

Lack of evidence for the association of ornithine decarboxylase (+316 G>A), spermidine/spermine acetyl transferase (-1415 T>C) gene polymorphisms with calcium oxalate stone disease

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Abstract. Urolithiasis is a complex and multifactorial disorder characterized by the presence of stones in the urinary tract. Urea cycle is an important process involved in disease progression. L-ornithine is a key amino acid in the urea cycle and is converted to putrescine by ornithine decarboxylase (ODC). Putrescine, spermidine and spermine are natural polyamines that are catabolized by a specific enzyme, spermidine/spermine acetyltransferase (SSAT). The single-nucleotide polymorphisms (SNPs) in the intron region of ODC (+316 G>A) and promoter region of SSAT (-1415 T>C) genes have been found to be associated with the polyamines expression levels. The aim of this study was to examine whether the ODC (+316 G>A) intron 1 region gene polymorphism and SAT-1 promoter region (-1415 T>C) gene polymorphisms are potential genetic markers for susceptibility to urolithiasis. A control group of 104 healthy subjects and a group of 65 patients with recurrent idiopathic calcium oxalate stone disease were enrolled into this study. Polymerase chain reaction (PCR)-based restriction analysis was performed for the ODC intron 1 (+316 G>A) region and SAT-1 (-1415 T>C) promoter gene polymorphisms by *Pst*I and *Msp*I restriction enzyme digestion, respectively. The genotype distribution of polymorphisms studied in the ODC intron 1 region (+316 G>A) and SAT-1 -1415 T>C promoter region did not reveal a significant difference between urolithiasis and the control groups ($P=0.713$ and 0.853), respectively. Furthermore, no significant difference was observed between the control and patient groups for ODC +316 G>A and SAT-1 -1415 T>C allele frequencies ($P=0.877$ and 0.644), respectively. In conclusion, results of the present study suggest that ODC + 316 G>A and

SAT-1 -1415 T>C gene polymorphisms might not be a risk factor for urolithiasis.

Introduction

Urolithiasis is a complex and multifactorial disorder (1) whose epidemiology fluctuates due to geographical, cultural and ethnic groups (2). Environmental, dietary and genetic factors may affect disease progression (3). Although various molecules and proteins, particularly ones involved in calcium metabolism are suspected of involvement in the developmental stages of stone formation, the genetic basis of stone formation remains to be clarified (4,5). Supersaturation and crystallization of urine ingredients affect the stone type, which may lead to progression of stone formation. Results of recent studies have shown that metabolic deficiency, which is effective in amino acid metabolism may be crucial for the formation of several types of stones including oxalate and uric acid types (6). In the same study, urine samples of calcium oxalate patients exerted the decreased levels of ornithine and tyrosine amino acids. L-ornithine is a dibasic key amino acid involved in the urea cycle and polyamine biosynthetic pathway (5,7). According to Kohri *et al* (8), plasma values of several amino acids including taurine, aspartic acid, hydroxyproline, glutamic acid, glycine, alanine, cystathionine, ornithine and lysine were found to be significantly higher in controls as compared to patients with stone formation (8). It was suggested that the regulation of amino acid metabolism in kidneys may promote stone formation by altering microenvironmental pH adjustment as well as the induction of reactive oxygen species (9). In addition, cystinuria, an autosomal recessive disease, which is caused by a defective tubular reabsorption of cystine and the three dibasic amino acids arginine, lysine and ornithine, result in a lifelong risk of renal stone formation due to the low solubility of cystine in urine (5).

Natural polyamines such as putrescine, spermidine and spermine, are a family of aliphatic amines that are metabolized in almost all living organisms (10). These polyamines have critical roles in various activities such as cell proliferation, differentiation and division, and stabilization of DNA, RNA and proteins (11). These polyamines also act as anti-oxidant and anti-inflammatory agents that most likely act

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as superoxide scavengers. Polyamines are also anti-glycating agents that reduce the formation of end-products of glycation processes (12). Clinical features of polyamines were determined in carcinogenesis, atherosclerosis and chronic diseases including chronic renal failure in patients who showed a higher incidence of stone formation (13-15). It is suggested that the alteration of polyamine metabolism increases toxic products of the catabolic pathway and cause significant uremic toxins such as acrolein to serve as a marker of chronic renal failure (13). The above findings demonstrated that the alteration of polyamine homeostasis might serve for risk evaluation in the etiology of acute or chronic degenerative diseases (16).

Putrescine synthesis from ornithine is regulated by the rate-limiting enzyme ornithine decarboxylase (ODC) (10), which is expressed from the ODC gene located at chromosome 2, band p25.1 (10). The expression of ODC may be altered by the presence of several single-nucleotide polymorphisms (SNPs) within the ODC gene intron 1 at position +316 (17). This SNP located between two c-myc transcription factor binding sites and rare allele (allele A) leads to a decrease in the ODC gene expression (18). However, Kohri *et al* (8) suggested that ODC polymorphism is significantly associated with whole blood polyamines in females who are A-allele homozygotes expressing more ODC than G-allele carrying females (19).

Spermidine/spermine acetyltransferase (SSAT) is the key polyamine catabolic enzyme that causes acetylation of spermidine or spermine (10). Its activity is required to provide polyamine homeostasis at tissue level (20). The SSAT enzyme is encoded by SAT-1 gene, which is located on the X chromosome, band p22.1 (21). Various SNPs have been determined within the SAT-1 gene near the polyamine-responsive element (PRE) region (22). The SNP within the SAT-1 gene is at position -1415 resulting in alteration of the T nucleotide to C nucleotide (23). Findings of previous studies (22-23) suggested that any chronic stress factors might increase the polyamine synthesis and metabolization of polyamines. It is hypothesized that due to proximity to the PRE and the recognition sites of the transcription factors acting on the promoter region of SAT-1 gene, the allelic variants of SAT-1 -1415T/C polymorphism may exert a significant impact on the expression rate of SAT-1. Due to the presence of SAT-1 on the X chromosome, the variance of allelic distribution of target SNP on SAT-1 was found to be associated with anxiety disorders in a male subpopulation (23). However, no studies are available with regard to the presence of SAT-1 -1415T/C polymorphism and the manner in which it affects other pathophysiological conditions including urolithiasis.

The aim of the present study was to examine the association of polyamine metabolism key enzymes ODC +316 G/A and SAT-1 -1415 T/C gene polymorphisms and the risk of developing urolithiasis.

Materials and methods

Study population. A total of 65 patients (42 male and 23 female) aged, 25-61 years (average age, 42.9±10.2 years) with recurrent idiopathic calcium oxalate stone disease were enrolled in this study. Blood and urine biochemistry tests were performed to evaluate the hypercalcemia, hyperuricemia, hyperoxaluria or hyperuricosuria cases for exclusion from the study. Patients

Table I. Study group characteristics.

	Control	Patients
Gender (n, %)		
Female	30 (29%)	23 (35%)
Male	74 (71%)	42 (65%)
Age (mean ± SD)	40.8±9.9	42.9±10.2
BMI (kg/m ²) (mean)	24.1	24.9
Smoking (n, %)	25 (24%)	32 (49%)
Recurrence (n)	0	44
Familial history of urolithiasis (n)	0	65
Blood chemistry (mean)		
Creatinine (mg/dl)	0.38	1.05
PTH (pg/dl)	38.5	66.94
Ca (mg/dl)	9.2	9.42
Mg (mg/dl)	1.8	2.22
P (mg/dl)	3.4	3.09
Urine chemistry (mean)		
Uric acid (mg/l)	5.8	72.81
pH	5.52	5.76
Creatinine (mg/24 h)	0.28 (male) 0.69 (female)	114.46
Oxalate (mg/day)	38	42
Citrate (mg/day)	118	230

who showed symptoms of urinary tract infections, pregnancy, vascular heart disease, acute or chronic infections, immunologic conditions and history of malignancies, neoplastic, coagulation disorders or chronic renal failure during the period of stone treatment were also excluded. There were 74 males and 30 females in the control group (age range, 20-58 years) with no family history for stone formation. These subjects were evaluated by renal ultrasonography and routine tests for urinary microscopic hematuria to exclude individuals with renal calcifications. Inclusion criteria for the two groups included use of non estrogen, progesterone preparation or menopausal drug or vitamin-D, antacid drugs, or heparin preparation within the previous 2 months (Table I). Patients and control subjects were informed about the study procedure. This study was approved by the Ethics Committee of Yeditepe University in Turkey (March 22, no. 2010/095). Human rights of the subjects were protected and any necessary approval was obtained from the ethics committee. Experiments performed on human subjects were conducted in accordance with the Declaration of Helsinki. All the procedures were carried out with the adequate understanding and written consent of the subjects.

Genotyping. A total of 169 venous blood samples were obtained and placed in EDTA tubes and genomic DNA content was isolated from blood mononuclear cells by using the salting-out method (24).

Genotypes of ODC +316 G/A polymorphism were determined by the polymerase chain reaction (PCR)-restriction

Table II. Genotype distribution of ornithine decarboxylase (ODC) +316 G/A gene polymorphism between urolithiasis cases and healthy controls.

Genotypes	Cases (n=65)		Controls (n=104)		Odds ratio (95% CI)	P-value
	No.	Percentage (%)	No.	Percentage (%)		
GG	36	55.4	56	53.8	1.00 (Reference)	0.713
GA	22	33.8	40	38.5	1.662 (0.522-5.291)	
AA	7	10.8	8	7.7	1.363 (0.453-4.096)	

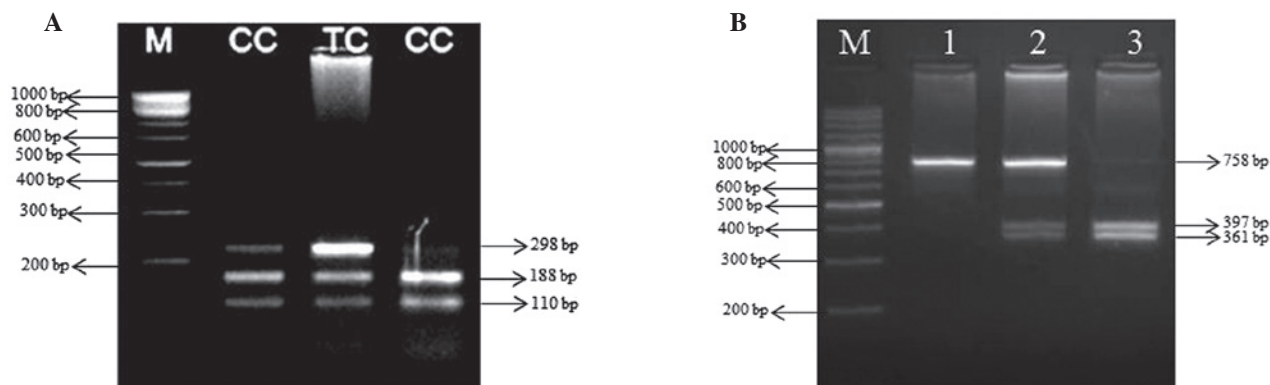


Figure 1. (A) Genotyping of ornithine decarboxylase (ODC) (+316 G>A) gene polymorphism. Lane 1 (M) is 100 bp DNA marker; Lane 2 is homozygous for G allele (758 bp); Lane 3 is heterozygous for G and A allele (758, 397 and 361 bp); Lane 4 is homozygous for A allele (397 and 361 bp). (B) Genotyping of SAT-1 (-1415 T>C) gene polymorphism. Lane 1 (M) is 100 bp DNA marker; Lane 2 is homozygous for G allele (758 bp); Lane 3 is heterozygous for G and A allele (758, 397 and 361 bp); Lane 4 is homozygous for A allele (397 and 361 bp).

fragment length polymorphism procedure. PCR reaction contained 1 μ g genomic DNA, 2 pmol each primer, 1X AmpliTaq 360 DNA Polymerase PCR buffer (PN4398818; Applied Biosystems, Foster City, CA, USA), 200 μ M dNTPs (R1121; Fermentas, Vilnius, Lithuania,) and 1 unit AmpliTaq 360 DNA polymerase in a volume of 25 μ l. The primers for the ODC +316 G/A region were 5'-ATCGTGGCTGGTTTGAGCTG-3' and 5'-GTCATCTGCTCTGTAGACACAGCG-3'. The protocol included an initial denaturation step at 94°C for 3 min, followed by 30 cycles with 30 sec of denaturation at 94°C, 30 sec of annealing at 55°C and 45 sec of elongation at 72°C, followed by a final elongation step 72°C for 10 min. PCR product (10 μ l of 757 bp long) of case and control groups was digested with 5 units *Pst*I (ER0611; Fermentas) restriction enzymes overnight at 37°C. The digestion products were subjected to 3.5% agarose gel electrophoresis. DNA from individuals carrying A allele yielded two fragments of 397 and 361 bp, whereas DNA from individuals carrying the G allele yielded only the uncut 758 bp. The presence of all three fragments was indicative of heterozygotes (Fig. 1A).

Genotypes of SAT-1 -1415 T/C were determined using PCR and restriction digestion with *Msp*I restriction enzyme. Primers for the amplification of SAT-1 -1415 T/C polymorphic site were 5'-GAAGGCCTTTTCCTCCTCTG-3' (forward) and 5'-GATAGGGCCTCACCATCTTG-3' (reverse). The protocol included an initial denaturation step at 94°C for 3 min, followed by 30 cycles with 30 sec of denaturation at 94°C, 30 sec of annealing at 60°C and 45 sec of elongation at 72°C, followed by a final elongation step 72°C for 10 min. PCR amplification

of a 298-bp fragment from the promoter of SAT-1 gene was digested with 5 units *Msp*I (ER1271; Fermentas) restriction enzymes overnight at 37°C. The digestion products were subjected to 2.5% agarose gel electrophoresis. Restriction fragments of 188 and 110 bp revealed the presence of C allele, whereas the T allele could not be determined at the *Msp*I recognition site (Fig. 1B).

Statistical analysis. The Hardy-Weinberg analysis was used to ensure that the allele frequencies of the controls and cases were in equilibrium within the population. Allele distribution and genotype frequencies among the cases and controls were compared with predictions using the χ^2 test. The odd ratios (OR) with 95% confidence intervals (CI) were calculated for the association between ODC +316 G/A and SAT-1 -1415 T/C genotypes and the risk of development of urolithiasis. $P < 0.05$ was considered to indicate a statistically significant difference. SPSS 13.0 software program was used for the statistical analysis.

Results

In this study, the genotype distribution and allele frequencies were evaluated according to the Hardy-Weinberg equilibrium for polymorphic sites of polyamine metabolic key enzymes ODC +316 G/A and SAT-1 -1415 T/C in the urolithiasis and disease-free control group. As shown in Table II, the genotype distribution of ODC +316 G/A SNP was not significantly different between urolithiasis patients and the control group

Table III. Allele frequencies of ornithine decarboxylase (ODC) +316 G/A gene polymorphism and urolithiasis.

Alleles	Cases (%)	Controls (%)	Odds ratio (95% CI)	P-value
G	0.723	0.731	1.011 (0.883-1.156)	0.877
A	0.277	0.269	0.972 (0.680-1.389)	

Table IV. Genotype distribution of SAT-1 -1415 T/C gene polymorphism among urolithiasis cases and healthy controls.

Genotypes	Cases (n=65)		Controls (n=104)		Odds ratio (95% CI)	P-value
	No.	Percentage (%)	No.	Percentage (%)		
TT	49	75.4	77	74	1.00 (Reference)	0.853
TC	6	9.2	8	7.7	1.313 (0.416-4.141)	
CC	10	15.4	19	18.3	1.581 (0.415-6.020)	

Table V. Allele frequencies of SAT-1 -1415 T/C gene polymorphism and urolithiasis.

SAT-1 -1415 alleles	Cases (%)	Controls (%)	Odds ratio (95 % CI)	P-value
T	0.800	0.779	0.974 (0.870-1.089)	0.644
C	0.200	0.221	1.106 (0.721-1.697)	

($\chi^2=0.676$, $df=2$, $P=0.713$). Similarly, the comparison of allele frequencies between the urolithiasis and control groups did not exert any association for ODC +316 G/A gene polymorphism alleles ($\chi^2=0.024$, $df=1$, $P=0.877$) (Table III).

Genotype distributions and allele frequencies of SAT-1 -1415 T/C in the urolithiasis and control groups are shown in Table IV and V. The genotype distribution of SAT-1 -1415 T/C did not exert any significant difference between urolithiasis patients and the control group ($\chi^2=0.318$, $df=2$, $P=0.853$). When the allelic distribution of SAT -1415 T/C was compared between the patients and control group, no significant difference was observed ($\chi^2=0.214$, $df=1$, $P=0.644$) (Table V).

The association of ODC +316 G/A and SAT-1 -1415 T/C gene polymorphism and urolithiasis cases were compared in gender subgroups. When the male population was compared according to ODC +316 G/A and SAT-1 -1415 T/C gene polymorphisms and allele frequencies between the urolithiasis cases and control group, the genotype distribution of ODC +316 G/A ($\chi^2=1.334$, $df=2$, $P=0.520$) and SAT-1 -1415 T/C ($\chi^2=0.975$, $df=2$, $P=0.614$) did not reach a statistically significant difference between the urolithiasis and control subjects. In accordance with genotype distribution, allele frequencies of ODC +316 G/A and SAT-1 -1415 T/C SNP were not significant between the urolithiasis and control groups ($\chi^2=0.851$, $df=1$, $P=0.607$, $\chi^2=1.752$, $df=1$, $P=0.255$), respectively (Table VI).

Discussion

Accumulating evidence suggests that in addition to inorganic substances, organic substances may be important in the

development urolithiasis, following their identification from calcium salts in the 1950s by Boyce and Sulkin (25). Those authors noted that in quantities of <3% of total stone weight, this organic 'matrix' directed the biomineralization process in an orderly manner (26). Urinary supersaturation with respect to stone salts is regulated by the urinary concentration of various participating ions such as calcium, oxalate and citrate, which lead to hypercalciuria, hyperoxaluria or hypocitraturia (4). Besides the urinary microenvironment, several damaging agents in kidney cells or dysfunction may affect the supersaturation and lead to stone formation.

There is no single theory that provides understanding of the molecular basis and pathogenesis of stone formation in kidneys. One of the identified mechanisms is free solution crystallization, which has been utilized in cystinuria patients who showed a higher recurrence rate for urinary stone formation (27). Cystinuria is an autosomal recessive disorder in renal tubular and intestinal transport of dibasic amino acids, which results in increased urinary excretion of cystine, ornithine, lysine and arginine (28). Ornithine and arginine are precursor amino acids of polyamine biosynthesis and ODC is the metabolic enzyme that produces putrescine from ornithine. Polyamine metabolism is regulated by a number of key enzymes, with ODC and SSAT and expression of these genes regulating the homeostatic tissue environment. According to previous studies (17,21) polyamine derivatives and metabolic conjugates are critical to provide balanced tissue environment. Circulating polyamines in blood and polyamine content of urine also serve as candidate biomarkers in the evaluation of disease progression for several chronic diseases and malignancies (20). In this study, we

Table VI. Genotype distribution and allele frequencies of ornithine decarboxylase (ODC) +316 and SAT-1 -1415 T/C gene polymorphisms in gender subgroups.

ODC +316

Genotypes	Male subgroup				Female subgroup			
	Cases	Controls	Odds ratio (95% CI)	P-value	Cases	Controls	Odds ratio (95% CI)	P-value
GG	25 (59.5%)	44 (59.3%)	1.00	0.520	11 (47.8%)	12 (40%)	1.00	0.825
GA	11 (26.2%)	24 (32.4%)	1.760 (0.513-6.042)		11 (47.8)	16 (53.3%)	0.219 (0.639-0.545)	
AA	6 (14.3%)	6 (8.1%)	2.182 (0.573-8.314)		1 (4.3 %)	2 (6.7%)	0.061 (0.804-0.727)	
G allele	0.726	0.757	1.042 (0.888-1.223)	0.607	0.717	0.667	0.929 (0.720-1.199)	0.674
A allele	0.274	0.243	0.888 (0.567-1.393)		0.283	0.333	1.179 (0.658-2.113)	

SAT-1 -1415

Genotypes	Male subgroup				Female subgroup			
	Cases	Controls	Odds ratio (95 % CI)	P-value	Cases	Controls	Odds ratio (95% CI)	P-value
TT	35 (83.3%)	56 (75.7%)	1.00	0.614	14 (60.9%)	21 (70%)	1.00	0.402
TC	2 (4.8%)	6 (9.1%)	0.667 (0.216-2.055)		4 (17.4%)	2 (6.7%)	0.680 (0.919-1.071)	
CC	5 (11.9%)	12 (16.2 %)	1.250 (0.185-8.444)		5 (21.7%)	7 (23.3%)	0.330 (0.566- 1.400)	
T allele	0.857	0.798	0.930 (0.826-1.048)	0.255	0.696	0.733	1.057 (0.825-1.346)	0.669
C allele	0.143	0.202	1.419 (0.768-2.621)		0.304	0.267	0.876 (0.478-1.606)	

investigated the possible association of the altered metabolism of diamine precursors of polyamine biosynthesis with urinary stone formation. The findings from this study reveal that the ODC gene +316 G/A and SAT-1 gene 1415 T/C polymorphisms cannot serve as candidate genetic markers for screening the causes of stone disease as the frequency of distribution in SNPs was similar in urolithiasis when compared with the disease-free control group. A recent study has shown that ODC >316 G/A polymorphism (rs2302615) was associated with the progression of sporadic breast cancer and that the presence of one copy of A allele has a protective role in the prevention of the disease (29). In addition, previous studies suggested that the G315A SNP in the ODC gene may be a genetic marker for risk of colorectal neoplasia and may also modify the association of aspirin use with risk (30,31). Individuals homozygous for the minor A allele of ODC were low risk for colorectal cancer recurrence compared to those with the major G allele. For the specific inhibitor of ODC, efflornithine or sulindac therapy received by colorectal cancer patients showed higher ototoxicity due to the presence of AA allele. However, A allele showed a protective effect against the recurrence of disease in aspirin users, and two copies of G allele reduced the risk of recurrence after treatment with efflor-

nithine and sulindac (32). Therefore the distribution of ODC-1 G/A alleles in association with disease progression should be evaluated using other genes that might be more effective.

Results of previous studies have shown that total polyamine content in blood and fibroblast cultures of patients who were suffering from schizophrenia were significantly higher compared to the control group (33-35). Although several reports indicated a strong association with reduced SAT-1 expression and SAT-1 342 C allele among male suicide completers (23), the allelic distribution of SAT-1 342 C did not exert any relationship with SAT-1 expression level in suicidal behavior as shown in a patient group from another study (36). Bermudo-Soriano *et al* (34) found that SAT-1 -1415 T/C has a strong association with anxiety but not schizophrenia. Therefore SAT-1 polymorphic alterations are unclear as to the possible association of SNPs on SAT-1 gene expression profile. For this reason, cell and animal models should be utilized to evaluate the functional role of dbSNPs at the promoter region of SAT-1 (SNPs=6526342 and 92893).

In conclusion, the results obtained from this study to determine ODC +316 G/A polymorphism and susceptibility to urinary stone disease provide no evidence that particular ODC

+316 G/A genotypes are associated with disease progression. In addition, the distribution of alleles of SAT-1 -1415 T/C gene polymorphism did not exert any significant association with urolithiasis. Although polyamines have critical roles in cellular homeostasis and are referred to as malignancy markers, other genotypic variations are required to understand the genetic basis of urolithiasis. To the best of our knowledge this is the first study to analyse the effect of the polymorphisms of polyamine metabolic enzymes genes in urolithiasis.

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