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ORIGINAL PAPER

*Apa*L1 urokinase and *Taq*1 vitamin D receptor gene polymorphisms in first-stone formers, recurrent stone formers, and controls in a Caucasian population

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Abstract The purpose of this study was to determine differences in genotype distribution and allele frequency of urokinase and vitamin D receptor (VDR) single nucleotide polymorphisms (SNPs) between first-stone formers, recurrent stone formers, and controls in a Caucasian population. A total of 86 first-stone formers, 78 recurrent stone formers, and 167 controls were included. Urokinase and VDR SNPs were tested by gene amplification followed by *Apa*L1 and *Taq*1 endonuclease digestion, respectively. Baseline variables, genotype, and allele frequencies were compared between the three groups, using descriptive statistics. Adjusted odds ratios were calculated to estimate the risk for recurrent urolithiasis associated with genotypes. We found that differences in the distribution of *Apa*L1 SNP and *Taq*1 SNP genotypes were statistically different between recurrent stone formers and first-stone formers, and between recurrent stone formers and controls. Allele frequency analysis showed that the T allele for *Apa*L1 SNP and the C allele for *Taq*1 SNP were significantly associated with recurrent urolithiasis. For *Taq*1 SNP, logistic regression analysis showed that the C/C genotype was associated with a more than threefold higher risk for recurrent urolithiasis. We conclude that *Apa*L1 and *Taq*1 SNPs of the urokinase and VDR genes are associated with recurrent urolithiasis in a Caucasian population.

Keywords Urolithiasis · Single nucleotide polymorphism · Urokinase · Vitamin D receptor

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Introduction

Urolithiasis, which reflects genetic and environmental factors, is a worldwide problem that is associated with substantial health and socioeconomic burdens. The prevalence of the disease in industrialized countries ranges from 5–9 % and is likely to increase in coming years [1]. Despite steady advances in shock-wave technology and endourological techniques, urolithiasis will recur in a majority of cases. It would be beneficial if, among patients with recurrent urolithiasis and their relatives, the clinician could predict patients at high risk that would benefit from tailored therapy and continual check-ups with sufficient scientific evidence.

In this context, single nucleotide polymorphism (SNP) gene analysis has been the focus of increased attention. The potential of SNP analysis in urolithiasis lies in its ability to map stone disease genes. One SNP of interest is the *Apa*L1 C/T SNP located at the 3′-untranslated region (UTR) of the urokinase gene on chromosome 10 [2]. It has been proposed that the fibrinolytic function of urinary urokinase may prevent the formation of organic matrix in the urinary tract [3], a major step in urinary stone formation [4]. Another candidate gene is the vitamin D receptor (VDR)

gene, which is involved in calcium metabolism, including calcium intestinal absorption and renal reabsorption [5].

Despite the increasing interest in gene–disease relationships, no prior study has investigated the urokinase gene *Apa*L1 C/T SNP in adult Caucasians. The aim of our study was to compare genotype distribution and allele frequency of the polymorphic urokinase and VDR genes between first-time stone formers, recurrent stone formers, and control patients, using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) technique.

Materials and methods

The study protocol was approved by the Ethical Committees of the Ondokuz Mayis and Uludağ Universities. A total of 164 stone formers (64 women and 100 men) aged 18 years and older were recruited from tertiary referral centers over the course of 2 years. Stone disease was confirmed by computed tomography, plain-film radiography, ultrasound examination, urinary stone emission, or endourological removal. There were 86/164 (52 %) first-time stone formers and 78/164 (48 %) recurrent (>1 episode) stone formers. The control group comprised 167 unrelated patients (80 women and 87 men) with normal urinalysis and absence of stone in ultrasound studies at general health screening. Among stone formers and controls, patients taking vitamin D and/or calcium supplements were excluded from the study. Furthermore, the control group did not include patients with known metabolic disorders and personal history and/or positive family history of urolithiasis. Clinical information on age at medical visit, menopausal status, recurrent stone episodes, and family history of urolithiasis was retrieved from medical charts. Family history was considered positive if any first- and/or second-degree relative had been affected by urolithiasis. Stone composition was analyzed by Fourier transform infrared reflectance spectroscopy. All participants provided informed consent to participate in the study.

For PCR–RFLP analysis venous blood samples were collected in EDTA tubes and genomic DNA was extracted by salt precipitation. The resulting DNA stock was stored at −20 °C. PCRs were carried out in a total volume of 25 µl, containing 50 ng of genomic DNA, 2–6 (50 for VDR) pmol of each primer, 200 mM deoxynucleotide triphosphates (MBI Fermentas, Amherst, New York, USA), 1.5 mM $MgCl_2$, 1xPCR buffer, 50 mM KCl, 1 unit Taq DNA polymerase (Promega, Madison, Wisconsin, USA). Primer sequences were: 5′-CCG CAG TCA CAC CAA GGA AGA G-3′ (forwards) and 5′-GAA CGA CAA TAG CTT TAC CCT CAG GCA-3′ (backwards) for urokinase SNP, and 5‘-CAG AGC ATG GAC AGG GAG CAA-3′ and 5′-CAC TTC GAG CAC AAG GGG CGT TAG C-3′ for VDR SNP (Iontek Inc., Maslak, Turkey). The primer sequences were checked using the University of California Santa Cruz genome browser (https://genome.ucsc.edu/cgi-bin/hgPcr). Detailed information on the two polymorphisms can be found in the NCBI SNP consortium database (http://www.ncbi.nlm.nih.gov/nuccore/G27040 for urokinase and http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=731236 for VDR). PCR products were amplified in a gradient thermal cycler (Techne, Burlington, New Jersey, USA). Cycling conditions were set as follows for urokinase/VDR: initial denaturation at 95/94 °C for 5 min, 35 cycles of denaturation at 95/94 °C for 60/30 s, annealing at 54/58 °C for 60/30 s, extension at 72 °C for 60/30 s, and final extension at 72 °C for 7 min.

PCR products were incubated with the restriction enzymes *Apa*L1 for urokinase SNP, *Taq*1 for VDR SNP (MBI Fermentas, Amherst, New York, USA), and restriction buffer at 37 °C for 16 h and 65° for 3 h, respectively. All digested products were loaded into 3 % agarose gel, electrophoresed and visualized with an appropriate gel imaging system (Vilber Lourmat®, Marne-la-Vallée, France). The genotypes were determined according to the size of DNA fragment. For *Apa*L1 urokinase SNP, digestion of the amplified 210 bp PCR product gave fragments of 185 and 25 bp if the product was excisable. C/C homozygotes yielded a 210 bp, undigested fragment, T/T homozygotes yielded 185 and 25 bp fragments, and C/T heterozygotes showed 210, 185, and 25 bp fragments (Fig. 1). For *Taq*1 VDR SNP, T/T homozygotes yielded a 501 bp fragment, C/C homozygotes yielded 208 and 293 bp fragments, and T/C heterozygotes showed 501, 293, and 208 bp fragments. For all experiments a 100 bp DNA ladder (MBI Fermentas, Amherst, New York, USA) was used. Laboratory personnel were blinded to all clinical data.

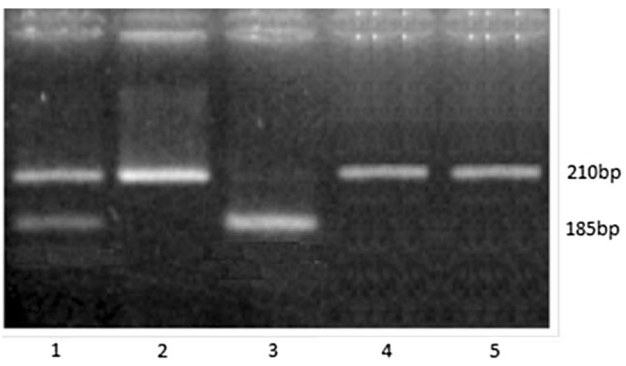

Fig. 1 PCR products obtained after digestion of the 210 bp amplified region of the urokinase gene 3′-untranslated region (UTR) with the restriction enzyme *Apa*L1shown on 3 % agarose gel. The amplified polymorphic region resulted in a digested fragment in lane 3 (185; 25 bp fragments not shown) (T/T), undigested fragments at *lanes 2,4,5* (210 bp) (C/C) and heterozygous bands at *lane 1* (C/T)

Table 1 Baseline characteristics of the patient population

	First-time stone formers ($n = 86$)	Recurrent stone formers ($n = 78$)	Controls ($n = 167$)	p value, recurrent vs. first-time	p value, recurrent vs. controls
Age, median (IQR)	45 (29–58)	41 (24–53)	45 (38–54)	0.3*	0.1*
Male gender, n (%)	50 (58)	50 (64)	87 (52)	0.4$^{\Phi}$	0.1$^{\Phi}$
Female gender, n (%)	36 (42)	28 (36)	80 (48)		
Menopause	15 (17)	8 (10)	27 (16)	0.2$^{\Phi}$	0.2$^{\Phi}$
Familial history +, n (%)	29 (34)	42 (54)	–	0.01$^{\Phi}$	–

* Kruskal–Wallis test

$^{\Phi}$ Chi-square test

Genotype and allele frequencies were obtained by direct counting and then dividing by the number of patients to determine genotype frequency and by the number of chromosomes to determine allele frequency. Goodness-of-fit to Hardy–Weinberg equilibrium was determined by χ^2-test using Online Encyclopedia for Genetic Epidemiology studies (OEGE) software (www.oege.org). Differences in baseline characteristics, genotype, and allele distribution between (1) recurrent stone formers and first-time stone formers, and (2) recurrent stone formers and controls were analyzed with the Chi-square test (or Fischer's exact test when greater than 20 % of the cells had an expected count of less than five) for categorical variables and the Kruskal–Wallis test for continuous variables. SNP data analyses were done for all patients and repeated for calcium-stone formers only. Multivariable logistic regression analysis was used to determine odds ratios (OR) and confidence intervals (CI) associated with a given genotype, adjusting for age and gender. All tests were performed using IBM SPSS 21 (IBM, Armonk, NY, USA), with a two-sided statistical significance set at $p < 0.05$.

Results

Median age of the patient population was 44 years (interquartile range [IQR] 35–54). Baseline characteristics of first-stone formers, recurrent stone formers, and controls are shown in Table 1. There was no statistically significant difference in terms of age and gender distribution between recurrent stone formers and first-time stone formers, and between recurrent stone formers and controls (all p values >0.05). A positive familial history was associated with recurrent urolithiasis ($p = 0.01$). Stone composition was available for 145/164 (88 %) patients and included 131/145 (90 %) calcium-containing stones, 9/145 (6 %) urate stones, 4/145 (3 %) infectious stones, and 1/145 (0.7 %) cystine stone.

Genotype distributions for *Apa*L1 urokinase SNP and *Taq*1 VDR SNP are shown in Table 2a and b. The control group was in Hardy–Weinberg equilibrium for *Taq*1 VDR SNP ($\chi^2 = 3.08$), not for *Apa*L1 urokinase SNP ($\chi^2 = 167$). Distribution of the *Apa*L1 urokinase SNP and *Taq*1 VDR SNP genotypes differed significantly between recurrent stone formers and first-stone formers ($p = 0.01$ for urokinase and $p = 0.003$ for VDR), and between recurrent stone formers and controls ($p = 0.002$ for urokinase and $p < 0.0001$ for VDR). When we analyzed only the 131 patients with calcium stone, similar results were found (Table 2b). Overall, the C/C genotype of *Apa*L1 urokinase SNP was found less frequently in recurrent stone formers than in first-time stone formers and controls. Moreover, the C/T genotype was detected only in recurrent stone formers. For *Taq*1 VDR SNP, the C/C genotype was found more frequently in recurrent stone formers than in first-time stone formers and controls. Allele frequency analysis showed that the T allele of *Apa*L1 urokinase SNP was significantly associated with recurrent stone formers ($p = 0.03$ vs. first-stone formers and $p = 0.002$ vs. controls). Furthermore, the C allele of *Taq*1 VDR SNP was significantly associated with recurrent stone formers ($p = 0.01$ vs. first-stone formers and $p = 0.001$ vs. controls).

For *Taq*1 VDR SNP, logistic regression analysis adjusted for age and gender showed that individuals that harbor the C/C genotype have a more than threefold higher risk for recurrent urolithiasis in comparison to individuals that harbor the T/T genotype (OR = 3.66, 95 % CI = 1.80–7.39, $p < 0.001$). This was also true for recurrent calcium-stone formers (OR: 3.76, 95 % CI = 1.78–7.99, p = 0.001). Due to the small number of patients harboring the T/T genotype, we lacked sufficient power to perform logistic regression analysis for *Apa*L1 urokinase SNP.

Table 2 Urokinase *Apa*L1 SNP and vitamin D receptor *Taq*1 SNP genotype distribution and allele frequency in first-stone formers, recurrent stone formers, and controls

	First-time stone formers ($n = 86$)	Recurrent stone formers ($n = 78$)	Controls (n = 167)	p value, recurrent vs. first-time	p value, recurrent vs. controls
(A) All stone formers and controls					
Urokinase *Apa*L1 SNP					
C/C, *n* (%)	81 (94)	64 (82)	155 (93)	0.01*	0.002*
C/T, *n* (%)	0	5 (6)	0		
T/T, *n* (%)	5 (6)	9 (12)	12 (7)		
C, *n* (%)	162 (94)	133 (85)	310 (93)	0.008	0.01
T, *n* (%)	10 (6)	23 (15)	24 (7)		
Vitamin D receptor *Taq*1 SNP					
T/T, *n* (%)	39 (45)	28 (36)	66 (40)	0.003	<0.0001
T/C, *n* (%)	37 (43)	24 (31)	86 (51)		
C/C, *n* (%)	10 (12)	26 (33)	15 (9)		
T, *n* (%)	115 (67)	80 (51)	218 (65)	0.004	0.003
C, n (%)	57 (33)	76 (49)	116 (35)		
	First-time stone formers ($n = 63$)	Recurrent stone formers ($n = 68$)	Controls ($n = 167$)	p value, recurrent vs. first-time	p value, recurrent vs. controls
(B) Calcium-stone formers and controls					
Urokinase *Apa*L1 SNP					
C/C, *n* (%)	58 (92)	54 (79)	155 (93)	0.047*	0.007*
C/T, *n* (%)	0	5 (7)	0		
T/T, *n* (%)	5 (8)	9 (13)	12 (7)		
C, *n* (%)	116 (92)	113 (83)	310 (93)	0.03	0.002
T, *n* (%)	10 (8)	23 (17)	24 (7)		
Vitamin D receptor *Taq*1 SNP					
T/T, *n* (%)	28 (44)	23 (34)	66 (40)	0.02	<0.0001
T/C, *n* (%)	26 (41)	21 (31)	86 (51)		
C/C, *n* (%)	9 (14)	24 (35)	15 (9)		
T, *n* (%)	82 (65)	67 (49)	218 (65)	0.01	0.001
C, *n* (%)	44 (35)	69 (51)	116 (35)		

Percentages may not sum due to rounding

* Fischer's exact test. All other p values were calculated the Chi-square test

Discussion

The mainstay for the prevention of urolithiasis includes dietary modifications, fluid intake, empiric medical therapy, or medical therapy based on specific biochemical anomalies [6]. Biochemical baseline is, however, limited in predicting drug efficacy and thus investigations into better predictors are needed [7]. In the present study, we show for the first time in an adult Caucasian population that *Apa*L1 urokinase SNP at the 3′-UTR is associated with recurrent urolithiasis. We also demonstrate that the T allele, which is frequent among Caucasian populations [8], is significantly associated with recurrent urolithiasis. In addition, we report that *Taq*1 VDR SNP is associated with recurrent urolithiasis. Our findings provide further support that in this Caucasian population genetic differences exist between non-stone formers, first-time stone formers, and recurrent stone formers.

Other authors have investigated *Apa*L1 urokinase SNP in smaller cohorts and different populations. Tsai et al., evaluating *Apa*L1 urokinase SNP in 258 Taiwanese patients, noted significant differences between calcium oxalate stone formers and healthy individuals [9]. Specifically, patients with recurrent calcium oxalate urolithiasis were more likely to harbor the C/T genotype. No T/T genotype was found. In agreement with our results, allele frequency analysis showed higher frequency of the T allele in patients with recurrent calcium oxalate urolithiasis (5.9 vs. 1.9 %, $p = 0.03$). Their findings and ours suggest that although the distribution of *Apa*L1 urokinase

SNP may modestly differ across some geographical areas, genetic differences between stone formers and non-stone formers in a given population remain. On the other hand, our results differ with findings from Mittal et al. In their study, *Apa*L1 urokinase SNP was compared between 130 patients with recurrent calcium oxalate stone disease and 150 healthy individuals in a north Indian population [10]. Although a marginal difference in the genotype distribution was detected, no difference was seen when analyzing allele frequencies. The discrepancy between this study and ours might reflect different patients' background, environmental factors, and selection criteria, as well as variations in laboratory techniques. Finally, Ozturk et al. investigated *Apa*L1 urokinase SNP in 120 patients from a Turkish pediatric population [11]. While differences in genotype distribution between recurrent stone formers and controls were reported, T allele frequency was similar. These results taken together with ours indicate that genetic factors involved in urinary stone formation in children and adults may be distinct for certain SNPs. Urokinase is an enzyme that may block the formation of organic matrix through its proteolytic function, thereby impeding the precipitation of minerals in the urinary tract environment [4]. Du Toit et al. reported lower urinary urokinase levels in men with urolithiasis compared to controls [12]. Hence, it could be advanced that urokinase SNPs may be involved in urinary urokinase downregulation.

SNPs of the VDR gene have been implicated in a variety of diseases, notably osteoporosis [13] and urolithiasis [14–18]. Moreover, suggestive linkages between calcium stone formation and several microsatellite markers located near the VDR locus have been demonstrated [19, 20]. *Taq*1 VDR SNP has been studied previously. In line with our findings, Seyhan et al. found that the C/C genotype and the C allele of *Taq*1 VDR SNP were associated with calcium stones in a children population with a similar ethnic background as ours [18]. Similarly, Nishijima et al. reported that differences in *Taq*1 genotype distribution in Japanese patients were associated with severe stone disease [14]. Conversely, Seo et al. found no significant risk for urolithiasis associated with *Taq*1 VDR SNP in Korean patients [21]. The association of other VDR SNPs with stone disease has been reported, including *Fok*I [15, 16] and *Bsm*I [17]. Upon binding of its primary ligand $1,25(OH)_2D_3$, VDR regulates calcium and phosphate metabolism, including renal calcium reabsorption. It has been shown in laboratory studies that SNPs or mutations of the VDR gene may alter transcriptional activity and VDR subcellular distribution [22]. It follows that SNPs may affect VDR activity in the urinary tract, and thus influence urinary calcium levels and stone formation. In addition, it has been reported that the VDR may play a role in regulating urinary citrate excretion [23]. Another explanation for our positive findings is that *Taq*1 VDR SNP may be in linkage disequilibrium with yet unidentified SNPs of the VDR gene and is not associated with urolithiasis per se [24].

We acknowledge the limitations of our study. At our institutions, complete metabolic evaluation is indicated from the third stone episode. Because of this, the small number of patients with available urinary biochemical profile precluded analyses to investigate the association of *Apa*L1 urokinase SNP and *Taq*1 VDR SNP with common conditions including idiopathic hypercalciuria and hyperoxaluria. It is notable that our results were valid for the entire cohort as well as for calcium-stone formers only. We chose to include all patients in the primary analysis of the SNPs because: (1) urokinase may not be solely associated with calcium urolithiasis [12], and (2) assuming that VDR activity influences urinary calcium levels, it has been shown that hypercalciuria is associated with a broad range of biochemical abnormalities, including hyperuricosuria, unduly acidic urine, and hypocitraturia [25]. These abnormalities are not exclusively associated with calcium stones [25]. Furthermore, our study included patients with a relatively homogenous ethnic background. This minimized the possibility of confounding due to population stratification. Conversely, it remains to be seen if our results can be extended to other populations, notably those from the northern hemisphere. Finally, our control group was in Hardy–Weinberg equilibrium for *Taq*1 VDR SNP, however not for *Apa*L1 urokinase SNP. This could be due to the selective criteria used to enroll healthy individuals or population stratification [26]. It may also be explained by the relatively small sample size of the control population, limiting the power for Hardy–Weinberg equilibrium testing [27].

Because urolithiasis is a heterogenous disease, identification of ideal candidates for medical therapy in the context of metaphylaxis remains suboptimal. Moreover, compliance with preventive measures is an important factor when evaluating therapy success [28]. It is obvious that a variety of genes and environmental factors add their phenotypic effect and interact to increase susceptibility to urinary stone formation. In this regard, gene mapping may help uncover genetic susceptibilities, identify patients and their relatives that will benefit from stone metaphylaxis, and guide patient counseling. Other candidate genes include, but are not limited to, calcium-sensing receptor, CLDN14, and melatonin receptor 1A [29–31]. Genome-wide SNP microarrays are commercially available for a number of conditions that include certain cancers and cardiovascular diseases [32]. Rapid improvements in genomics are likely to lead to a better understanding of the pathogenesis of urolithiasis, provide practical tools at reduced cost for gene mapping, and therefore serve the purpose of reducing the health and socioeconomic burdens of the disease.

Conclusion

*Apa*L1 urokinase and *Taq*1 VDR SNPs result in increased susceptibility to recurrent urolithiasis. Our findings indicate that gene mapping may have a role in identifying high-risk patients, leading to tailored therapeutic strategies and follow-up that may ultimately reduce the health and socioeconomic burdens of urolithiasis. However, further research is warranted to identify candidate genes in a given population that would be suitable genetic markers.

Compliance with the ethical standards

Conflict of interest There is no financial arrangement or other relationship that could be constructed as a conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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