

Table 6: Allelic distribution of CAG repeats polymorphism according to presence and absence of 21 repeat in patients and controls in male and female gender

Allele	Men		Women	
	Control	Stone patient	Control	Stone patient
AR				
21- repeat	23 (16.7 %)	10 (9.7%)	6 (50%)	9 (40.9%)
No 21- repeat	115 (83.3 %)	93 (90.3%)	6 (50%)	13 (59.1%)
Total	138	103	12	22
*P; risk (95%CI)	0.133; 1.860 (0.84-4.1)		0.724; 1.444 (0.35-5.94)	

*Fisher's exact test

genotype in our population is accord to (25). In another study by (26) no association was observed with stone formation for VDR (Fok-I). In one of our previous study we reported a significant association of VDR (Fok-I) polymorphism with pediatric as well as adult nephrolithiasis. (27). Besides Fok I, there are other known genetic polymorphisms in the VDR gene that cluster at the 3' end like *Apal* polymorphisms in the intron 8 and *TaqI* variant in intron 9. Association of *Taq I* and *Apal* have also been reported with renal stone disease (28, 29). In our population we observed that Tt genotype of *Taq-I* showed significant risk for stone formation, similar with (28), while no association was seen for *Apal* genotypes. A recent report by Sezgin et al having no association between urolithiasis and VDR (*Apal*, *Bsml*, and *TaqI*) gene variants (30).

Association of AR gene with stone formation has been proved by animal studies showing that castrated male rats have a

marked reduction in stone formation (31) leading to the hypothesis that androgen has a positive association with stone disease (32). We examined the CAG repeat length in AR gene and observed that mean CAG repeats did not differ significantly in stone patients and controls (21 repeats in both groups). This observation is in accord to study by Chen et al (17).

It is apparent that men have higher stone incidence rates than women, implying that there could be a relationship between urolithiasis among men and hormonal variations (33). We therefore evaluated the association of VDR and AR genes according to gender also. We observed a significant difference in Ff of Fok-I and Aa of *Apal* genotypes in males showing higher risk while no association was seen in female group. Though short CAG repeat showed higher risk in males however the P-value did not reach significant. Chen et al observed a significant association of CAG repeat with stone

Table 7: Frequencies of VDR (Fok-I, *Taq-I* & *Apal*) genotypes in hypercalciuric vs. normocalciuric stone formers

	VDR (Fok-I) Genotypes		
	FF (%)	Ff (%)	ff (%)
Hypercalciuric patients (40)	11 (27.5)	28 (70.0)	1 (2.5)
Normocalciuric patients (85)	14 (16.5)	70 (82.3)	1 (1.2)
P, OR at 95% CI	1.0 (Ref)	0.143, 0.509 (0.20-1.25)	0.870, 1.273 (0.07-22.72)
	VDR (<i>Taq-I</i>) Genotypes		
	TT (%)	Tt (%)	tt (%)
Hypercalciuric patients (40)	18 (45.0)	20 (50.0)	2 (5.0)
Normocalciuric patients (85)	35 (41.2)	44 (51.8)	6 (7.0)
P, OR at 95% CI	1.0 (Ref)	0.755, 0.884 (0.40-1.92)	0.617, 0.648 (0.11-3.54)
	VDR (<i>Apal</i>) Genotypes		
	AA (%)	Aa (%)	aa (%)
Hypercalciuric patients (40)	17 (42.5)	20 (50.0)	3 (7.5)
Normocalciuric patients (85)	26 (30.6)	51 (60.0)	8 (9.4)
P, OR at 95% CI	1.0 (Ref)	0.211, 0.600 (0.26-1.33)	0.456, 0.574 (0.13-2.47)

Table 8: Binary logistic regression analysis of combined effect of CAG repeats length with VDR genes predicting the risk of stone formation (Variables in bracket are taken as reference i.e. 1.00)

Variable (reference group)	P-value	OR, 95% CI
VDR FokI (FF)-repeat (>21)		
Ff	0.203	1.371, 0.84-2.23
ff	0.503	0.563, 0.10-3.02
VDR TaqI (TT)-repeat (>21)		
Tt	0.019	1.830, 1.10-3.03
tt	0.537	0.750, 0.30-1.87
VDR ApaI (AA)-repeat (>21)		
Aa	0.250	1.348, 0.81-2.24
AA	0.425	0.724, 0.32-1.60

formation and showed that long repeats are protective in men (17).

Absorptive hypercalciuria is a common cause of kidney stones and cause may be an excessive intestinal absorption due to increase vitamin-D synthesis or alteration in the VDR gene. The involvement of VDR in calcium kidney stones has been speculate because half of the patients have idiopathic hypercalciuria, most of them with increase intestinal calcium absorption and low BMD (26). When we stratified our patients according to hypercalciuria and normocalciuria with all the three loci of VDR, it showed no significant difference, however, the mean CAG repeat was significantly higher in hypercalciuric patients showing higher urinary calcium excretion.

Short CAG repeats increases AR transactivation activity and thus increased efficiency to transmit growth signals (15), which may maintain intestinal calcium absorption and urinary calcium excretion. We further evaluated the number of polyglutamine (CAG) repeats of the AR gene influence stone formation in conjunction with VDR since AR signaling and VDR action have been shown to be linked (7, 34). Interestingly, the combined effect demonstrated 1.8-folds higher risk in patients with Tt genotype of Taq-I and short CAG repeat. Different polymorphisms of the VDR gene may interact with AR. It is possible that the transactivation efficiency associated with the various VDR genotypes is heterogeneous with respect to downstream genes, such that some genes are more efficiently transactivated by one genotype and other genes by another (6). Currently there is not a single report showing the synergistic effect of these two steroidal hormones with urolithiasis. Epidemiological data support genetic susceptibility of urolithiasis (23, 12) but association of genetic

polymorphisms with risk of urolithiasis is still incomprehensible. Both AR and VDR polymorphisms appear to preferentially confer risk for stone disease; however, larger and multicentric studies are suggestive to confirm these results.

The study, for the first time from the Indian subcontinent, represents our efforts to investigate the association of vitamin D receptor (Fok-I, Taq-I, Apa-I) and Androgen receptor gene variants in relation to urolithiasis. Thus, our results provided evidence to genetic association of FokI and TaqI VDR gene variants with renal stone formation. Fok-I and Apa-I showed higher risk for stone disease among male population. Long CAG repeat length was associated with hypercalciurea. Finally the combined effect of VDR and AR gene showed that patients with Tt genotype of Taq-I and short CAG repeat had higher risk for stone formation. Though this report confirms association of VDR polymorphisms with urolithiasis warrants further investigation in large cohort for susceptibility as well as functions allelic variants. In addition, measurement of the VDR and androgen levels in serum as well as their expression profiles in kidney tissue samples would have help in getting insights into their functional implications.

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