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Original Article

Associations between *IL-1RN* variable number of tandem repeat, *IL-1β* (−511) and *IL-1β* (+3954) gene polymorphisms and urolithiasis in Uighur children of China

Jiefeng Xiao, Shukai Zheng, Zhaolong Qiu, Kusheng Wu*

Department of Preventive Medicine, Shantou University Medical College, Shantou, Guangdong, China

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Abstract *Objective:* Interleukin-1 (IL-1) is a pro-inflammatory cytokine which may be related to urolithiasis. Genetic polymorphisms of the interleukin-1beta (*IL-1β*) have been proposed as markers for urolithiasis in some areas. Due to the high incidence of urolithiasis in Uighur children (Xinjiang, China) and existence of ethnic difference, our aim is to explore the potential of IL-1 gene polymorphisms and urolithiasis among these children.
Methods: Genomic DNA extracted from peripheral blood of 115 patients and 98 controls were used for genotype polymorphisms analyses. IL-1 receptor antagonist (*IL-1RN*) gene variable number of tandem repeat (VNTR) gene polymorphisms were analyzed by PCR method. PCR-based restriction analysis was done for the *IL-1β* (−511) and *IL-1β* (+3954) gene polymorphisms by endonucleases Ava I and Taq I, respectively. The genotype distribution, allele frequencies, carriage rate, and haplotype frequencies were statistically analyzed.
Results: No significant differences were observed in genotypic frequencies between pediatric urolithiasis patients and control group for *IL-1RN* gene ($\chi^2=1.906$, $p=0.605$), *IL-1β* (−511) gene ($\chi^2=0.105$, $p=0.949$), or *IL-1β* (+3954) gene ($\chi^2=3.635$, $p=0.169$). There were yet no significant differences of the allele frequencies of *IL-1RN* VNTR gene ($p=0.779$), *IL-1β* (−511) gene ($p=0.941$), and *IL-1β* (+3954) gene ($p=0.418$) in the case and control groups, as well as the carriage rate and haplotype of them (all $p>0.05$).
Conclusions: The associations between *IL-1RN* VNTR, *IL-1β* (−511) and *IL-1β* (+3954) genes polymorphisms and urolithiasis were not significant in Uighur children. The results need to be confirmed in studies with larger population sample size, as well as in other ethnic groups.

* Corresponding author.
E-mail address: kswu@stu.edu.cn (K. Wu).
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1. Introduction

Urolithiasis is one of the three major diseases of the human urinary system and is common in the worldwide. However, pediatric stone disease is easily neglected because of different distribution. In northern America, 7% of kidney stones occur in children who are under 16 years old. Edvardsson et al. [1] reported a high incident of pediatric stone disease in Iceland as compared to western countries. Xinjiang, a multi-ethnic region that is the main residence of the Uighur people, has a high incidence of children urolithiasis [2]. The preliminary statistics of Kashi area in Xinjiang showed that the incidence of urolithiasis among Uighur children is higher than other ethnic group, accounting for 98% [3]. Clinical diagnosis has revealed that urolithiasis is more prevalent in Uighur patients than in patients of other races living in the same region [4].

Urolithiasis is affected by numerous factors, including genetic and environmental factors, abnormal metabolism, race, and living habits [5]; the specific mechanism by which urinary calculi form in Uighur children is still unknown. The single nucleotide polymorphisms are widely used to be a tool for identifying genetic alteration to map the complex disease genes [6]. Some studies have reported that cytokine gene polymorphisms, including those of the genes encoding interleukin-1 (IL-1) beta (IL-1β), IL-1 receptor antagonist (IL-1RN), and tumor necrosis factor (TNF)-alpha have demonstrated an association with inflammatory disease and glomerulonephritis [7]. Genes encoding the IL-1 family of proteins include those for IL-1α, IL-1β, IL-1 receptor types I and II, and IL-1RN; these genes are clustered on human chromosome 2q and are known to be polymorphic and are in linkage disequilibrium [8]. Different polymorphisms have been reported about *IL-1β*. There are two mainly influencing protein productions: One is located within the promoter region at position −511 and the other in Exon 5 at +3954, where it is responsible for a cytosine (C) to thymine (T) transition [9,10]. The region within the second intron of IL-1RA contains variable number of tandem repeat (VNTR) of 86 base pairs; however, the alleles do not correspond exactly to the repeats because some extra bases are amplified along with the repeats. These three polymorphisms directly affect protein structure and alter its function, not by affecting the sequence of amino acids in polypeptide chains, but rather at different transcriptional or post-transcriptional levels by altering the structure of the gene's regulatory elements with binding sites for transcription factors, transcription rate, mRNA splicing, and mRNA stability [11]. As *IL-1β* gene plays a major role in inflammation, the aim of this study was to determine the association of specific polymorphisms of pro-inflammatory *IL-1* (both exon-5 and promoter region) and *IL-1RN* (anti-inflammatory) genes with susceptibility for risk of stone formation in pediatric urolithiasis patients.

2. Methods

2.1. Patients

All the participants were Uighur children from Kashi city, Xinjiang, China. Urolithiasis was confirmed by ultrasonography, abdominal radiography or computerized tomography. Blood and urine biochemistry tests were performed to evaluate the hyperuricemia, hypercalcemia, hyperuricosuria or hyperoxaluria cases of both groups to exclude them from the study. Patients who showed symptoms of urinary tract infections, birth defects in the urinary tract, vascular heart diseases, acute, chronic, infectious, immunologic conditions, history of malignance, neoplastic diseases, coagulation disorders or chronic renal failure were also excluded. The same criteria were also used in the control group. Meanwhile, the control group had no family history of stone and was evaluated by renal ultrasonography and routine tests for urinary microscopic hematuria in order to exclude individuals who may have had renal calcification. The written informed consents were obtained from all the parents or guardians of the children. This study was performed with the approval of the Human Ethical Committee of Shantou University Medical College (Approval number: SUMC 2016XM-0017).

2.2. DNA extraction and genotyping

Blood samples were collected in ethylenediamine tetraacetic acid-containing tubes storing at −80 °C. Genomic DNAs were extracted from peripheral blood leukocytes using blood genomic DNA extraction kits (TIANGEN, Beijing, China). Polymerase chain reaction (PCR) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) were used to identify the genotypes of IL-1 related genes. The PCR products were showed on agarose gel electrophoresis with different concentrations according to its size. The specific analysis about each gene polymorphism is listed in Table S1 (supplementary information). *IL-1RN* VNTR gene has six alleles which can be directly analyzed. As both −511 and +3954 sites involve C to T polymorphism, therefore, *IL-1β* (−511) genotypes are designated as C/T while *IL-1β* (+3954) as E_1/E_2. *IL-1β* (−511) gene and *IL-1β* (+3954) gene were separately analyzed by PCR amplification followed by restriction fragment length polymorphism (RFLP) analysis after Ava I digestion and Taq I digestion. The size of fragments was estimated by comparing with 100-bp DNA ladder (TIANGEN, Beijing, China).

2.3. Statistical analysis

The Hardy-Weinberg equilibrium test was done for each polymorphism. The χ^2-test was used to analyze gene frequency, allele frequency, carriage rates and haplotype distributions in patients and controls. The results were expressed as odds ratio (OR) and 95% confidence interval (CI). Probability of values of $p<0.05$ (two-sided) was considered statistically significant. All analyses were performed using an SPSS 22.0 software program (IBM Corp., Armonk, NY, USA).

3. Results

A total of 115 urolithiasis patients including 92 boys and 23 girls were recruited from People's Hospital of Kashgar,

Xinjiang and enrolled in this hospital-based case-control study. The control group included 69 boys and 29 girls, who were also recruited from the same hospital but without urolithiasis. In this study, the age of children was from 0.1 years old to 13.3 years old and the average age was 5.4±3.1 years old. Levels of uric acid, creatinine and serum Ca^{2+}, P, Mg^{2+}, K^{+}, Na^{+}, Cl^{-} and retinol binding protein (RBP) did not differ among the 115 patients and 98 controls (all $p>0.05$), but urea was higher for children with stones than controls ($p<0.001$) while CO_2 combining power (CO_2CP) and cystatin C level were lower (both $p<0.001$) (Table S2, supplementary information).

The PCR analysis revealed the presence of polymorphisms at the specific sites. For *IL-1RN* gene, wild type I/I (410 bp), mutant type II/II (240 bp), and heterozygote I/II, I/III, I/IV, II/IV (410 bp, 240 bp, 500 bp, and 325 bp) phenotypes were observed (Fig. 1A); for *IL-1β* (−511) gene, wild gene C/C (190 bp and 114 bp), mutant type T/T (304 bp) and heterozygote T/C (304 bp, 190 bp, and 114 bp) phenotypes were observed (Fig. 1B); for *IL-1β* (+3954) gene, wild type E_1/E_1 (135 bp and 114 bp), mutant type E_2/E_2 (249 bp) and heterozygote E_1/E_2 (135 bp, 114 bp, and 249 bp) phenotypes were observed (Fig. 1C).

The distributions of genotypes, allele frequency, carriage rate and haplotype in patients and controls are shown in the following tables. No significant differences were observed in genotypic frequencies between pediatric urolithiasis patients and control group for *IL-1RN* gene ($\chi^2=1.906$; $p=0.605$), *IL-1β* (−511) gene ($\chi^2=0.105$; $p=0.949$), or *IL-1β* (+3954) gene ($\chi^2=3.635$; $p=0.169$) (Table 1). For the allele frequency, there were yet no significant differences at the three sites. The allele frequency of *IL-1RN* gene ($\chi^2=1.369$; $p=0.799$), *IL-1β* (−511) gene ($\chi^2=0.005$; $p=0.941$), and *IL-1β* (+3954) gene ($\chi^2=0.655$; $p=0.418$) are shown in Table 2. The carriage rates of *IL-1RN* VNTR gene ($p=0.810$), *IL-1β* (−511) gene (OR=1.01, 95% CI=0.65–1.57), and *IL-1β* (+3954) gene (OR=0.79, 95% CI=0.46–1.37) do not exist significant differences in patients and controls (Table 3). We also analyzed haplotype further, and the results showing that all of the eight possible haplotypes were common in patients and controls, and no significant difference was observed ($\chi^2=2.518$; $p=0.926$).

4. Discussion

Urolithiasis affects many people globally, with a 1%–5% prevalence and a 50% recurrence rate over 10 years [12]. Generally, the incidence of urinary tract stones in children was distinctly lower than in adults. Children with urinary tract stones account for 1% in the whole population of urolithiasis from developed countries, while the proportion is up to 30% in developing countries especially Turkey and Far East area [13–15]. The prevalence of urolithiasis in Uighur children is higher than Han children living in the same area [3], which means the internal factors work in addition to environmental factors. The racial differences and genetic background may play important roles in the development of pediatric urolithiasis.

The formation of urinary stones in children has close relationship with their abnormal metabolism, urinary tract abnormalities and urinary tract infection. Abnormal metabolism is the main reason to cause urinary stones in children, which accounts for 42%. The proportion of urinary tract infection is about 34%–58% [16]. Some urinary system diseases such as urethral malformations and urinary obstruction will cause abnormal urine excretion resulting in urethral infection which eventually leads to urethral calculi. In the whole process, the effect of gene and inheritance can not be ignored. In previous study, Bid et al. [17] explored the role of vitamin-D receptor (VDR) gene, calcitonin receptor (CTR) gene and IL-1 related genes polymorphism in pediatric nephrolithiasis from north Indian population. Mittal et al. [18] also investigated the relationship of *IL-1RN* gene, *IL-1β* (−511) gene, and *IL-1β* (+3954) gene with urolithiasis in adults. Chen et al. [19] suggested that polymorphism in IL-1 was to be associated with development and severity of adult urolithiasis. Coker Gurkan et al. [12] reported the relationships of *IL-1RN* gene, *IL-1β* (−511) gene and urolithiasis in Turkish population. These results suggest some genes may involve in abnormal metabolism of urinary system and contribute to the formation of urolithiasis eventually.

Based on the findings above, we evaluated the potential of *IL-1RN* VNTR, *IL-1β* (−511) and *IL-1β* (+3954) gene polymorphisms and their relationships with urinary stone

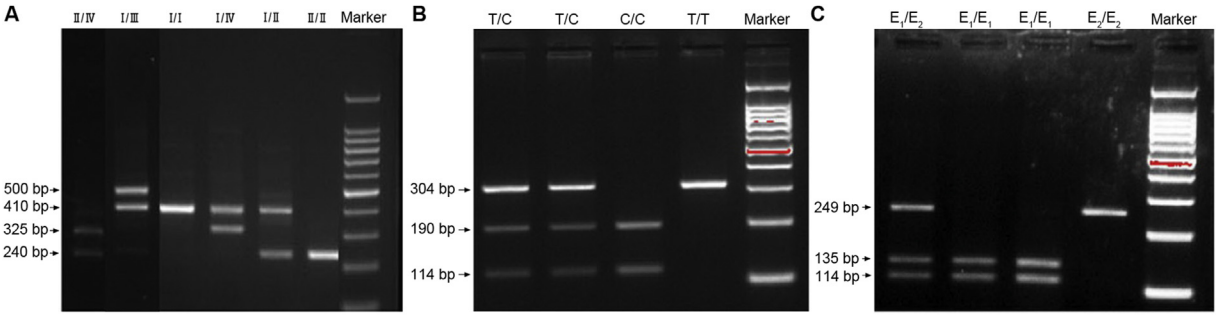

Figure 1 The results of agarose gel electrophoresis. (A) *IL-1RN* VNTR gene; (B) *IL-1β* (−511) gene; (C) *IL-1β* (+3954) gene. I/I, *IL-1RN* VNTR gene wild type; II/IV, I/III, I/IV and I/II, *IL-1RN* VNTR gene heterozygote; II/II, *IL-1RN* VNTR gene mutant type; T/C, *IL-1β* (−511) gene heterozygote; C/C, *IL-1β* (−511) gene wild type; T/T, *IL-1β* (−511) gene mutant type; E_1/E_2, *IL-1β* (+3954) gene heterozygote; E_1/E_1, *IL-1β* (+3954) gene wild type; E_2/E_2, *IL-1β* (+3954) gene mutant type; IL-1β, interleukin-1beta; VNTR, variable number of tandem repeat.

Table 1 Genotype distribution of *IL-1RN* VNTR gene, *IL-1β* (−511) gene, and *IL-1β* (+3954) gene polymorphism between patients and controls.

Gene	Genotype	Patient, *n* (%)	Control, *n* (%)	*p*-Value	OR (95% CI)
IL-1RN VNTR	I/I	71 (61.7)	59 (60.2)	0.605	1.00
	I/II	36 (31.3)	27 (27.6)		0.90 (0.49—1.66)
	II/II	5 (4.4)	7 (7.1)		1.69 (0.51—5.59)
	Others	3 (2.6)	5 (5.1)		2.01 (0.46—8.74)
IL-1β (−511)	C/C	31 (27.0)	25 (25.5)	0.949	1.00
	T/C	55 (47.8)	49 (50.0)		1.11 (0.58—2.12)
	T/T	29 (25.2)	24 (24.5)		0.97 (0.46—2.07)
IL-1β (+3954)	E_1/E_1	71 (61.7)	69 (70.4)	0.169	1.00
	E_1/E_2	41 (35.7)	24 (24.5)		0.60 (0.33—1.10)
	E_2/E_2	3 (2.6)	5 (5.1)		0.58 (0.13—2.53)

IL-1RN VNTR, interleukin-1 receptor antagonist variable number of tandem repeat; IL-1β, interleukin-1beta; OR, odds ratio; CI, confidence interval; I, *IL-1RN* VNTR gene wild type; II, *IL-1RN* VNTR gene mutant type; III and IV, *IL-1RN* VNTR gene heterozygote; C, *IL-1β* (−511) gene wild type; T, *IL-1β* (−511) gene mutant type; E_1, *IL-1β* (+3954) gene wild type; E_2, *IL-1β* (+3954) gene mutant type.

Table 2 Allele frequencies of *IL-1RN VNTR* gene, *IL-1β* (−511) gene, and *IL-1β* (+3954) gene in patients and controls.

Gene	Allele	Patient, *n* (%)	Control, *n* (%)	*p*-Value	OR (95% CI)
IL-1RN VNTR	I	180 (78.3)	149 (76.4)	0.799	1.00
	II	47 (20.4)	41 (21.0)		1.05 (0.66—1.69)
	III	2 (0.9)	4 (2.1)		2.42 (0.44—13.38)
	IV	1 (0.4)	1 (0.5)		1.21 (0.08—19.48)
IL-1β (−511)	C	117 (50.9)	99 (50.5)	0.941	1.00
	T	113 (49.1)	97 (49.5)		0.99 (0.67—1.44)
IL-1β (+3954)	E_1	183 (79.6)	162 (82.7)	0.418	1.00
	E_2	47 (20.4)	34 (17.3)		1.22 (0.75—2.00)

IL-1RN VNTR, interleukin-1 receptor antagonist variable number of tandem repeat; IL-1β, interleukin-1beta; OR, odds ratio; CI, confidence interval; I, *IL-1RN* VNTR gene wild type; II, *IL-1RN* VNTR gene mutant type; III and IV, *IL-1RN* VNTR gene heterozygote; C, *IL-1β* (−511) gene wild type; T, *IL-1β* (−511) gene mutant type; E_1, *IL-1β* (+3954) gene wild type; E_2, *IL-1β* (+3954) gene mutant type.

disease among Uighur children from Kashi city, Xinjiang, China. The three genetic sites belong to cytokine family. Cytokines are the components of urine and they are shown to be associated with the stone formation in kidneys [20,21]. It has been known to have a specific endogenous functional regulator in the form of a receptor antagonist (IL-1RN). Genes encoding the IL-1 family of proteins include those for IL-1α, IL-1β, the IL-1 receptor types I and II, and *IL-1RN* gene; these genes are clustered on human chromosome 2q and are known to be polymorphic and are in linkage disequilibrium [8]. IL-1RN is a competitive inhibitor of IL-1 and a powerful anti-inflammatory agent. Genetic variation in this region is manifested as single nucleotide polymorphism [22]. We chose to concentrate on cytokines as candidate genes because they have several proteins that are key components in the pathogenesis of many diseases. For example, The IL-1RN protein is a natural competitive inhibitor of IL-1, acting by occupying the IL-1 cell-surface receptor without triggering signal transduction, thus reducing inflammation [23]. Studies of several cytokine gene polymorphisms, including those on genes encoding IL-1β, IL-1RN have demonstrated that an association with various inflammatory diseases and the estimation of cytokines is suggestive of their association with stone disease [8,19,23].

The results suggested that *IL-1RN* VNTR, *IL-1β* (−511), and *IL-1β* (+3954) genes may not be an influential marker for susceptibility to Xinjiang Uighur pediatric urolithiasis. There are some differences between our results and previous researches. In our study, genotypic frequencies between pediatric urolithiasis patients and control group for *IL-1RN* have no statistical difference. The allele frequency of *IL-1RN* and the carriage rate of *IL-1RN* also show no significant difference, while Coker Gurkan et al. [12] found that the difference of genotypic frequencies between urolithiasis patients and control group is statistically significant ($\chi^2=6.131$; $p=0.047$). In addition, urolithiasis patients had a significantly higher frequency of the allele II in the *IL-1RN* ($\chi^2=13.156$; $p=0.007$). Their analysis revealed that *IL-1RN* VNTR allele II carriers combined with IL-1β (-511) C carriers were significantly higher in frequency among urolithiasis cases than disease-free healthy control patients ($\chi^2=13.156$; $p=0.004$) [12]. Mittal et al. [18] indicated significant differences were observed in the genotype frequencies for *IL-1RN* gene polymorphisms ($\chi^2=14.778$; $p=0.039$). Chen et al. [19] observed that the distribution of *IL-1RN* polymorphism had significant difference between the control group and stone patient group ($p<0.01$) [19]. In addition to the ethnic difference, the patients of above studies are adults while our study focused

Table 3 Carriage rate of *IL-1RN VNTR* gene, *IL-1β* (−511) gene and *IL-1β* (+3954) gene in patients and controls.

Gene	Carriage	Patient, *n* (%)	Control, *n* (%)	*p*-Value	OR (95% CI)
IL-1RN VNTR	I	109 (70.8)	91 (70.0)	0.810	—
	II	42 (27.3)	34 (26.2)		
	III	2 (1.3)	4 (3.1)		
	IV	1 (0.6)	1 (0.7)		
IL-1β (−511)	C	86 (50.3)	74 (50.3)	0.965	1.00
	T	85 (49.7)	73 (49.7)		1.01 (0.65–1.57)
IL-1β (+3954)	E_1	112 (71.8)	93 (76.2)	0.404	1.00
	E_2	44 (28.2)	29 (23.8)		0.79 (0.46–1.37)

IL-1RN VNTR, interleukin-1 receptor antagonist variable number of tandem repeat; IL-1β, interleukin-1beta; OR, odds ratio; CI, confidence interval. I, *IL-1RN* VNTR gene wild type. II, *IL-1RN* VNTR gene mutant type. III and IV, *IL-1RN* VNTR gene heterozygote. C, *IL-1β* (−511) gene wild type. T, *IL-1β* (−511) gene mutant type. E_1, *IL-1β* (+3954) gene wild type. E_2, *IL-1β* (+3954) gene mutant type. —, not available.

on children. Adults may have more complicated factors to cause urolithiasis compared with children. In Indian children, Mittal et al. [18] reported that the distribution of *IL-1RN* polymorphism on comparing two groups demonstrated significant difference ($\chi^2=7.58$; $p=0.023$). Pediatric nephrolithiasis patients had a significantly higher frequency of the allele I (410 bp) in the *IL-1RN*. The carriage rate of this allele was also more frequent in patients as compared to controls (40.0% *vs.* 26.7%) [19,24]. This result found in Indian children is different from our finding in Uighur children. Studies have reported that haplotypes rather than alleles are superior for risk estimation in multifactorial diseases. The importance of IL-1 haplotypes may reflect differential regulation of IL-1RN expression by IL-1β [25]. For the haplotype, our study is similar to Mittal et al.'s [24] finding on Indian children. When haplotypes were constructed combining the three sites of IL-1, all the eight possible haplotypes were common in patients. Especially, the haplotype T-E_1-I was associated with a slightly higher risk of the disease, but there is no statistical difference. Furthermore, IL-1RA is a hormone receptor coupled with a G protein, a single change in the intracellular domain that may occasionally cause disease. It is considered possible that allelic variants of the *IL-1RN* may have an influence over the variation of intracellular signal pathways and may be associated with some diseases [26]. Major cause of pediatric nephrolithiasis is renal tubular acidosis, which is mainly caused by inflammation. Allele I (410 bp) is associated with low production of IL-1RN, a natural antagonist of IL-1β and its low production may not be able to counteract the inflammation [24]. Although several studies have shown the possible correlation between urolithiasis and *IL-1RN* polymorphism among different population, apparently, they are not consistent with our study targeting Uighur urolithiasis children. The related mechanism mentioned before could not explain the occurrence of Uighur urolithiasis children. Many external factors such as environmental factors, life style, feeding style and dietary may be need to interact with genetic polymorphism to explore the reasons of high incidence of urolithiasis in Uighur children in China.

Obviously, the patients of all the studies are totally different. They have different races, living and eating habits, geographical and financial conditions. These extrinsic factors, to some extent, could influence the results. *IL-1RN* polymorphism may not affect pediatric urolithiasis in Uighur population according to our study but further investigation should be performed. Since the similarity of gene exists in the same race, more comparisons need to be done among different ethnic groups. Kashi, as a multi-ethnic settlement, has many ethnic groups. Other ethnic groups in this area can be involved in to compare with Uighur children in order to find the difference of *IL-1RN* polymorphism. For the *IL-1β* (−511) gene polymorphism, only Mittal et al. [24] found significant difference between urolithiasis patients and the control group in Indian adults. The rest of studies including ours, reported no statistically significant findings. For the *IL-1β* (+3954) gene polymorphism, we have consistent results with other studies. All of these negative results might present that the three sites have no effects on pediatric urolithiasis in Uighur population from different perspectives.

5. Conclusions

Urolithiasis in children has a geographical variability both in terms of incidence and etiology. We did not detect an association between the polymorphisms of *IL-1RN* VNTR, *IL-1β* (−511), and *IL-1β* (+3954) and urolithiasis in Uyghur children, which still need studies with larger sample size to confirm, as well as in other ethnic groups. The phenomenon we found in Uighur children may hint that some of cytokine genes have indecisive effect on the specific people, which should be considered by doing more comparisons among different ethnic children. In addition, we should also focus on the interaction between the gene and environment to have a better understanding of the reasons of high incidence of urolithiasis in Uighur children of China.

Author contributions

Study design: Kusheng Wu.
Data acquisition: Jiefeng Xiao, Shukai Zheng, Zhaolong Qiu.
Data analysis: Jiefeng Xiao, Shukai Zheng, Zhaolong Qiu.
Drafting of manuscript: Jiefeng Xiao.
Critical revision of the manuscript: Kusheng Wu.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajur.2021.04.009.

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