Association Study of Klotho Gene Polymorphism With Calcium Oxalate Stones in The Uyghur Population of Xinjiang, China

Abdusamat Ali[1†], Halmurat Tursun[2†], Alim Talat[1],Akpar Abla[1], Erpan Muhtar[1],Tao Zhang[1], Murat Mahmut[1]*

Purpose: The aim of the present study was to investigate the correlation between Klotho gene polymorphisms and calcium oxalate stones in Xinjiang Uyghur people.

Materials and Methods: We compared 128 patients with calcium oxalate stones (case group) and 94 healthy people (control group), detected the genotype and allele distributions of single-nucleotide polymorphisms (SNPs) of the Klotho gene (rs3752472, rs650439, and rs1207568) by reverse transcription polymerase chain reaction.

Results: The distributions of the genotype and allele frequencies of the SNPs were consistent with the Hardy–Weinberg equilibrium in the two groups. There were statistically significant differences between the genotype and allele distributions of rs3752472 between the case and control groups; the allele frequencies in the case/control groups were C = 240 (93.7%)/151 (80.3%) and T = 16 (6.3%)/37 (19.7%). There was no statistically significant difference in the genotype distribution of rs650439 between the case and control groups, but there was a difference in the allele distribution; the allele frequencies in the case/control groups were A = 202 (78.9%)/143 (57.2%) and T = 54 (21.1%)/107 (42.8%). There were no statistically significant differences in genotype and allele distributions between the case and control groups of rs1207568; the allele frequencies in the case/control groups were C = 194 (71.3%)/145 (77.1%) and T = 78 (28.7%)/43 (22.9%). In rs3752472, the risk for patients with the C and A alleles increased by 3.675 and 2.799 times, respectively.

Conclusion: The rs3752472 and rs650439 SNPs are related to the risk of calcium oxalate stones in Xinjiang Uyghur people, and might be one of the risk factors.

Keywords: case-control studies; genotype; Klotho gene; polymorphism; urolithiasis.

INTRODUCTION

Urolithiasis is one of the three major diseases of the human urinary system and it is common in the worldwide. Etiology of urolithiasis is affected by numerous factors, including genetic and environmental factors, abnormal metabolism, race, and living habits[(1)]; therefore, the exact mechanism by which urinary calculi form remains unclear. With the development of molecular biology techniques such as polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), the molecular pathogenesis of urolithiasis has become a focus of research, and the correlation between susceptibility to urolithiasis and gene polymorphism has received particular attention.

Xinjiang, a multi-ethnic region that is the main residence of the Uyghur people, has a high incidence of urolithiasis. Clinical diagnosis has revealed that urolithiasis is more prevalent in Uyghur patients than in patients of other races living in the same region[(2)]. Research has shown that levels of the Klotho enzyme are related to the risk of urolithiasis[(3,4)]. In order to investigate the correlation between Klotho gene polymorphisms and the risk of renal calculi in Xinjiang Uyghur people, we studied the distribution of three SNPs (rs3752472, rs650439, and rs1207568) in Uyghur people and investigated the correlation between polymorphic loci and calcium oxalate stones, as well as the molecular mechanisms that cause them.

MATERIALS AND METHODS

Subjects

Between January 2013 and September 2013, patients with urinary calcium oxalate stones who were treated at the Department of Urinary Surgery of the First Affiliated Hospital of Xinjiang Medical University and the Second Affiliated Hospital of Xinjiang Medical University were enrolled in the study. The study population was composed of 128 Uyghur patients and 94 healthy volunteers. All the patients and volunteers were biologically unrelated Uyghur people. The patients had an av-

[1]Department of Urology, The Second Affiliated Hospital of Xinjiang Medical University, Urumqi ,Xinjiang, China.

[2]Department of Urology, The First Affiliated Hospital of Xinjiang Medical University, Urumqi ,Xinjiang, China.

†These authors contributed equally to this work.

*Correspondence: Department of Urology, The Second Affiliated Hospital of Xinjiang Medical University, Urumqi ,Xinjiang, China.

Tel: +86- 13999118109. Fax: +86-13999118109. E-mail: mekit@126.com.

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Table1. General Characteristics of the subjects (n = 222).

Variables	Patients(n=128)	Controls(n=94)	*P* Value
Age, years, mean ± SD	43 ± 10	46 ± 9	.536
Gender, no (%)			.349
Male	83(64.8)	66(70.2)	
Female	45(35.2)	28(29.8)	
BMI, kg/m^2, mean ± SD	24.9±3.7	24.1 ± 3.1	.552
Smoking, no (%)	52 (40.6)	44 (46.8)	.511
Family history, no (%)	67 (52.3)	----	----
Recurrence (n)	44 (34.3)	----	----
Serum calcium (mg/dL)	9.2 ± 0.6	9.1 ± 0.5	.039*
Serum phosphate (mg/dL)	3.5 ± 0.6	3.6 ± 0.6	.0001*
urine calcium (mg/24h)	242.8 ± 145.3	107.5 ± 87.0	.0001*
urine phosphate(mg/24h)	355.0 ± 239.8	291.6 ± 132.5	.045*
Urine pH	6.1 ± 0.6	6.0 ± 0.6	.400
Creatinine (µmol/L)	91.6 ± 31.8	82.4 ± 23.5	.027*

Abbreviations: BMI, body mass index; SD, standard deviation.
* $p < .05$ generated by comparison between healthy controls and stone patients.

erage age of 43 ± 10 years. Seventy-six patients (59%) harbored kidney stones, thirty-two patients (25%) had ureteral stones, and the other thirty patients (16%) were diagnosed with bladder stones. Sixty-seven patients 67 (52.3%) reported a family history of stones and forty-four patients (34.3%) had recurrent stones. According to clinical symptoms, X-ray plain film results, and B-mode analyses, patients with urinary calculi received surgery or extracorporeal shock wave lithotripsy treatment, and the samples taken from them contained calcium oxalate stones. Stone analysis was performed using Shimadzu Fourier Transform Infrared Spectrophotometer 8300 manufactured by Shimadzu Corporation, Japan. The control group consisted of 94 healthy volunteers with an average age of 46 ± 9 years without diagnosis or history of urinary calculi. There were no significant differences in the sex or age distribution between the case and control groups (all $P > .05$).

We measured serum concentrations of total creatinine (Cr), lactate dehydrogenase (LDH), calcium, phosphate, sodium, potassium, magnesium and PTH (parathyroid hormone) as well as 24-hour urine excretions of Cr, calcium, phosphate, sodium, potassium and magnesium in both groups. In the present study, we only analyzed the levels of serum calcium, phosphate, and Cr and the 24-hour urinary excretions of calcium and phosphate. Laboratory data and clinical characteristics of 222 subjects were presented in **Table 1**. Patients with calcium oxalate stones were included in this study. Exclusion criteria for patients and controls were the presence of chronic urinary tract infections, renal tubular acidosis, renal failure, hyperparathyroidism, osteoporosis, cancer and using drugs that effect calcium and hormone metabolism, such as diuretics, calcium and vitamin D supplements. Patients using anti-diabetic and anti-hypertensive agents were also excluded from the study. The study protocol was approved by the Ethics Committee of the First and Second Affiliated Hospital of Xinjiang Medical University (approval number: IACUC-20140221097). Written informed consent for research was obtained from the participants.

Genomic DNA extraction

Blood (3 mL) was drawn from the antecubital veins of subjects in the case and control groups using ethylenediaminetetraacetic acid as an anticoagulant, and the samples were stored at -20°C. DNA was extracted using a blood genomic DNA extraction kit (K5017500; BioChain Science & Technology, Inc, Beijing, China). Agarose gel electrophoresis was used to confirm genomic DNA integrity.

Table 2. PCR Primer sequences.

	Upstream primer (5′-3′)	Downstream primer (5′-3′)	Length of gene segment
rs3752472	CCTCCTTTACCTGAAAATCGG	GGCTTGGTGAGACTGCTGATT	104 bp
rs650439	AGGACGACCAGCTGAGGGTGTAT	TCTGGTGACATAACCTTCAGGAGCT	104 bp
rs1207568	TGGACGCTCAGGTTCATTCT	CCTCTAGGATTTCGGCCAGT	243 bp

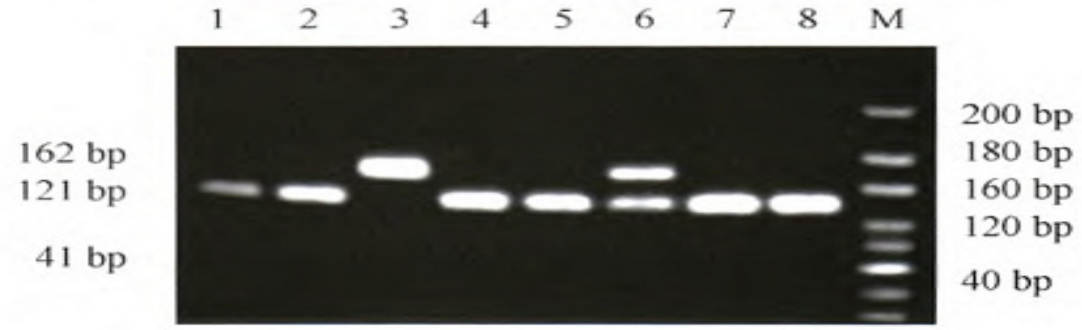

Figure 1. Genotyping of the rs 3752472 polymorphism site in the Klotho gene.

PCR amplification primers

The PCR amplification primers were designed according to the complete sequences in GenBank and the study by (Xu et al. 2013) (**Table 2**).

PCR amplification and genotype detection

The total volume of the PCR reactants was 15 μL, which comprised the following: the upstream and downstream primers (0.2 μL each), template DNA (0.5 μL), deoxyribonucleotide triphosphate (dNTP) (10 mM), and distilled H2O (11 μL). The PCR amplification conditions were as follows: pre-degeneration at 95°C for 5 min; degeneration at 95°C for 30 s, annealing at 68°C for 45 s, and extension at 72°C for 60 s for a total of 40 cycles; and extension at 72°C for 6 minutes. The lengths of the amplified molecules were 122 bp (rs3752472), 104 bp (rs650439) and 243bp (rs1207568); they were analyzed using 3% agarose gel electrophoresis and an imaging system. The products of the PCR were sequenced by the Life Sciences Corporation.

Statistical analysis

The polymorphism allele frequencies were found to be consistent with Hardy-Weinberg equilibrium. Genotypes, differences in genotype frequency distribution, and the correlation of specific genotypes with the occurrence and development of calcium oxalate stones were analyzed by the chi-squared test. The measurement data was expressed as mean ± standard deviation (SD). All data was analyzed on the SPSS software platform (v.17.0; SPSS Inc., Chicago, IL, USA). *P* value < .05 was considered to be statistically significant.

RESULTS

Genotype detection

The PCR analyses revealed the presence of polymorphisms at the specific sites rs3752472 (mutant T/T (162 bp), wild type C/C (121,41 bp), and heterozygote C/T (162, 121, 41bp) phenotypes were obtained; **Figure 1**, and rs650439 (mutant T/T (97,47 bp), wild type A/A(144 bp), and heterozygote A/T (144, 97, 47 bp) phenotypes; **Figure 2**, and rs l207568 (mutant T/T (147, 96 bp), wild type C/C (243 bp), and heterozygote C/T (243, 147, and 96 bp) phenotypes ; **Figure 3**.

Comparison of genotype and allele frequency

For the rs3752472 SNP, the results of genotype detection were as follows: OR = 3.707, 95%CI: 1.861-7.384, $X^2 = 14.912$; v = 1; and $P < .01$. People carrying the C/C genotype had a significantly higher risk of developing calcium oxalate stones (3.707 times higher) than people carrying the C/T+T/T genotype. There was a significant difference in the genotype frequency between the case and control groups. People carrying the C allele had a higher risk of developing calcium oxalates stones (**Table 3**).

For the rs650439 SNP, the results of genotype detection were as follows: OR = 1.169, 95%CI: 0.677-2.020, $\chi^2 = 0.315$, v = 1, $P = .575$. There was a significant difference in the distribution of A and T alleles between the case and control groups. People carrying the A allele were at higher risk of developing calcium oxalate stones (2.799 times higher) than those carrying the T allele (**Table 4**).

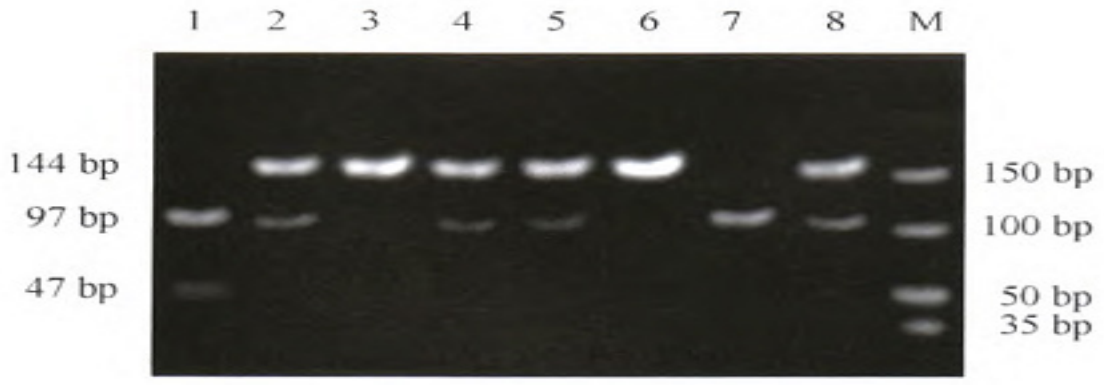

Figure 2. Genotyping of the rs 650439 polymorphism site in the Klotho gene.

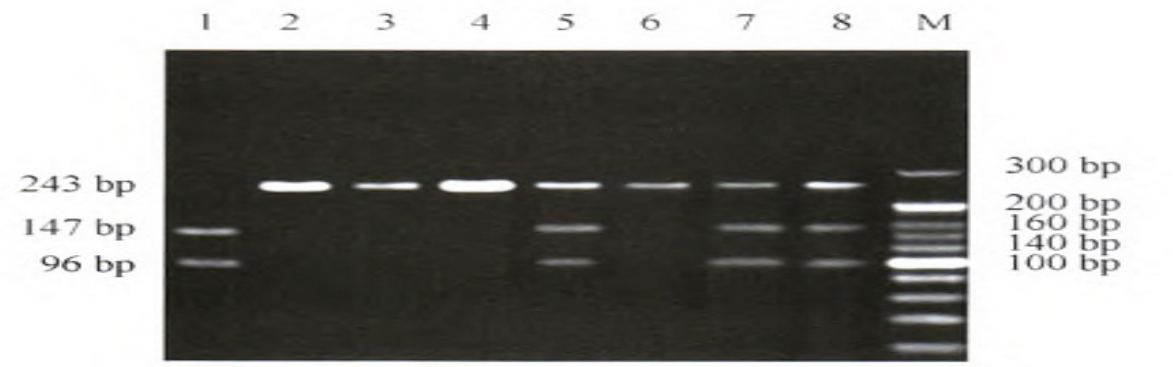

Figure 3. Genotyping of the rs l207568 polymorphism site in the Klotho gene.

For the rs1207568 SNP, the results of genotype detection were as follows: OR = 0.741, 95%CI: 0.428–1.285, χ^2 = 1.140, v = 1, *P* = .286. There was no significant difference between the two groups (**Table 5**).

DISCUSSION

Urinary calculi affect many people globally, with a 1–5% prevalence and a 50% recurrence rate over 10 years. Extensive research has shown that a disorder in calcium phosphate metabolism is related to urinary calculi, particularly calculi containing calcium. Many genes, including the Klotho gene, are involved in the development of urinary calculi. Klotho was discovered by Kuro-o et al.[5], and is mainly expressed in the renal tubules and choroid plexus as a type-I trans-membrane protein containing 1014 amino acids.[6-7] To a lesser extent, the protein is also expressed in the pituitary, parathyroid, pancreas, ovary, testis, and placenta. The Klotho protein is not only found in the organs that regulate calcium balance, such as in the renal and parathyroid systems, and the choroid epithelial cells of the ventricles, but is also present in the blood and cerebrospinal fluid because its extracellular domain is usually involved in digestion.[6,8] Human klotho is a type-I trans-membrane glycoprotein with β-glucuronidase catalytic activity, and is a regulator of calcium and phosphate levels.[9-10]

There has been intensive international research into Klotho gene polymorphisms and the calcium–phosphorus metabolic balance. Xu et al.[4] found that there was a significant association between the rs3752472 SNP in Klotho and the risk of calcium oxalate nephrolithiasis. The risk attributed to the homozygote CC genotype was twice that of the heterozygote CT and homozygote TT genotypes, while the rs650439 SNP was not related to the risk of calcium oxalate nephrolithiasis. We investigated Klotho gene polymorphisms in Xinjiang Uyghur people by comparing case and control groups; in the rs3752472 SNP, there was a significant difference between the risk for people carrying the C/C genotype (3.707 times more likely) compared with those carrying the T/T+T/C genotype (OR = 3.707, 95%CI = 1.861–7.384, *P* < .01). The risk of developing calcium oxalate nephrolithiasis was 3.675 times greater for people carrying the C allele than for those carrying the T allele. However, in the rs650439 SNP there was no significant difference between the genotype frequencies, but the distribution of A and T alleles was significantly different. The risk of calcium oxalate nephrolithiasis for the people carrying the A allele was 2.799 times higher than for those carrying the T allele (OR = 2.799, 95%CI: 1.893–4.138, *P* < .01).

Telci et al.[3] studied the G395A, F252V, and C1818T polymorphisms of the Klotho gene and found that only G395A was significantly associated with the risk of calcium oxalate nephrolithiasis. People carrying the GG genotype of the G395A Klotho polymorphism were twice as likely to develop calcium oxalate nephrolithiasis as those with the homozygous AA or the heterozygote GA genotypes, while those with the A allele were at higher risk of the disorder and presented more obvious symptoms such as hypercalcemia and hypophosphatemia. Yamada et al.[11] considered that the Klotho gene G395A polymorphism was associated with pre-

Table 3. Comparison of genotype and allele frequencies of rs3752472.

	Cases (N = 128)	Control (N = 94)	OR	95%CI	*P*
Genotype (%)					
C/C	113 (88.3)	63 (67.0)	1		
C/T	14 (10.9)	25 (26.0)			
T/T	1 (0.78)	6 (7.0)			
C/T+T/T	15 (11.7)	31 (33.0)	3.707	1.861–7.384	*P* < .01
Allele (%)					
C	240 (93.75)	151 (80.3)	1		
T	16 (6.25)	37 (19.7)	3.675	1.976–9.838	*P* < .01

Table 4. Comparison of genotype and allele frequencies of rs650439.

	Cases (N = 128)	Control (N = 94)	OR	95%CI	*P*
Genotype (%)					
A/A	81 (63.3)	56 (59.6)	1		
A/T	40 (31.3)	31 (33.0)			
T/T	7 (5.4)	7 (7.4)			
A/T+T/T	47 (36.7)	38 (40.4)	1.169	0.677–2.020	.575
Allele (%)					
A	202 (78.9)	143 (57.2)	1		
T	54 (21.1)	107 (42.8)	2.799	1.893–4.138	*P* < .01

menopausal and postmenopausal bone mineral density, and the GG genotype was a risk factor for its reduction. Xu et al.[12] found no significant differences in allele and genotype frequencies of the Klotho gene G395A polymorphism (rs1207568) in their experimental and control groups. Moreover, we also found no relationship between the Klotho gene G395A polymorphism (rs1207568) and the risk of calcium oxalate nephrolithiasis when we compared patients and healthy volunteers from the Xinjiang Uyghur region. These results are influenced by race, the methods used, and the sample size, and further research is required.

We found that polymorphisms resulting from Klotho gene mutations had an effect on normal calcium and phosphorus metabolism, and led to a loss of Ca2+, an increase in urinary calcium, an interaction with urinary matrix proteins, and high risk of lithogenesis. Alapont Pérez et al.[13] found that the risk of calcium oxalate nephrolithiasis was related to hot and dry climates. People living in the Xinjiang region experience long days and consequently high levels of vitamin D. The Klotho gene[14] and Klotho mRNA expression[15] are regulated by vitamin D receptors and vitamin D receptor elements, leading to an increase in intestinal calcium absorption and urinary calcium excretion. All of these factors have an effect on calcium and phosphorus metabolism. The situation is exacerbated by urinary concentration, which is one of the causes of calcium oxalate nephrolithiasis in Xinjiang Uyghur people.

In this study, we found that the Klotho gene rs3752472 and rs650439 SNPS are associated with the risk of calcium oxalate nephrolithiasis in Uyghur people from the Xinjiang region, and might be risk factors for the disorder.

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CONFLICT OF INTEREST

The authors report no conflict on interest.

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Table 5. Comparison of genotype and allele frequencies of rs1207568.

	Cases (N = 128)	Control (N = 94)	OR	95%CI	*P*
Genotype (%)					
C/C	74 (57.8)	61 (64.9)	1		
C/T	46 (35.9)	23 (24.5)			
T/T	8 (6.3)	10 (20.6)			
C/T+T/T	54 (42.2)	33 (35.1)	0.741	0.428–1.285	.286
Allele (%)					
C	194 (71.3)	145 (77.1)	1		
T	78 (28.7)	43 (22.9)	0.738	0.480–1.134	.165

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