Association between *OPN* genetic variations and nephrolithiasis risk

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Abstract. Osteopontin (*OPN*) has an important role in urolithiasis. However, few studies have explored the association between *OPN* genetic variants and urolithiasis risk. In the present study, three single-nucleotide polymorphisms (SNPs) (rs28357094, rs11439060 and rs11730582) located on the promoter of *OPN* were genotyped in a total of 480 individuals, including 230 nephrolithiasis patients and 250 matched healthy controls, and the associations between these SNPs and nephrolithiasis risk in different genetic models was assessed. No significant differences were identified in the genotype and allele frequencies of OPN rs28357094 or rs11730582 (P=0.805 for rs28357094; P=0.577 for rs11730582, respectively). However, carriers with the *OPN* rs11439060 insertion (ins) types (ins/deletion and ins/ins) were overrepresented in urolithiasis patients compared with the controls [odds ratio (OR), 1.55; 95% confidence interval (CI), 1.08-2.22]. In the stratified analysis, the increased risk was more evident among younger subjects (adjusted OR, 1.68; 95% CI, 1.01-2.81), females (2.15; 1.14-4.08), overweight subjects (1.80; 1.07-3.05), normotensive subjects (2.48; 1.02-6.00), abnormal blood sugar subjects (1.58; 1.08-2.30), smokers (1.63; 1.02-2.60), and ever-drinkers (1.98; 1.10-3.60).. These findings revealed that the *OPN* rs11439060 polymorphism may act as genetic biomarker for the detection of high-risk nephrolithiasis patients.

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Introduction

Urolithiasis is a common urinary disease with an increasing incidence worldwide, of which the occurrence varies among geographical regions and ethnicities. Kidney stone formation is reported to contribute to obesity, type 2 diabetes and hypertension (1-3). A previous study demonstrated a series of events leading to kidney stone formation, including oxalate/crystal-induced oxidative stress, tubular cell injury, inflammation and fibrosis (4). A positive family history predisposes an individual to nephrolithiasis, which suggests that genetic factors may have a critical role in the development of kidney stone formation (5).

Osteopontin (OPN), also known as or secreted phosphoprotein 1, is a secreted phosphoprotein expressed in various human body fluids, notably plasma, bile and urine (6). The OPN protein is synthesized within the kidney and is subsequently secreted into the urine by epithelial cells, including the papillary epithelium, distal convoluted tubule and loop of Henle (7). However, several studies have showed a controversial function of OPN, among which OPN could have a role in repressing the nucleation and aggregation of calcium oxalate crystals *in vitro* (8); however, OPN knockdown in the kidneys of hyperoxaluric rats could lead to reduction in renal calcium oxalate crystal deposition (9). OPN, a constituent of stone matrix, is also known as a specific monocyte chemoattractant to the renal interstitium (10). The expression of *OPN* mRNA is unregulated in stone-forming rats (11). In addition, OPN could promote the adherence of calcium oxalate crystals to Madin-Darby canine kidney cells (11). Furthermore, OPN could increase the formation and aggregation of calcium oxalate crystals in several experimental systems (12). Taken together, inhibitory and stimulatory roles in calcium stone formation have been proposed for OPN.

Single-nucleotide polymorphisms (SNPs) have been proposed as a worthwhile tool for identifying key genes associated with complex disease, such as those involved in urolithiasis (13). Polymorphisms in the *OPN* gene have been reported to exhibit functional implications and have been evaluated in several conditions (14), including the associations with autoimmune lymphoproliferative syndrome (15), type 1 diabetes (16), multiple sclerosis (17), systemic lupus

erythematosus (18), rheumatoid arthritis (19,20) and Crohn's disease (21). However, the associations between *OPN* gene polymorphisms and urolithiasis are not widely reported (22,23), and have not been investigated in Chinese populations.

Considering OPN is linked to formation of calcium oxalate crystal deposition in the kidneys, we hypothesized *OPN* as a candidate gene linked to the genetic predisposition to develop nephrolithiasis. Additionally, the genetic variants in the promoter affecting the transcriptional level has attracted increasing attention; therefore in the present study, three SNPs (rs28357094 -170 T>G, rs11439060 -260 del/ins and rs11730582 -546 T>C) located on promoter of *OPN* were genotyped in a total of 480 individuals, including 230 nephrolithiasis patients and 250 matched healthy controls, and the associations between these SNPs and nephrolithiasis risk in different genetic models in the Chinese population were assessed.

Materials and methods

Study population. The present study was approved by the Institutional Review Board of Huaiyin Hospital (Huai'an, Jiangsu, China), and all subjects signed the informed consent form. There were 230 nephrolithiasis cases and 250 controls in this hospital-based case-control study. Briefly, all cases were confirmed to have nephrolithiasis at the time of enrollment in the ongoing study, and were recruited from Huaiyin Hospital between March 2010 and January 2013, of which 91% were calcium oxalate kidney stones. Those seeking general physical examinations at the outpatient department were recruited as the controls in the same hospital between March 2010 and January 2013. Controls with renal diseases were excluded. Individuals who smoked daily for >1 year were defined as a smoker. The status of body mass index (BMI, kg/m^2), hypertension and diabetes are according to the World Health Organization standards. Hypertension is presented if the resting blood pressure is persistently ≥140/90 mmHg for most adults. The World Health Organization definition of diabetes is for a single raised glucose reading with symptoms, otherwise raised values on two occasions, of either: Fasting plasma glucose ≥7.0 mmol/l (126 mg/dl) or with a glucose tolerance test, 2 h after the oral dose a plasma glucose ≥11.1 mmol/l (200 mg/dl). The informed consent was obtained from the eligible subjects prior to recruitment. Through face-to-face interviews, the information of individual demographics was obtained.

Genotyping. The genomic DNA samples from cases and controls were isolated from peripheral blood lymphocytes. The ABI 7900HT Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used for the TaqMan SNP genotyping assay. The PCR was performed in a 10 μl per reaction on the genomic DNA (10 ng) using a TaqMan universal PCR master mix (Applied Biosystems, Thermo Fisher Scientific, Inc.). Forward, reverse primers, FAM and VIC labeled probes were designed by Applied Biosystems (ABI Assay-by-Designs) Genotype analysis was carried out by two independent investigators in a blind manner. Approximately 10% of all the samples were selected randomly for confirmation, and the results were 100% concordant.

Table I. Characteristic descriptions of patients with nephrolithiasis and the controls.

Variables	Cases, n=230	Controls, n=250	P-value[a]
Mean age ± SD, years	46.7±12.5	45.9±12.5	0.174
≤46, n (%)	109 (47.4)	134 (53.6)	
>46, n (%)	121 (52.6)	116 (46.4)	
Gender, n (%)			0.785
Male	150 (65.2)	166 (66.4)	
Female	80 (34.8)	84 (33.6)	
Body mass index, n (%)			0.578
≤24	111 (48.3)	127 (50.8)	
>24	119 (51.7)	123 (49.2)	
Hypertension, n (%)			0.055
Yes	59 (25.7)	46 (18.4)	
No	171 (74.3)	204 (81.6)	
Diabetes, n (%)			0.931
Yes	17 (7.4)	19 (7.6)	
No	213 (92.6)	231 (92.4)	
Smoking status, n (%)			0.001
Ever	103 (44.8)	76 (30.4)	
Never	127 (55.2)	174 (69.6)	
Drinking status, n (%)			0.811
Ever	88 (38.3)	93 (37.2)	
Never	142 (61.7)	157 (62.8)	

[a]P-value for two-sided χ^2 test. SD, standard deviation.

Statistical analysis. The Pearson's χ^2 test and Student's t-test were used to assess the differences in the enrolled variables between cases and controls. The associations between the risk of nephrolithiasis and genotypes were estimated using adjusted odds ratios (ORs) and corresponding 95% confidence intervals (CIs) from unconditional logistic regression with the adjustment of age and gender. Hardy-Weinberg equilibrium (HWE) was constructed to evaluate the population accuracy for the controls. All the statistical analyses were constructed with SAS software version 9.1 (SAS Institute, Inc., Cary, NC, USA) and $P<0.05$ for two-sided analysis was considered to indicate a statistically significant difference.

Results

Study characteristics. A total of 230 kidney calculi cases and 250 healthy controls were recruited in the analysis, and the demographic characteristics are summarized in Table I. The cases and controls appeared to be well-matched on age, gender and BMI. There were more individuals smoking in

Table II. Genotype and allele frequencies of *OPN* polymorphisms among cases and controls, and their associations with nephrolithiasis risk.

Genotype	Cases/controls, n	P-value[a]	OR (95% CI)[b]	P-value (HWE)
rs28357094				0.832
TT	124/137	0.805	Ref	
TG	94/97		1.08 (0.74-1.57)	
GG	12//16		0.83 (0.38-1.83)	
TG+GG	106/113	0.846	1.04 (0.73-1.49)	
TT+TG	218/234		Ref	
GG	12//16	0.581	0.81 (0.37-1.74)	
Trend		0.957		
rs11439060				0.532
del/del	99/135	0.035	Ref	
ins/del	108/100		1.46 (1.00-2.13)	
ins/ins	23/15		2.14 (1.06-4.33)	
ins/del+ins/ins	131/115	0.016	1.55 (1.08-2.22)	
del/del+ins/del	207/235		Ref	
ins/ins	23/15	0.105	1.79 (0.91-3.54)	
Trend		0.010		
rs11730582				0.989
TT	114/134	0.577	Ref	
TC	101/98		1.23 (0.85-1.79)	
CC	15/18		0.97 (0.47-2.02)	
TC+CC	116/116	0.377	1.19 (0.83-1.71)	
TT+TC	215/232		Ref	
CC	15/18	0.769	0.89 (0.44-1.81)	
Trend		0.554		

[a]P-value for two-sided χ^2 test; [b]adjusted for age and gender in the logistic regression model. *OPN*, osteopontin; OR, odds ratio; CI, confidence interval; HWE, Hardy-Weinberg equilibrium; ref, reference; del, deletion; ins, insertion.

cases compared to controls. No significant difference in the distributions of age, gender, BMI, hypertension, diabetes and drinking status were observed (P=0.174, 0.785, 0.578, 0.055, 0.931 and 0.811, respectively).

Genotype and allele frequencies of OPN polymorphisms in the nephrolithiasis cases and controls. Allele frequencies and genotype distributions of *OPN* polymorphism in patients and controls are listed in Table II. Analyzed SNPs showed no deviation from the HWE for controls (P>0.05). No significant differences in genotype and allele frequencies of *OPN* rs28357094 or rs11730582 were observed between nephrolithiasis and controls (P=0.805 for rs28357094; P=0.577 for rs11730582, respectively), except *OPN* rs11439060 (P=0.035). Carriers of the *OPN* rs11439060 insertion (ins) allele genotypes [ins/deletion (del) and ins/ins] were overrepresented in the kidney calculi patients (53.25%) compared with the controls (46.75%). Individuals with the *OPN* rs11439060 ins allele genotypes (ins/del + ins/ins) had a significantly increased risk of nephrolithiasis compared to those with the ins/ins genotype (P=0.016; OR, 1.55; 95% CI, 1.08-2.22).

Association and stratification analysis between OPN rs11439060 polymorphisms and nephrolithiasis. Furthermore, the stratified analyses were conducted by age, gender, BMI, hypertension status, drinking and smoking status. As presented in Table III, increased risks of nephrolithiasis for the ins allele genotypes (ins/del + ins/ins) were found among younger subjects (adjusted OR, 1.68; 95% CI, 1.01-2.81), females (2.15; 1.14-4.08), overweight subjects (1.80; 1.07-3.05), normotensive subjects (2.48; 1.02-6.00), abnormal blood sugar subjects (1.58; 1.08-2.30), smokers (1.63; 1.02-2.60), and ever-drinkers (1.98; 1.10-3.60).

In silico analysis. Subsequently, whether rs11439060 could perform biological function was detected. Thus, the RegulomeDB (http://regulome.stanford.edu/) was utilized and the ENCODE database was implemented to predict the effect of rs11439060. The results showed 2b of RegulomeDB score, which indicated that rs11439060 was involved in affecting transcriptional factors binding, motifs change, chromatin structure modification and histone modifications. All these indicated that rs11439060 may participate in gene expression by multiple tracks.

Table III. Stratified analyses on the association between the OPN rs11439060 single-nucleotide polymorphism and nephrolithiasis risk.

	Cases (n=230), n (%)		Controls (n=250), n (%)				
Characteristics	Del/del	Ins/del+ins/ins	Del/del	Ins/del+ins/ins	OR (95% CI)[a]	P-value[a]	P-value[b]
Age, years							
≤46	47 (43.1)	62 (56.9)	75 (48.7)	59 (51.3)	1.68 (1.01-2.81)	0.046[c]	0.585
>46	52 (43.0)	69 (57.0)	60 (51.7)	56 (48.3)	1.44 (0.86-2.42)	0.164	
Gender							
Male	67 (44.7)	83 (55.3)	86 (51.8)	80 (48.2)	1.34 (0.86-2.08)	0.201	0.654
Female	32 (40.0)	48 (60.0)	49 (58.3)	35 (41.7)	2.15 (1.14-4.08)	0.018[c]	
BMI, mg/k^2							
≤24	53 (47.7)	58 (52.3)	70 (55.1)	57 (44.9)	1.34 (0.79-2.25)	0.273	0.185
>24	46 (36.1)	73 (63.9)	65 (52.8)	58 (47.2)	1.80 (1.07-3.05)	0.028[c]	
HP							
Yes	25 (42.4)	34 (57.6)	28 (60.8)	18 (39.2)	2.48 (1.02-6.00)	0.035[c]	0.163
No	74 (38.7)	97 (61.3)	107 (52.5)	97 (47.5)	1.42 (0.94-2.14)	0.098	
Diabetes							
Yes	8 (47.0)	9 (53.0)	9 (47.4)	10 (52.6)			0.101
No	100 (46.8)	113 (53.2)	122 (53.0)	108 (47.0)	1.58 (1.08-2.30)	0.021[c]	
Smoking status							
Ever	41 (39.8)	62 (60.2)	34 (44.8)	42 (55.2)	1.63 (1.02-2.60)	0.041[c]	0.367
Never	58 (45.7)	69 (54.3)	101 (58.0)	73 (42.0)	1.26 (0.68-2.33)	0.454	
Drinking status							
Ever	34 (38.7)	54 (61.3)	52 (55.9)	41 (44.1)	1.98 (1.10-3.60)	0.021[c]	0.141
Never	65 (45.8)	77 (54.2)	83 (52.9)	74 (47.1)	1.34 (0.85-2.11)	0.206	

[a]Adjusted for age and gender in the logistic regression model; [b]P-value for the heterogeneity test; [c]P<0.05. BMI, body mass index; HP, hypertension; del, deletion; ins, insertion.

Discussion

Genetic variations are involved in the development and progression of nephrolithiasis (22). In this candidate gene study, polymorphisms in the *OPN* gene were associated with the presence of nephrolithiasis. A total of three polymorphisms were genotyped. This case-control study identified an association between a 1-base pair ins/del polymorphism rs11439060 (-/G) within the *OPN* promoter and nephrolithiasis susceptibility, and found that individuals with the rs11439060 variant genotypes (ins/del + ins/ins) had a significantly increased nephrolithiasis risk compared with the del/del genotypes.

OPN, an acidic phosphorylated glycoprotein, has been demonstrated to cause multiple biological processes such as the regulation of normal and abnormal calcification (12,24). An animal experiment revealed that overexpressed OPN could cause diabetic glomerulosclerosis (25). However, with regards to the urinary tract, OPN, mainly synthesized within the kidney and subsequently secreted into the urine, serves as a normal element for urine (7,26). Previous studies performed in cell lines have approved the inhibitory activities of OPN on aggregation, calcium crystal nucleation and adhesion to renal epithelial cells (8,27). Additional *in vivo* studies discovered that mice with *OPN*-knockdown were more susceptible to calcium urolithiasis compared to wild-type mice (28). Furthermore, emerging evidence has revealed that the products of the *OPN* gene, including mRNA and protein expression levels could be elevated and assembled in renal epithelial cells results of calcium oxalate crystals exposure (29,30), and the increased products of the *OPN* gene were considered to prevent urolithiasis formation (8,27). In addition, the imbalanced effect among the urinary-promoting/-inhibiting factors has been widely considered as more important in urinary stone formation compared to the disturbance of any other single substance (31).

Considering OPN has a crucial role in stone formation, polymorphism in the gene controlling the synthesis of *OPN* may significantly affect the risk of urolithiasis. The studies for the association between *OPN* genetic variants and the calcium urolithiasis risk are limited. There is an association of two common SNPs of *OPN* [non-synonymous amino acid located at positions +9402 (Arg/His) and +9171 (Asn/Ser) in exon 7] with calcium urolithiasis risk, and the SNP at position +9402 is significantly associated with the increased risk of calcium urolithiasis (32). Furthermore, a novel SNP at -156 (delG/G) of the *OPN* gene has been considered as a candidate genetic marker for calcium urolithiasis risk (33). Recently, Gao *et al* (34) have performed sequencing analysis

on the entire *OPN* gene for the stone cases and normal controls of Japanese ancestry, and identified that two novel SNPs (-145T/G and -144G/T) resided at the *OPN* promoter region were also significantly associated with the risk of calcium urolithiasis. Additionally, Gögebakan *et al* (35) identified the significant association between the T-593A and C6982T polymorphism and nephrolithiasis risk in the Turkish population; however, the small sample size limited its extensive use as biomarkers. Even though the present study did not detect the expression of *OPN*, the findings show the notable association between SNP rs11439060 and the increased nephrolithiasis risk. It is biologically plausible that the allele of rs11439060 could affect the expression level of *OPN*, and contribute to the development of nephrolithiasis. Furthermore, the association between smoking and the nephrolithiasis risk was also identified, suggesting that there may be a risk effect of smoking on nephrolithiasis development and progression. In the subsequent stratified analysis, smokers with rs11439060 ins/del + ins/ins have a higher risk of nephrolithiasis, indicating the interaction effect of SNP rs11439060 and smoking on nephrolithiasis.

Considered the complex process for kidney stone formation, it is impossible that any single SNP or gene would exert a clear influence on nephrolithiasis risk. There was a limitation in the present study; it is unclear that aberrant expression of *OPN* is the cause or the consequence of crystal deposition. Certain studies have found the association between expression of *OPN* and development of nephrolithiasis *in vivo*, but the present results are not substantial in themselves to clarify the causal link between rs11439060 and nephrolithiasis risk. Although a strong correlation between rs11439060 and nephrolithiasis risk was observed, how genetic variability at this locus influences *OPN* function remains to be fully elucidated at functional levels. Additional large well-designed studies in multiple populations of different ethnicities and further functional studies are warranted.

In conclusion, the present study has provided the initial evidence that individuals carrying the SNP rs11439060 ins/del + ins/ins have a significantly increased nephrolithiasis risk compared with the del/del genotypes, indicating that the ins G allele was a deleterious effect on the nephrolithiasis. The results suggest that the SNP rs11439060 may serve as a potential marker to predict the nephrolithiasis risk in Chinese populations.

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