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Short communication

Klotho gene polymorphism of rs3752472 is associated with the risk of urinary calculi in the population of Han nationality in Eastern China

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ABSTRACT

Background/aims: The incidence of urolithiasis has considerably increased throughout the world in the last two decades. Clinical researches have showed an association between oxidative stress and stone formation. Emerging evidence indicated a novel function for klotho protein in anti-oxidative stress. In this study, we aimed at investigating a possible relationship between klotho gene polymorphisms and the risk of calcium oxalate urolithiasis in the population of Han nationality in Eastern China.
Methods: Klotho gene polymorphisms rs3752472 in exon3, rs650439 in intron 4 and rs577912 in intron 1 were investigated in 426 patients with calcium oxalate stones compared with 282 age-matched healthy volunteers with no history of stone formation, using TaqMan SNP Genotyping Assays.
Results: Significant differences were found between rs3752472 and the risk of nephrolithiasis as CC genotype of rs3752472 klotho polymorphism had almost 2-fold increased stone risk compared with the heterozygote genotype CT and homozygous genotype TT(95% CI = 1.013–2.255, OR = 1.512,p = 0.043).
Conclusion: Our results showed that the rs3752472 polymorphism of klotho gene is associated with the risk of calcium oxalate urolithiasis and may act as a risk factor during stone formation in our study population.

1. Introduction

Urolithiasis is a common urinary disease caused by both genetic and environmental factors (Mittal et al., 2008). 75% to 80% of renal stones are composed of calcium oxalate (CaO_x), including monohydrate (COM) and dehydrate (COD) forms (Moe, 2006). As a key element in the most common form of renal stones, calcium oxalate stones have been reported to be injurious to the renal epithelial cells through oxidative stress (OS), which is considered as a major risk factor for crystallization and crystal deposition in the kidneys (Scheid et al., 1996; Thamilselvan et al., 1997). Study by Mauricio Davalos et al. showed that COM is cytotoxic to LLC-PK1 cells through oxidative stress, leading to the cell viability reduction, which eventually attributes to the stone formation (Davalos et al., 2010). Reactive oxygen species (ROS) was produced and increased exclusively when tubular cells were exposed to a high concentration of oxalate. It could cause cell death and crystal deposition in renal tubules. Clinical researches showed that OS caused by ROS might lead to the formation of renal stones (Huang et al., 2003; Thamilselvan et al., 2000; Tungsanga et al., 2005). Furthermore, excessive ROS aggravated the progression of OS, resulting in the loss of renal cell integrity, which in reverse promoted the growth of the calcium oxalate stones.

Klotho was originally identified as an anti-aging protein (Kuro-o et al., 1997). In addition to the transmembrane form, klotho also exists as a soluble form, which can be derived from the body fluid including urine, blood and cerebrospinal fluid (Shiraki-Iida et al., 1998). While acting as a regulator of calcium and phosphate homeostasis, soluble klotho (s-klotho) also plays an important role in suppression of insulin/IGF-1 signaling and OS (Kuro-o, 2009; Moe, 2006; Razzaque and Lanske, 2007).

Given the relationship between OS and the formation of calcium oxalate stones and the role of klotho in the suppression of OS, we aimed to investigate whether the polymorphisms of the klotho gene (rs3752472, rs650439, rs577912) were associated with the risk of calcium nephrolithiasis in the population of Han nationality in Eastern China.

Abbreviations: SNP, single nucleotide polymorphisms; CaO_x, calcium oxalate; COM, calcium oxalate monohydrate; COD, calcium oxalate dehydrate; OS, oxidative stress; ROS, reactive oxygen species; Insulin/IGF-1, insulin/insulin-like growth factor-1; ESWL, extracorporeal shock wave lithotripsy; PCNL, percutaneous nephrolithotomy; Cr, creatinine; LDH, lactic dehydrogenase; DNA, deoxyribose nucleic acid; EDTA, ethylene diamine tetraacetic acid; PCR, polymerase chain reaction; ORs, odds ratios; CI, confidence interval; pH, potential of hydrogen; FOXO, forkhead box; SOD2, manganese-superoxide dismutase; ESRD, end-stage renal disease; mRNA, messenger ribose nucleic acid.

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2. Methods and materials

2.1. Patient selection

We conducted a case–control study from September 2010 to May 2012 in Jiangsu province hospital, Nanjing, China. A total of 426 unrelated patients (299 men and 127 women, aged 18–70, mean age 47.7 ± 12.0) of Han nationality in Eastern China with calcium oxalate stone regardless of family history were enrolled in our study. All these stone samples were obtained either from extracorporeal shock wave lithotripsy (ESWL) or surgery (such as percutaneous nephrolithotomy (PCNL), laparoscopy and ureteroscopy), which were analyzed through the Infrared Spectroscopy method postoperatively. Nothing but pure calcium oxalate stones (COM or COD) or combined forms could be selected in our study. 556 patients met specifications after stone analysis while only 426 patients were recruited into the final research as many other urogenital diseases such as ureterostenosis and ureteropelvic junction obstruction were found postoperatively.

A control group was drawn up of 282 healthy unrelated volunteers (212 men and 70 women, aged 18–71, mean age 48.4 ± 13.0) who resided in the same geographic area as the stone patients and with no history or radiological finding of stone disease. Urine and serum biochemical tests were performed in both patients and healthy volunteers in order to exclude any individuals with hyperparathyroidism, hypercalcemia, hyperphosphatemia, hyperuricemia, hypocitricuria, gout, cancer, and other diseases that might affect calcium or oxalate metabolism.

We measured the serum concentrations of total creatinine (Cr), lactic dehydrogenase (LDH), calcium, phosphate, sodium, potassium, magnesium and PTH (parathyroid hormone) as well as 24-hour urine excretions of creatinine, calcium, phosphate, sodium, potassium and magnesium in both two groups. In the present study, we only analyzed the levels of serum calcium and phosphate, LDH, Cr and the 24-hour urine excretions of calcium and phosphate.

Our study was approved by the ethics review board of Nanjing Medical University.

2.2. Genotyping

10 ml of blood was drawn from each participant via EDTA as anticoagulant and stored at −80 °C for genotyping. All subjects' genomic DNA was extracted from fasting venous blood samples through the genomic DNA extraction kit (Genomic DNA kit; Tiangen, Beijing, China). Genotyping was performed by the TaqMan probe assay from Applied Biosystems Inc (Foster City, CA). The sequences of primer and probe for each SNP were available on request. Based on the sequences of the klotho gene available from the GeneBank, the following primers were designed. For each SNP we used the following set of primers: rs3752472_forward 5′-AAATGGCTTCCCTCCTTTACCT-3′, rs3752472_reverse 5′-CAAGCAAAGTCACAGGGAAATG-3′, rs577912_forward 5′-AGTACACTGTAACAACCAGGCAAGA-3′, rs577912_reverse 5′-TAAGCCCTTTATTGTCACCTACTTTG-3′, rs650439_forward 5′-AAAC GAAGCTCTCAAAGGTAAGGA-3′, rs650439_reverse 5′-CCCTCTGGTGA CATAACCTTCAG-3′. According to the manufacturer's instruction, amplifications were performed in the 384-well ABI 7900HT Real Time PCR System (Applied Biosystems, USA). PCR amplification was performed in 10 μL final volume with 20 ng of genomic DNA at the following conditions: 95 °C for 20 s, and 40 cycles each of 95 °C for 3 s and then 60 °C for 30 s. SNP variation was assessed by means of the allelic discrimination assay employing the Applied Biosystems Software Package SDS 2.4. All genotyping ambiguity was resolved by checking raw fluorescence data and repeating the genotyping again. Four blank controls were included in each plate to ensure accuracy of the genotyping. About 8% to 10% of the samples were randomly selected for repeated assays, and the results were in agreement with the results of the first assays.

2.3. Statistic analysis

Statistical analysis was performed with SPSS statistics package, version 19.0 (IBM Corporation, Armonk, NY). Continuous variables were expressed as mean ± SD. ANOVA test was performed to compare quantitative variables by klotho genotypes. Differences in allele frequency and genotype distribution between groups and the relationship between the risk of urolithiasis and klotho gene polymorphisms was compared with binary logistic regression. Odds ratios (ORs) were given in 95% confidence interval (CI) and $p < 0.05$ was considered statistically significant.

3. Results

Laboratory data and clinical characteristics of 708 subjects were presented in Table 1. Compared with the controls, serum phosphate and 24 h-urine calcium levels were significantly higher in the stone disease group. While the serum LDH and urine pH were similar between the two groups, significant differences were detected in the levels of serum calcium and creatinine. ($p = 0.039$ and 0.027, respectively).

Genotype and allele frequencies of klotho polymorphisms in 426 stone patients and 282 controls and their association with the risk of nephrolithiasis were shown in Table 2. Klotho genotype frequency distributions were not deviated from Hardy–Weinberg equilibrium in patients and control subjects ($p > 0.05$). In the stone disease group, the allele frequencies of rs3752472 polymorphism were 0.924 for C allele and 0.076 for T allele, while frequencies of C and T allele were 0.894 and 0.106, respectively, in the control group. The allele frequencies of polymorphism at position rs577912 (A/C) were 0.250/0.750 in patients group and 0.215/0.785 in the control group, while the frequencies of the A allele and T allele of rs650439 polymorphism (0.688 and 0.312, respectively) in patients with nephrolithiasis turned to be similar to these of control subjects (0.676, 0.324, respectively). Allele frequencies of all the three polymorphisms were not significantly associated with the risk of nephrolithiasis. (For rs3752472, OR = 1.413, 95% CI = 0.953–2.097, $p = 0.086$; for rs650439, OR =1.048, 95% CI = 0.809–1.358, $p = 0.723$; for rs577912, OR = 0.862, 95% CI = 0.648–1.146, $p = 0.306$.)

Statistical comparisons for genotype distribution were performed with logistic regression modeling. Among the three polymorphisms, statically significant differences were found only between rs3752472 and the risk of nephrolithiasis. When compared to the CC genotype, the OR for nephrolithiasis risk of patients with TT+CT genotype was 1.512 (95% CI = 1.013–2.255, $p = 0.043 < 0.05$).

In order to examine the potential influence of klotho genotypes on the risk factors of nephrolithiasis, phosphate and calcium levels of serum and 24 h-urine, the levels of serum Cr, LDH and urine pH were analyzed in both two groups independently (Table 3). No

Table 1
General characteristics of the subjects (n = 708).

Group	Patients of urinary calculi	Healthy controls	p value
Gender (male/female)	299/127	212/70	0.147
Age (years)	47.7 ± 12.0	48.4 ± 13.0	0.426
Serum calcium (mg/dL)	9.3 ± 0.6	9.2 ± 0.5	0.039*
Serum phosphate (mg/dL)	3.4 ± 0.6	3.7 ± 0.6	0.0001*
24 h urine calcium (mg)	241.8 ± 145.3	108.5 ± 87.0	<0.0001*
24 h urine phosphate (mg)	356.0 ± 239.8	290.6 ± 132.5	0.055
Creatinine (μmol/L)	91.5 ± 31.8	82.4 ± 23.5	0.027*
Urine pH	6.1 ± 0.6	6.0 ± 0.6	0.400
lactic dehydrogenase (U/L)	162.5 ± 30.3	170.4 ± 40.2	0.124

Data represent means ± SD unless otherwise indicated.
* $p < 0.05$ generated by comparison between healthy controls and stone patients.