Genetic polymorphism (rs6776158) in CaSR gene is associated with risk of nephrolithiasis in Chinese population

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Abstract
The objective of this study is to find about the association between calcium-sensing receptor (CaSR) genetic variants and susceptibility to nephrolithiasis in the Chinese Han population.

This hospital-based case-control study included 319 nephrolithiasis cases and 378 healthy controls subjects. Two SNPs in CaSR were genotyped using the TaqMan assay. We found that subjects carrying the G allele of rs6776158 (AG and GG) had significantly higher risk of nephrolithiasis compared to the AA genotype (P = .015 and .009, respectively). Our results indicate that rs6776158 polymorphism that might elevate the risk of nephrolithiasis in the Chinese population.

Keywords: CaSR, nephrolithiasis, polymorphism

1. Introduction
Nephrolithiasis is a major clinical problem leading to medical care expenses and public health burden worldwide. The incidence of nephrolithiasis is about 5% among females and 12% among males. Based on final phenotype, nephrolithiasis can be considered as a multifactorial disorder, homogeneous but heterogeneous for intermediate phenotypes, and pathogenic mechanisms. Factors such as lifestyle and obesity seem to be involved in its development, but its pathogenesis may be influenced by hormonal, genetic, or anatomical factors. Studies showed that the incidence of nephrolithiasis was higher among the first-degree relatives of patients with stones than healthy individuals. Meanwhile, concordance of stones was greater among monozygous than dizygous twins. Both indicated an involvement of a genetic factor.

Calcium nephrolithiasis represents 70% to 85% of cases of nephrolithiasis. One of the main risk factors for calcium nephrolithiasis is hypercalcemia. The most important risk factors for calcium nephrolithiasis are considered to be perturbations in calcium homeostasis. Familial hypocalciuric hypercalcemia (FHH) leads to lifelong hypercalcemia. An inactivating mutation of calcium-sensing receptor (CaSR) causes FHH has been shown by a previous study. In addition, Liu et al observed that single nucleotide polymorphisms (SNPs) of CaSR were associated with hypercalcemia. Both studies suggested that CaSR may influence calcium delivery to the kidney.

The human CaSR gene is located on chromosome 3q13-21.1. It codes for a 1078-amino acid membrane protein ubiquitously expressed as a homodimer. We hypothesized that genetic variants of CaSR could influence the risk of nephrolithiasis, given the important role of CaSR in the calcium metabolism. In this study, we select 2 single nucleotide polymorphisms in CaSR (rs6776158 and rs10190) to evaluate their associations with the risk of nephrolithiasis in a Chinese population.

2. Materials and methods
2.1. Study population
The study was approved by the Institutional Review Board of the Nanjing Medical University, Nanjing, China. A total of 319 nephrolithiasis cases and 378 controls subjects, who were free from metabolic syndrome, were recruited from the First Affiliated Hospital of Nanjing Medical University, Nanjing, China. All the
subjects were blood unrelated Han Chinese. All the patients were diagnosed based on ultrasonographic and radiographic findings. Patients who showed symptoms of urinary tract infections, renal failure, chronic diarrhea, or cancer were excluded. Control subjects were recruited from those who were seeking routine outpatient care and had no history of familial urinary stone disease and no presentation of renal calcification at health screening. Each subject donated 5 mL of blood for genomic DNA extraction after having given their written informed consent. The institutional review board of Nanjing Medical University approved the research protocol.

2.2. SNP selection
Based on HapMap data (http://hapmap.ncbi.nlm.nih.gov/) and PubMed data (http://www.ncbi.nlm.nih.gov/projects/SNP/), we selected 4 SNPs located in the CaSR 5’ Untranslated Region (UTR), 5’ near gene, exon or 3’UTR (rs6776158, rs2270916, rs1042636, rs10190). Minor allele frequency (MAF) of all these genes is more than 5% in the Han Chinese population. Considering a complete linkage disequilibrium (r’ = 1) between rs2270916, rs1042636 with rs10190, only rs10190 was selected for genotyping.

2.3. Genotyping
Genomic DNA was extracted from anti-coagulated peripheral blood leukocytes by proteinase K digestion and phenol/chloroform extraction. The polymorphisms were genotyped using the TaqMan MGB technology (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. The PCR reactions were carried out in a total volume of 5 μL containing TaqMan Universal Master Mix, 80X SNP Genotyping AssayMix, Dnaase-free water and 10-ng genomic DNA. The PCR conditions were 50°C at 2 minutes, 95°C at 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The 384-well ABI 7900HT Real-Time PCR System (Applied Biosystems) was used for the genotyping assay, according to the manufacturer’s instructions and the Sequence Detection Systems software (SDS 2.3). Applied Biosystems was used to automatically collect and analyse the data and to generate the genotype calls. Four negative controls were included in each plate to ensure accuracy of the genotyping. Two people performed genotyping independently, in a blinded manner, to ensure quality control. Approximately 5% of the samples were randomly selected for repeated genotyping and the results were 100% concordant.

2.4. Statistical analysis
Pearson’s chi-square (χ²) test was used to analyse differences in frequency distributions of genotype frequencies between nephrolithiasis cases and controls. Quantitative variables included serum phosphate; serum calcium, serum creatinine, and urinary pH were reported in the text as mean value ± SD and compared between groups by Student t test or 1-way ANOVA with Tukey post-hoc test. Using an unconditional logistic regression, we estimated the association between the polymorphisms and risk of nephrolithiasis by computing odds ratios (ORs) and their 95% confidence intervals (CIs). All ORs were adjusted for age, sex, level of serum phosphate, calcium, and creatinine as well as urinary pH. All statistical analyses were 2-sided and performed with Statistics Analysis System software (Version 9.1.3; SAS Institute, Inc., Cary, NC) and P < .05 was considered statistically significant.

3. Results
3.1. Characteristics of the study population
The demographic characteristics and clinical information of nephrolithiasis cases and controls are outlined in Table 1. There were no significant differences between the cases and controls in the terms of sex (P = .49) and age (P = .76). There was also no significant difference in the levels of serum phosphate (P = .55), Urinary pH (P = .18) and Serum Uric Acid level (P = .23) between the nephrolithiasis patients and the healthy subjects. However, the patients had significantly higher level of serum calcium (2.313 ± 0.170 mmol/L vs 2.286 ± 0.136 mmol/L, P = .018) and serum creatinine (99.49 ± 82.87 μmol/L vs 82.34 ± 39.58 μmol/L P < .001) than that in the controls.

3.2. Distribution of the CaSR genotype between the cases and controls
Genotype of the 2 polymorphisms among the nephrolithiasis patients and control subjects, and their associations with risk of nephrolithiasis are shown in Table 2. The observed genotype frequencies of the polymorphisms in the control group were consistent with Hardy–Weinberg equilibrium (HWE) (P > .05). As shown in Table 2, we observed significant differences in the distribution of the genotype of rs6776158 between the patients and control subjects (P < .05). The frequencies of the AA, AG, and GG genotypes were 15.7%, 45.7%, and 38.5% among nephrolithiasis cases and 24.1%, 42.3%, and 33.6% among controls, respectively (P = .021). Based on logistic regression analysis, when using the rs6776158 AA genotype as the reference, both the AG (P = .015, adjusted OR = 1.67, 95% CI = 1.09–2.55) and GG (P = .009, adjusted OR = 1.64, 95% CI = 1.06–2.55) genotypes were associated with a significantly increased risk of nephrolithiasis compared with the AA genotype. Furthermore, a significantly increased risk was found in the combined genotypes AG/GG compared with the AA genotype (P = .006, adjusted OR = 1.64, 95% CI = 1.11–2.43). The rs6776158 G allele frequency was 61.4% among the cases and 51.8% among the controls (P = .009). However, we found no significant association between rs10190 and risk of nephrolithiasis (P = .556).

4. Discussion
In this study, we investigated whether CaSR polymorphisms were associated with the risk of nephrolithiasis in a Chinese population. We found that rs6776158 was associated with an elevated risk of nephrolithiasis. Compared to individuals who carried the rs6776158 AA genotype, individuals who carried the
rs6776158 AG/GG genotype had an increased risk of nephrolithiasis. At the same time, rs6776158 G allele was more frequent in stone formers than in controls. We suggested that rs6776158 G allele was a risk allele of nephrolithiasis.

CaSR is a plasma membrane protein that regulates parathyroid hormone (PTH) secretion by parathyroid cells and calcium reabsorption by kidney tubular cells. It is activated by the increase of extracellular concentration of calcium ions, which bind to the large extracellular N-terminal domain of the CaSR molecule. CaSR exerts its cellular activity through the stimulation of a G protein by its intracellular tail, which leads to the inhibition of PTH production and tubular calcium reabsorption. In addition, CaSR influences intestinal Ca absorption, bone remodeling, and even nervous transmission.

Nadimuthu Vinayagamoorthy, et al observed that CaSR polymorphisms have a potential to be related to serum calcium levels in East Asians. The analysis of the described CaSR functions in the kidney suggests that CaSR is sensitive to serum calcium. Primary hyperparathyroidism is a disease characterized by excessive parathyroid cell proliferation and PTH secretion and occurs frequently in postmenopausal women. Kidney stones that are generally related to hypercalciuria are a common complication in PHPT patients. Studies had suggested that risk of nephrolithiasis in primary hyperparathyroidism was associated with polymorphisms of the CaSR gene. Considering the influence of CaSR to calcium metabolism, we believed that CaSR polymorphisms also associated with nephrolithiasis in non-hyperparathyroidism group.

Recently, the association between CaSR SNPs and the risk of various diseases, including nephrolithiasis, has been clarified by genetic formation approaches using single amino acid mutations and by molecular epidemiological studies. Guha M, et al found out that polymorphisms in CaSR and Claudin 14 Genes Associated with Increased Risk of Kidney Stone Disease in Patients from the Eastern Part of India. Chou YH, et al suggested that the genetic effects of CaSR influenced the pathogenesis of calcium nephrolithiasis in Taiwanese. CaSR down-regulation has been hypothesized to be involved in stone formation. Vezzoli G, et al suggested that an alteration of tubular CaSR activity may amplify the risk of calcium-phosphate precipitation in the tubular lumen and predispose to stone formation and SNPs decreasing tubular CaSR expression may be involved in calcium nephrolithiasis. Accordingly, the level of the CaSR mRNA was lower in kidney medulla samples isolated from homozygotes for the G allele than those carrying AA or AG genotypes. The conclusion regarding CaSR SNPs in our study is supported by the results of previous studies. Otherwise, Kang Liu, et al observed significant associations between rs10190 and the risk of nephrolithiasis in Caucasian subgroups but not in Asian population in a meta-analysis. This conclusion is supported by our study.

Some limitations of this study should be noted. First, the sample size was small, which limited the statistical power of analysis. Second, the lack of detailed information on nephrolithiasis risk factors such as diet, BMI, and serum PTH further limited evaluating the associations between those factors and nephrolithiasis risk. Moreover, our study was a retrospective hospital-based study so that the inherent selection bias could not be entirely excluded. However, all genotype frequencies of the 2 polymorphisms that occurred in patients and in controls subjects in this study agreed with HWE, suggesting that the selection bias was unlikely to be substantial.

5. Conclusions

In summary, the present study provides evidence to elucidate the genetic effects of the CaSR SNPs on the pathogenesis of nephrolithiasis. We found rs6776158 polymorphism that might elevate the risk of nephrolithiasis in the Chinese population. Further epidemiological studies with larger sample size and more environmental and risk factors are needed in order to confirm our findings.

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Author contributions

All authors participated in the study conception, design and coordination. HZ, HH, ZY, XW and KS participated in the conception and initial design as well as acquisition of data in the trial. QC, PL, ZQ, YS, MZ, DR, KS, and YC participated in

Table 2

| Polymorphisms | Cases (n = 319) | Controls (n = 378) | P | Adjusted OR (95% CI) |
|--------------|----------------|------------------|---|-------------------|
| rs6776158    |                |                  |   |                   |
| AA          | 50             | 91               |   | 0.021             |
| AG          | 146            | 160              |   | 0.015             |
| GG          | 123            | 127              |   | 0.009             |
| AG/GG       | 269            | 287              |   | 0.06             |
| A           | 246            | 342              |   | 0.012             |
| G           | 392            | 414              |   | 1.32             |
| rs10190     |                |                  |   |                   |
| CC          | 98             | 103              |   | 0.556             |
| CT          | 149            | 190              |   | 0.79             |
| TT          | 72             | 85               |   | 0.81             |

*Two-sided χ² test for either genotype distributions or allele frequencies between the cases and controls.

Adjusted for sex, serum phosphate, serum calcium, serum creatinine, and urinary pH in logistic regression model; 95% CI: 95% confidence interval.
the design of this study and analysis of data. HZ, YW, SF, KS, and XW participated in interpretation of data. HZ and KS drafted the manuscript and QC, XW, DR participated in critical revision and final approval of the manuscript.

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