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Original

Vitamin D receptor gene polymorphisms in children with kidney stone disease

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Kidney stone disease has a multifactorial etiology involving the interaction of genetic and environmental factors. There is an increased risk of stone formation in the relatives of idiopathic stone patients, which can be explained up to 60% by genetic factors. This study was conducted to explore the association of vitamin D receptor (VDR) gene polymorphisms with the risk of urolithiasis (UL) in Turkish children.

We investigated the VDR gene polymorphisms: *ApaI, BsmI, TagI, Cdx2, FokI,* in 52 children (26 boys, 26 girls) with UL and in 51 healthy children (22 boys, 29 girls) without UL. *Apa I, BsmI, TagI, Cdx2, FokI* genotypes were analyzed by *Apa I, BsmI, TagI, Cdx2, FokI* restriction enzyme digestion, respectively. The resulting alleles are designated as ABTCF (*ApaI, BsmI, TagI, Cdx2,* and *FokI* restriction site is absent), or abtcf (*ApaI, BsmI, TagI, Cdx2, FokI* restriction site is present), respectively. Genotype and allele frequencies were calculated, and the association with UL, hypercalciuria and hypocitraturia was investigated.

Our data provide no statistically significant evidence for an association between UL and VDR *ApaI, BsmI, TagI, Cdx2,* and *FokI genotype* and allele frequencies. Patients with hypocitraturia and hypercalciuria were compared with the control group and no statistically significant difference was detected in terms of VDR gene *ApaI, BsmI, TagI, Cdx2,* and *FokI* polymorphisms and allele frequencies.

Our data suggest that the VDR *ApaI, BsmI, TagI, Cdx2,* and *FokI* polymorphisms do not indicate a significant risk for UL.

Key words: urolithiasis, hypercalciuria, hypocitraturia, vitamin D receptor.

Urolithiasis (UL) is a multifactorial disease, the onset and severity of which is influenced by both genetic and environmental factors. It is seen that 50-70% stone disease patients have a first degree relative with UL and a family history was reported to increase the disease risk, 2.57 times in males.[1,2] Additionally, studies of kidney stone-forming twins demonstrated a higher concardance for kidney stones in monozygotic than in dizygotic twins.[3] It is still not clear whether the increased risk is attributable to genetic factors, environmental factors or some combination. The genes responsible for heritability of UL are still not determined; however, several genetic loci that appear to have a minor contribution to UL have been identified like vitamin D receptor (VDR), vascular endothelial growth factor, E-cadherin, p21, calcium-sensing receptor, calcitonin receptor and osteopontin[4]. Among them, the VDR gene is the most widely studied.

The VDR gene is located on chromosome 12 and encodes VDR. The effect of vitamin D which is a key player in calcium metabolism is mediated by the interaction between its active metabolite, calcitriol, and its cellular receptor, VDR, on target cells.[5] In view of their potential influence on the hormonal signal, VDR gene polymorphisms have been studied in

disorders of calcium metabolism and in urinary calcium stone disease.[6-10] Four single nucleotide polymorphisms (SNPs) of the VDR gene, *ApaI, BsmI, TagI,* and *FokI,* which were hypothesized to influence the expression and/or function of the VDR protein have been widely studied to investigate the associations between these polymorphisms and the risk of UL, however, the results were inconclusive.

To evaluate a more precise estimation of the relationship, to date, four meta-analyses were performed[11-14] (Table I). Lin et al.[11] found a significant associations of *FokI* and *TagI* polymorphisms with UL risk. Zhang et al.[12] found associations between the *ApaI and TagI* SNPs and UL. Liu et al.[13] demonstrated that only *TagI* polymorphisms was associated with UL risk, whereas *ApaI, BsmI,* and *FokI* polymorphisms were not. The most recent study by Zhou et all.[14] indicated that VDR *ApaI, BsmI, TagI,* and *FokI* polymorphisms were not associated with UL risk for overall populations and in Caucasions. However, the *FokI f* allele and *ff* genotype, *TagI TT* genotype, *ApaI* gene polymorphisms were associated with the risk of UL in Asians. The genetic effect may be different in different populations. Therefore the present study was conducted to explore the association of VDR gene *ApaI, BsmI, TagI, Cdx2,* and *FokI* polymorphisms with the risk of UL in a Turkish population.

Material and Methods

Patient selection and urinary metabolic examinations. The present study is a case-control study. Fifty-two children with UL and fifty-one age and sex matched children were enrolled in this study. A diagnosis of UL was based on imaging (ultrasonography (US) or computed tomography). All of the cases were re-evaluated in our centre with serial US examinations 1-3 months later to confirm the diagnosis, exclude artefacts and follow UL status. A metabolic evaluation was performed including serum electrolytes and tests for metabolic risk factors, including hypercalciuria, hypocitraturia, cystinuria, hyperoxaluria, hyperuricosuria and hyperphosphaturia were carried out. Urinary solute abnormalities was detected by abnormal values in two consecutive 24-hour urine collections or spot urine samples. Hypercalciuria was diagnosed if the amounts of calcium in the urine exceeded 4 mg/kg per 24 h (0.1 mmol/kg/ 24 h) and/or the urinary calcium/creatinine ratio exceeded 0.8 mg/mg for 0–12 months, 0.53 mg/mg for 1–3 years, 0.4 mg/mg for 3-5 years, 0.3 mg/mg for 5-7 years and 0.21 mg/mg for children older than 7 years of age.[15,16] The normal value for urine citrate was accepted as >1.6 mmol/1.73m^2/24-hour and/or a urine citrate/creatinine ratio >0.1 mol/mol creatinine.[17] All control subjects were screened for UL by urinary US. Those who had UL were excluded. The exclusion criteria for patients and controls were the presence of recurrent urinary tract infection, renal failure, other metabolic diseases, hypercalcemia, hyperphosphatemia, primary and secondary hyperparathyroidism, hypervitaminosis D, chronic diarrhea, cancer, and osteoporosis. This study was approved by the local ethics committee and informed consent was obtained in all cases.

DNA extraction. Genomic DNA isolations were performed from 200 μl peripheral blood (EDTA-anticoagulated) leukocytes by using QIAamp DNA Blood Mini Kit (Qiagen Inc.) according to the manufacturer's protocol and stored at -20^0 C until the PCR (polymerase chain reaction) step.

Determination of Genotypes. Screening of polymorphisms was carried out by using SNaPshot® multiplex system (Applied Biosystems Inc.). For this purpose, 3 primers were designed for each amplicons; 2 for PCR and 1 for SNaPshot® reaction for polymorphisms Cdx2, FokI, BsmI, ApaI and Taq . Four PCRs were set for each sample (ApaI and TaqI were amplified with same reaction). Briefly, 2μl of genomic DNA and 20.2 μl of water were amplified in an automated thermal cycler. PCRs were checked by using 2% agarose gel electrophoresis. All 4 reactions for one patient's sample were mixed and purified by using NucleoFast® 96 PCR kit (MACHEREY-NAGEL GmbH). Purified PCRs were used in SNaPshot® assay which carried out according to the manufacturer's recommendations. Capillary electrophoresis of the SNaPshot® reactions were carried out by using ABI 3130 capillary electrophoresis instrument (Applied Biosystems Inc.). Genotyping of the polymorphisms were demonstrated on GeneMapper 4.0 software (Applied Biosystems Inc.), by analyzing the

Table I. Summary of Previous Meta-analyses that have Investigated the Association Between Vitamin D Receptor Gene Polymorphisms and Urolithiasis.

Author, year	N	VDR* gene polymorphisms associated with urolithiasis	VDR gene polymorphisms not associated with urolithiasis	Comment
Lin, 2011[11]	17 studies	TagI	ApaI and BsmI	Increased risk associated with TagI tt +Tt, FokI ff + Ff in Asians
Zhang, 2013[12]	23 studies	TagI and ApaI	FokI and BsmI	Increased risk associated with TagI tt +Tt and ApaI AA + Aa among Asians but not among Caucasians
Liu, 2014[13]	20 studies	TagI	FokI , BsmI and ApaI	Increased risk associated with TagI tt + Tt in Asians
Zhou, 2015[14]	6 studies	None	TagI, FokI , BsmI and ApaI	Increased risk associated with TagI TT and FokI ff Asians
Present study, 2015	52 children	None	TagI, FokI , BsmI, ApaI and Cdx2	

*VDR: vitamin D receptor

electrophoregrams obtained during capillary electrophoresis. The resulting alleles are designated as ABTCF (*ApaI, BsmI, TagI, Cdx2,* and *FokI* restriction site is absent), or abtcf (*ApaI, BsmI, TagI, Cdx2, FokI* restriction site is present), respectively. Genotype and allele frequencies were calculated, and the association with UL, hypercalciuria and hypocitraturia was investigated.

Statistical analysis. Differences in the frequencies of the VDR polymorphisms between patients with UL, hypercalciuria, hypocitraturia and the control group were analyzed using the Chi-square test. Hardy Weinberg equilibrium was tested for all polymorphisms for control group. Descriptive data are presented as mean ± standart deviation. The relative associations between patients with UL, hypercalciuria, hypocitraturia and controls were assesed by calculating odds ratios and 95% confidence intervals. A p-value less than 0.05 was considered as significant. SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

Results

Fifty-two children (26 boys, 26 girls), aged 106.15±41.12 (60-204) months with UL were evaluated. The control group included fifty-one children (22 boys, 29 girls), aged 125.88±43.22 months. The groups were similar with respect to age and gender (Table II). Urinary metabolic examination revealed that most common pathologic findings were hypocitraturia and hypercalciuria. Only 30% of the cases were normal for all parameters (Table II). Our data provide no statistically significant evidence for an association between UL and VDR *ApaI, BsmI, TagI, Cdx2,* and *FokI genotype* and allele frequencies (Table III, Table IV). On the other hand, although not statistically significant we found higher genotypic (tt genotype: 44% and 35% for patients and controls respectively, p:0.2) and allelic frequencies (t allele: 68% and 59% for patients and controls respectively, p:0.15) for *TaqI* in children with UL compared to controls (Table III, Table IV). Patients with hypocitraturia and hypercalciuria were compared with the control group and no statistically significant difference was detected in terms of VDR gene *ApaI, BsmI, TagI, Cdx2,* and *FokI* polymorphisms and allele frequencies.

Discussion

Urolithiasis is a complex disease resulting from the interaction between environmental influences, hormanal and genetic factors. The VDR gene influence hormonal signal and regulates calcium homeostasis by affecting bone resorption and incresing calcium absorption. The allelic variations in the 3' UTR region may alter the messenger RNA stability or change vitamin D activity by affecting the regulation of translation, hence predisposing

Table II. Demographic Characteristics of Patients with Urolithiasis and Control Group and Urinary Metabolic Examination of Study Group.

	Urolithiasis (n=52)	Control (n=51)	P
Sex n, (%)			>0.05
Female	26 (50)	29 (57)	
Male	26 (50)	22 (43)	
Age (months)*	106.15 ± 41.12	125.88 ± 43.22	>0.05
Urinary metabolic examination n, (%)			
Hypocitraturia	12 (23)		
Hypercalciuria	11 (21)		
Hyperoxaluria	4 (8)		
Cystinuria	3 (6)		
Hypercalciuria and hypocitraturia	3 (6)		
Hypercalciuria and hyperoxaluria	3 (6)		
Normal	16 (30)		

* Data are shown as mean ± standart error

to stone disease.[18,19] A lot of genetic epidemiological studies have been conducted to explore the relationship between SNPs and UL.[11-14] However, studies yielded conflicting results. Taken together, all 4 meta-analyses exhibited significant associations between *TagI* polymorphism and UR risk in Asian population but not in Caucasian population (Table I), suggesting that ethnic differences may play a role in UL susceptibility.[11-14] It is possible that different UL risks in Asians and Caucasions were due to exposure to various environmental factors in addition to genetic susceptibility.

Turkey which is a country that constitutes a bridge between Europe and Asia has experienced major population movements and one of the significant countries in the world from the genetic diversity (variability) point of view. In a recent study by Hodoğlugil and Mahley[20], population structure and genetic relatedness of samples from three regions of Turkey, using over 500,000 SNP genotypes, were compared together with Human Genome Diversity Panel data. Principal component analysis, in this study, showed a significant overlap between Turks and Middle Easterners and a relationship with Europeans and South and Central Asians; however, the authors concluded that Turkish genetic structure is unique. As previously mentioned the genetic effect may be different in different populations. Therefore, in our study, we examined VDR gene polymorphisms, *ApaI, BsmI, TagI, Cdx2, FokI* and their relationship to UL, hypercalciuria and hypocitraturia in a Turkish population having uinique genetic structure and high genetic variability. In addition, to the best of our knowladge, present study is the first study examining the role of Cdx2 polymorphism of the VDR gene in the etiology of UL.

In our study, we did not observe significant evidence for an association between UL and VDR *ApaI, BsmI, TagI, Cdx2,* and *FokI genotype* and allele frequencies. There are few studies on the correlation of VDR polymorphism with UL in Turkish children.[6] Gunes et al.[6] demonstrated that the VDR *ApaI, BsmI* and *TagI* polymorphisms do not confer a significant risk for UL. Our data is consistent with the findings of Gunes et al.[6]. Although not statistically significant, we found higher genotypic and allelic frequencies for *TaqI* in children with UL (Table III, Table IV). Similarly largest 3 of 4 meta-analyses showed an increased risk associated with the *TagI tt + TT* polymorphism in Asians (Table I). Findings in meta-analyses and our study indicates that the *TagI tt* genotype has a close relationship to UL risk especially

Table III. Vitamin D Receptor Gene *Bsml, Apal, Fokl, Taql,* and *Cdx2* Polymorphisms in Children with Urolithiasis and the Control Group.

Gene	Allel	Patients n (%)	Controls n (%)	p
Bsml	BB	28 (54)	20 (39)	0.302
	Bb	19 (36)	23 (45)	
	bb	5 (10)	8 (16)	
Apal	AA	18 (35)	22 (43)	0.134
	Aa	24 (46)	14 (28)	
	aa	10 (19)	15 (29)	
Fokl	FF	23 (44)	26 (51)	0.770
	Ff	25 (48)	21 (41)	
	ff	4 (8)	4 (8)	
Taql	TT	4 (8)	9 (18)	0.280
	Tt	25 (48)	24 (47)	
	tt	23 (44)	18 (35)	
Cdx2	CC	39 (75)	34 (67)	0.602
	Cc	10 (19)	12 (23)	
	cc	3 (6)	5 (10)	

Table IV. Allelic Frequencies of Vitamin D Receptor Gene *Bsml, Apal, Fokl, Taql,* and *Cdx2* Polymorphisms in Children with Urolithiasis and the Control Group.

Gene	Allel	Patients n (%)	Controls n (%)	p
Bsml	B allel	75 (72)	63 (62)	0.114
	b allel	29 (28)	39 (38)	
Apal	A allel	60 (58)	58 (57)	0.904
	a allel	44 (42)	44 (43)	
Fokl	F allel	71 (68)	73 (72)	0.606
	f allel	33 (32)	29 (28)	
Taql	T allel	33 (32)	42 (41)	0.159
	t allel	71 (68)	60 (59)	
Cdx2	C allel	88 (85)	80 (78)	0.335
	c allel	16 (15)	22 (22)	

in certain ethnic groups. The mechanism by which the *TagI* gene polymorphism affects the development of UL is unclear because the *TagI* polymorphism of the VDR gene locating in the 3′ UTR region is considered to be silent and does not cause any amino acid change in the protein.[19] Since the 3′ UTR region may be involved in the regulation of mRNA stability, the *TagI* polymorphism may change the level of VDR mRNA and affect the individual responsiveness of vitamin D[18,19]. Further large-scale studies with more ethnic groups should be conducted to better clarify the association of the *TagI* polymorphism with the risk of UL.

The present study has some limitations. First, the sample size was relatively small along with the small number of patients with hypercalciuria and hypocitraturia to draw any conclusion. This study was also a single center based study which could be unrepresantative

of patients with UL for all ethnic groups in Turkish population. Secondly, measurement of enviromental factors such as UV exposure and nutrition effecting vitamin D levels was lacking, therefore, vitamin D gene and environment interactions could not be investigated. These limitations could be improved by enrolling a greater number of patients from all over the country and assessing various environmental and nutritional risk factors.

In conclusion, our data suggest that the VDR *ApaI, BsmI, TagI, Cdx2,* and *FokI* polymorphisms do not indicate a significant risk for UL in Turkish population and *TagI* gene polymorphism may be associated with increased risk in certain ethnic groups.

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