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Distribution of *APOE* gene polymorphism in the Chinese Uyghur children & its association with urolithiasis

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***Background & objectives*: This study was to survey the apolipoprotein E (*APOE*) gene polymorphism distribution among Chinese Uyghur children and to explore the relationship between *APOE* gene polymorphism and the occurrence of urolithiasis.**

***Methods*: A total of 144 Uyghur children with urolithiasis and 274 without the history of urolithiasis were enrolled in this study. Venous blood samples were collected from all participants, and *APOE* genotyping, derived from rs429358 and rs7412, was performed using Sanger sequencing.**

***Results*: Among the 418 children, the most prevalent genotype was E3/3, accounting for 71.3 per cent in the urolithiasis group and 71.4 per cent in the control group, followed by E3/4 and E2/3. Higher frequencies of the ε2 and ε4 alleles and lower frequencies of the *ε*3 allele were observed in the test group, and the unusual allele *ε*1 was also found in them. However, there were no significant differences between cases and controls at both rs429358 and rs7412 genotype and allele frequencies [odds ratio (OR)=0.98, 95% confidence interval (CI): 0.57-1.67; 0.98 (0.59-1.63); 1.43 (0.75-2.74) and 1.40 (0.74-2.62), respectively]. Likewise, none of significant differences was found between cases and controls at both APOE genotype and allele frequencies [OR=0.88, 95% CI: 0.51-1.53; 0.74 (0.33-1.64); 1.10 (0.73-1.66); 1.13 (0.76-1.67) and 1.14 (0.76-1.70), respectively].**

***Interpretation & conclusions*: The present study does not support any association between *APOE* genotyping and urolithiasis in Uyghur children.**

Key words Apolipoprotein E - gene polymorphism - urolithiasis - Uyghur children

There are various reports of a globally increasing incidence of urolithiasis, of which 2-3 per cent is represented by the paediatric population[1,2]. There is considerable cause for public concern because urolithiasis is associated with to significant pain, decreased renal function and increased costs[3,4]. Clinical data have documented the youngest patient with urolithiasis as a three-month-old Uygur child from Xinjiang, China[5]. Literature suggests that the younger the patient is, the higher the recurrence rate will be, with about 50 per cent of children presenting with recurrent symptomatic urolithiasis within three

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years after the initial treatment. Once urolithiasis has recurred, the recurrence frequency is suggestively increased significantly and recurrence interval is also shortened[6-8].

Urolithiasis is a complex polygenic, multifactorial disease where genetic and environmental factors together significantly contribute to the pathophysiology and pathogenesis of urolithiasis[9]. Epidemiological investigations have suggested environmental factors, such as climate, lifestyle and dietary habits, influence the development of urolithiasis[10-12]. Nevertheless, with the development of molecular biology and the in-depth analysis of urinary calculi, genetic factors have started receiving increased attention over the year, also the study of single-nucleotide polymorphisms (SNPs) has emerged as a tool to identify the association of genes with various diseases and to prevent diseases[13,14]. Previously published studies have shown the association of some genes such as those encoding lipoproteins, [paraoxonase-1 and apolipoprotein E (APOE)], with urolithiasis[15,16].

The *APOE* gene is polymorphic, resulting from two amino acid substitutions, cysteine (Cys) and arginine (Arg) at positions 112 (rs429358) and 158 (rs7412)[17,18]. The *APOE* gene is located on the chromosome 19q13 and possesses three alleles in general, designated ε2, ε3 and, ε4, which vary in the presence of either C or T nucleotides at positions 112 and 158[17-20]. Of these, the allele ε3 is the most prevalent and the rare allele ε1 presents only in special populations[21]. The three alleles encode three common isoforms, namely E2 (Cys112/Cys158), E3 (Cys112/Arg158) and E4 (Arg112/Arg158), giving rise to six different genotypes three homozygous (E2/E2, E3/E3 and E4/E4) and three heterozygous (E2/E3, E2/E4 and E3/E4)[17,18,20,22]. Although there are only one or two amino acid changes, *APOE* variants transform *APOE* structure and function[23]. Likewise, the distribution of *APOE* polymorphism varies in different populations and ethnics groups[20,22,23].

A study on Uyghur adults demonstrated that urolithiasis was associated with *APOE* gene polymorphism, and the E3/4 genotype and ε4 allele might be susceptibility factors for this condition[16]. However, little is known about the relationship between the *APOE* gene polymorphism and Uyghur children urolithiasis and, the main influencing factors for children urolithiasis may differ from those of adult urolithiasis. Thus, there is a clear need for studies exploring the distribution of *APOE* gene polymorphism in the Chinese Uyghur children and risk of urolithiasis in them.

Material & Methods

Subjects: A total of 418 Uyghur children under 14 yr of age from the First People's Hospital of Kashi, Xinjiang Uyghur Autonomous Region, People's Republic of China, were included in the study between April 2016 and February 2017, comprising 144 cases (109 males) and 274 controls (175 males).

Inclusion criteria for the cases were defined as follows: (*i*) Uyghur children under the age of 14 yr; (*ii*) outpatients and inpatients with a diagnosis of urolithiasis; (*iii*) urolithiasis was confirmed by ultrasound, abdominal X-ray or computerized tomography (CT); (*iv*) no other chronic renal failure or chronic urinary infection diseases and; (*v*) no other metabolic diseases. Inclusion criteria for the controls were defined as follows: (*i*) Uyghur children under the age of 14 yr; (*ii*) coming from the same region as the cases; (*iii*) no history of urolithiasis; (*iv*) no chronic renal failure or chronic urinary tract infections; and (*v*) no other metabolic diseases. The study was approved by the Ethics Committee of Shantou University Medical College Shantou, China, and written informed consent was obtained from the children's parents or guardians of the children.

Parameters studied: Basic demographic information (age, gender, height and weight) and some clinical mineral profiles [serum potassium (K), sodium (Na), chlorine (Cl), calcium (Ca), phosphate (P) and magnesium (Mg)] were collected from medical records.

DNA isolation and apolipoprotein E genotyping: Two millilitres of venous blood was collected in anticoagulant tubes, and DNA was extracted using blood genomic DNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China) as per manufacturer's protocol. The purity and concentration of extracted DNA were determined by NanoDrop 2000 Ultramicro Spectrophotometer (Thermo Scientific, USA), and the quality was identified by agarose gel electrophoresis.

Polymerase chain reaction (PCR) amplification for *APOE* genotyping was performed using an optimization method of a high guanine–cytosine (GC) fragment[24,25]. The primers were synthesized BGI Tech Co. Ltd. Shenzhen, China, and referring to the related literature, their sequences were as follows[25] forward:

5'-AACAACTGACCCCGGTGGCG-3' and reverse: 5'-ATGGCGCTGAGGCCGCGCTC-3'. A total of 25 µL PCR mixture contained 12.5 uL 2×premix Taq™ (Takara Bio, Inc., Tokyo, Japan), 0.5 µL forward and reverse primer each (10 µmol/µL), 1 µL DNA template, 2.5 µL dimethyl sulfoxide and 8.5 µL ddH_2O. PCR amplification was performed in a thermal cycler (Bio-Rad, California, USA), and cycling parameters were set as follows: 95°C denaturation for five minutes, and first five cycles (95°C for one minute and 72°C for three minutes), and then 30 cycles (95°C for one minute, 65°C for one minute, and 72°C for one minute), and finally extension at 72°C for 10 min. After amplification, the PCR products of 292 bp were identified by 2.0 per cent agarose gel electrophoresis, and *APOE* genotyping was performed (BGI Tech, Shenzhen, China) by Sanger sequencing.

Statistical analysis: The genotype distributions of two SNPs (rs429358 and rs7412) were analyzed using Chromas and MegAlign, and *APOE* genotypes were assigned derived from the genotype distribution of the two SNPs[20,26,27] (Table I). The APOE alleles were defined as follows[20,26,27]: ε1=rs429358(C)+rs7412(T), ε2 =rs429358(T) +rs7412(T), ε3=rs429358(T)+rs7412(C) and ε4=rs429358(C)+rs7412(C). Hardy–Weinberg equilibrium (HWE) of two SNPs (rs429358 and rs7412) was evaluated using online encyclopaedia for Genetic Epidemiology studies (OEGE) software (*(http://www.oege.org/software/hardy-weinberg.html)*[28].

Continuous data were presented as mean ± standard deviations and categorical data as numbers (percentages). Normal distribution of data was assessed by Kolmogorov–Smirnov and Shapiro–Wilk tests. For comparing basic demographic characteristics and mineral profiles, an independent sample t test was used for continuous variables normally distributed, and otherwise, Mann–Whitney U test was used. Chi-square test was used for categorical variables. All genotype and allele frequency distributions were analyzed with Chi-square test, and unconditional binary logistic regression was used for odds ratios (ORs) and confidence intervals (CIs). All analyses were conducted using IBM SPSS 22.0 software (IBM, Armonk, NK, USA), and two-sided $P<0.05$ was defined as statistical significance.

Table I. Apolipoprotein E (*APOE*) genotype distribution based on two single-nucleotide polymorphisms (rs429358 and rs7412)

rs429358	rs7412	*APOE*
C/C	C/C	E4/4
T/C	C/C	E3/4
T/T	C/C	E3/3
T/T	T/C	E2/3
T/C	T/C	E2/4
T/T	T/T	E2/2

Results

Characteristics of the participants: The summary statistics of demographic characteristics and mineral profiles between the urolithiasis group (n=144) and the control group (n=274) are described in Table II. There was no statistical difference in the mean age between the urolithiasis and the control group; the mean body mass index was also not different between these two groups, but there were more boys in the urolithiasis group. As for mineral profiles, serum Cl and Ca levels were higher for children with urolithiasis, but serum Mg level was lower for children with urolithiasis. Levels for serum K, Na and P were not found to be different in either group.

Apolipoprotein E (APOE) rs429358 and rs7412 gene polymorphisms: The genotyping results of *APOE* rs429358 and rs7412 are shown in Figure, and the summary statistics of two-SNP genotype and allele frequency distributions are presented in Table III. The genotype frequency distributions of the two SNPs were consistent with the Hardy-Weinberg equilibrium (HWE) in both the case and control groups. Only within the control group, five children were detected with rs429358 CC genotype and one child with the rs7412 TT genotype in this study. Thus, during subsequent analysis, individuals with the rs429358 CC genotype or the rs7412 TT genotype were excluded. However, there was neither any statistical difference between cases and controls with rs429358 and rs7412 genotypes, nor in allele frequencies [OR=0.98, 95% CI: 0.57-1.67; 0.98 (0.59-1.63); 1.43 (0.75-2.74) and 1.40 (0.74-2.62), respectively].

APOE gene polymorphism: *APOE* polymorphism derived from rs429358 and rs7412 is presented in Table IV. In theory, six genotypes can be generated based on both SNPs (rs429358 and rs7412)[17,18,20,22]. However, three common genotypes in the children, namely E3/4, E3/3 and E2/3, were identified in our study. Only in the control group, five, six and one

Table II. Comparison of demographic characteristics and mineral profiles between the case and control groups (SD)

Parameters	Case group (n=144)	Control group (n=274)	*P*
Age (yr)	4.48±3.19	4.55±3.57	0.661
Gender, n (%)			
Male	109 (75.7)	175 (63.9)	0.014
Female	35 (24.3)	99 (36.1)	
BMI, kg/m^2	17.54±6.84	18.56±8.16	0.515
Serum K, mmol/L	4.43±0.60	4.21±0.77	0.725
Serum Na, mmol/L	138.49±3.86	137.59±5.55	0.130
Serum Cl, mmol/L	105.08±4.48	103.67±6.42	0.031
Serum Ca, mmol/L	2.32±0.13	2.29±0.20	0.020
Serum P, mmol/L	1.70±0.36	1.68±0.43	0.645
Serum Mg, mmol/L	0.86±0.10	0.90±0.15	0.003

Data are presented as mean±SD unless indicated. SD, standard deviation; BMI, body mass index; K, potassium; Na, sodium; Cl, chlorine; Ca, calcium; P, phosphate; Mg, magnesium

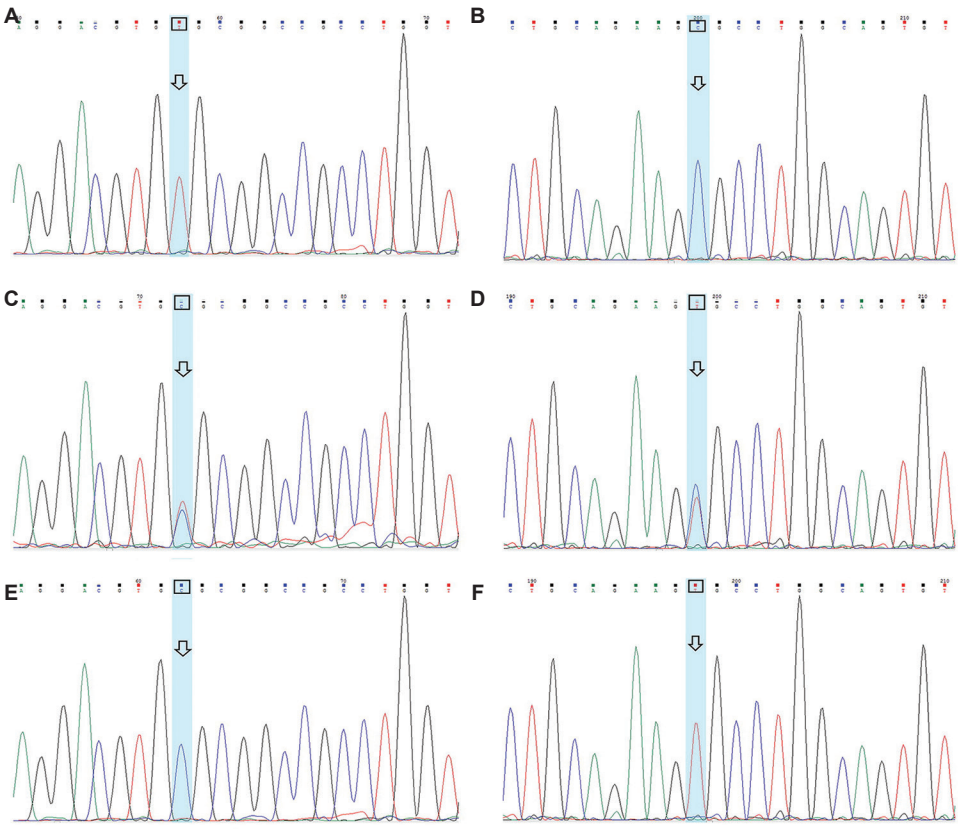

Figure. The sequencing map of *APOE* rs429358 and rs7412, the arrows indicate the mutation s namely, **A**: rs429358 TT; **B**: rs7412 CC; **C**: rs429358 TC; **D**: rs7412 CT; **E**: rs429358 CC; **F**: rs7412 TT.

children were identified with E4/4, E2/4 and E2/2 genotypes, respectively. Therefore, individuals with the E4/4, E2/4 or E2/2 genotype were removed from the subsequent analysis. For most human populations, three common *APOE* alleles (ε2, ε3 and ε4) can be detected[17-20]. In the present study, the unusual allele ε1 was also detected. As shown in Table IV, both cases and controls showed the highest frequencies for E3/3 genotype (71.3 and 71.4%) and ε3 allele (46.4 and 46.0%). However, none of the significant

Table III. Associations of rs429358 and rs7412 gene polymorphisms with children urolithiasis

APOE gene polymorphisms	Patient group n (%)	Control group n (%)	OR (95%CI)
rs429358[a]			
TT	111 (81.6)	218 (82.0)	0.98 (0.57-1.67)
TC	25 (18.4)	48 (18.0)	1.00
T	247 (90.8)	484 (91.0)	0.98 (0.59-1.63)
C	25 (9.2)	48 (9.0)	1.00
rs7412[b]			
CC	122 (89.7)	232 (85.9)	1.43 (0.75-2.74)
CT	14 (10.3)	38 (14.1)	1.00
C	258 (94.9)	502 (93.0)	1.40 (0.74-2.62)
T	14 (5.1)	38 (7.0)	1.00

[a]Individuals (n=5) with *CC* genotype in the control group were excluded from the analysis; [b]Individual (n=1) with *TT* genotype in the control group was excluded from the analysis. OR, odds ratio; CI, confidence interval

Table IV. Association of apolipoprotein *E* gene polymorphism with children urolithiasis

APOE gene polymorphisms	Patient group (n=136) n (%)	Control group (n=259) n (%)	OR (95% CI)
Genotype			
E3/4 (*ε4* carrier[a])	25 (18.4)	42 (16.2)	1.00
E3/3	97 (71.3)	185 (71.4)	0.88 (0.51-1.53)
E2/3 (*ε2* carrier[a])	14 (10.3)	32 (12.4)	0.74 (0.33-1.64)
Allele			
ε1	39 (3.6)	86 (4.0)	1.00
ε2	261 (24.0)	522 (24.3)	1.10 (0.73-1.66)
ε3	505 (46.4)	986 (46.0)	1.13 (0.76-1.67)
ε4	283 (26.0)	550 (25.7)	1.14 (0.76-1.70)

[a]Individuals (n=5, 6, 1) with the *E4/4*, *E2/4* and *E2/2* genotype in the control group were removed from our analysis, respectively. OR, odds ratio; CI, confidence interval

differences was found between cases and controls at both *APOE* genotype and allele frequencies [OR=0.88, 95% CI: 0.51-1.53; 0.74 (0.33-1.64); 1.10 (0.73-1.66); 1.13 (0.76-1.67) and 1.14 (0.76-1.70), respectively].

Discussion

In the present study, for *APOE* genotype distribution derived from both SNPs (rs429358 and rs7412), six genotypes, namely E3/4, E3/3, E2/3, E4/4, E2/4 and E2/2 were found. E3/3 was observed as the predominant genotype, found in 71.3 per cent in the urolithiasis group and 71.4 per cent in the control group. In case of *APOE* allele distribution, three common alleles, namely *ε*2, *ε*3 and *ε*4, and the rare allele *ε*1 were observed.

Among all participants, the most prevalent genotype was E3/3, followed by E3/4 and E2/3, which is in accordance with the results of Han Chinese population living in other areas[22,29]. Furthermore, a comparison of *APOE* genotype frequencies in nine populations (Tyrolean, Sudanese, Indian, Chinese, Japanese, Hungarian, Icelandic, Finnish and Malay) has suggested the major *APOE* genotypes as E2/3 (frequency range: 7.0 to 16.9%), E3/3 (frequency range: 39.8 to 72.1%) and E3/4 (frequency range: 11.3 to 35.9%)[30]. The results of the present study were within this frequency range. However, previous published studies on Uygur men in Xinjiang, China, have shown that in addition to E3/3, E3/4, and E3/3 genotypes, E4/4 genotypes also have a high frequency[23,31]. Our results showed some differences, possibly due to a different population set and a different history of disease.

Interestingly, compared with the studies of Han Chinese populations in the north and southwest[22,29],

higher frequencies of the ε2 and ε4 alleles and lower frequencies of the ε3 allele were found in the present study. Archaeological and DNA sequencing researches have suggested that Uyghur was a unique ethnic group, originating from Europe, currently living in the Tarim Basin of Xinjiang region, China, and ethnically assimilated and integrated with the Han population for a long time[32,33]. The present finding can provide additional evidence that Uyghurs are unique. Another study of correlation between *APOE* polymorphisms and urolithiasis in the Uyghur population has also reported similar finding[16]. Besides, the literatures suggests that ε2 and ε4 alleles were more frequent in Afro-Caribbeans and African-Americans compared with Caucasians and Asians.

The present study also found an unusual allele ε1. The rare ε1, inherited as a recessive, is mainly found in Caucasian populations[21,25]. Based on archaeological finds, the Uyghur population suggestively belonged to the Caucasian race[32,33]. Previous studies demonstrated ε3 was designated as wild type and its mutants were designated ε1 (Gly_{127} →Asp, Arg_{158}→Cys), ε2 (Arg_{158}→Cys) and ε4 (Cys_{112}→Arg)[25,36]. Further functional research found ε4 allele was associated with elevated low-density lipoprotein (LDL) level while ε1 allele was associated with decreased LDL level similar to the ε2 allele[21]. The functional analysis of these alleles indicated that there was no difference between the alleles ε1 and ε2 (Arg_{158}→Cys), and the glycine/aspartic acid interchange at residue 127 may not be of functional significance[21,36]. Therefore, particular attention should be paid to the pathological impact of the ε1 allele and the individuals who carry the ε2 and ε4 mutants at higher frequency than the wild allele ε3.

However, the present study does not support any association between *APOE* genotyping and urolithiasis in Uyghur children. In contrast, a similar study of Uyghur adults suggested urolithiasis was related to *APOE* gene polymorphism and the E3/4 genotype, and ε4 allele might be potential risk factors for urolithiasis[16]. This inconsistency in findings may be due to the difference in sample selection. Besides, some mineral profiles (such as serum Cl, Ca and Mg) were found to differ in both the groups, which indicate that occurrence of urolithiasis may be associated with mineral metabolism in the body.

There are, however, some limitations in the present study. Firstly, the blood lipid profiles of Uyghur children were not measured, so further analysis of the association of blood lipid levels with *APOE* gene polymorphism in order to perform a deep exploration of the risk factors of urolithiasis susceptibility could not be achieved. Second, compared with the number of adult urolithiasis cases, the sample size in the present study was relatively small.

Overall, the APOE genotype and allele frequency distributions in Uyghur children are unique compared with Han Chinese and Caucasian populations. However, the present study does not support any association between *APOE* genotypes and urolithiasis in Uyghur children.

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***Conflicts of Interest*:** None.

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