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Association of Polymorphism at 3′-UTR of Urokinase Gene with Risk of Calcium Kidney Stones

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ASTRACT

Urokinase might play a role in the formation of kidney stones. This study was done to determine the association between +4065 T/C polymorphism at the 3′-untranslated region of urokinase gene and calcium kidney stones. This Case-Control study was carried out on 70 cases with a history of calcium kidney stones and 70 controls from the Baqiyatallah hospital of Tehran in 2013. The study of polymorphism was performed by Allele-specific PCR method. Mutant T allele frequency was %41 in cases and %18 in controls. Hardy-Weinberg equilibrium (HWE) was tested using chi-square test. The chi-square showed that the control population is in HWE, and as expected, the patient group does not follow HWE. The odds ratio for the risk of the T allele was 1.7 (%95 confidence interval 0.2988-1.158). A significant association was found between the urokinase gene T/C polymorphism and the formation of calcium kidney stones. Also, effect modification was examined for both sexes and different age groups. According to the results, T polymorphism in the 3'-UTR of urokinase gene may increase with age.

Keywords: Single nucleotide polymorphism, 3'-Untranslated region, AS-PCR, Hardy-Weinberg equilibrium

INTRODUCTION

Urolithiasis is a universal health problem and it is the third most common urologic disease. It is more common among men [1]. The male-female sex ratio changed from 1.7:1 to 1.3:1, which is due to lifestyle related risk factors. In Iran, the prevalence was estimated as 5.7%, slightly more frequent in males (6.1%) than females (5.3%) whereas the annual incidence of urolithiasis in 2005 was 145.1 [2,3].

Calcium oxalate and calcium phosphate stones are the most prevalent type of urolithiasis, occurring in 70-80% of kidney stone patients [4]. It is a heterogeneous disease involving both genetic and environmental factors. This polygene and complex disorder results from the interaction between environmental influences and hormonal and genetic factors [5]. Rendina *et al.* reported that genetic factors are important determinants for kidney stone [6].

Single nucleotides of polymorphisms (SNPs) are responsible for inter-individual variability in mediating genetic predisposition to the complex disease [7]. The urokinase gene is located on chromosome 10q24 [8]. Three polymorphic sites have been commonly reported; a T/C substitution in exon 6, a C/T change in intron 7 [9] and a T/C polymorphism at the +4065 nucleotide in the 3'-untranslated region (3'-UTR) [1]. Of the three sites, the 3'-UTR T/C polymorphic site is the most widely studied, and it has been associated with many complex diseases [10-15]. T/C polymorphism at the +4065 nucleotide was previously reported (STS Accession number: G27040) by Tripputi *et al.* [9]. Tsai *et al.* reported that the 'T' allele in 3'-UTR increases the risk of calcium stone disease [16]. Ozturk *et al.* demonstrated that 3'-UTR T/C polymorphism play a role in childhood recurrence urolithiasis [17]. Li *et al.* proved that T/C polymorphism in 3'-UTR of urokinase gene increases the risk of calcium stone disease [1]. By contrast, two studies did not find any association between 3'-UTR T/C polymorphism and nephrolithiasis [18,19].

For the first time we investigated whether this polymorphism is associated with calcium stone disease in Iran. The study of polymorphism was performed by Allele-specific PCR (AS-PCR) method. Allelic frequencies in a normal population were compared with those in patients

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with a recurrent calcium stone disease. Also, HWE was tested using the chi-square test. Control genotypes should be in HWE when they are selected in large size and they have random mating. If the SNP has a true genetic effect that is not controlled by a multiplicative model, the cases will not be in HWE [20]. Moreover, the effect modification was examined for both sexes and different age groups .When we analyze the association of an exposure with disease incidence, an effect modifier is a variable over which the effect of exposure on disease risks various. We can test whether ORs are significantly different using the chi-square test for Heterogeneity.

MATERIALS AND METHODS

Case and Control Groups' Selection

This case-control study was carried out at the genetic research laboratory of Baqiyatallah hospital in 2013. A community of 140 people, consisting of 70 patients (mean age 40.0 ± 7.0 years) with a history of calcium kidney stones and 70 controls (mean age 40.0 ± 7.0 years) with no history of stone disease or renal calcification were selected randomly from the Baqiyatallah hospital. In collaboration with hospital urinary tract experts, patients and controls were selected from the same areas of Tehran province in terms of features such as climate and drinking water. Because 70 to 80 percent of kidney stones have calcium compounds, and studies have shown the importance of genetics in the development of this kind of stone [1,17,18], calcium stones were evaluated. People who drank little liquid (less than 1.5 liters per day) or people with a high calcium intake (greater than 1500 mg per day) or those with predisposing diseases were excluded from the population. The diagnosis of urinary tract calculus was documented by a plain X-ray film, intravenous pyelography and renal ultrasound. Patients with urinary tract infection were excluded from the study. Controls were selected from general population in terms of matching age, gender and ethnicity.

DNA Extraction

Five milliliters of blood was collected in EDTA vials from cases and controls. The genomic DNA was prepared from peripheral blood using Gene All; an extraction column kit.

Polymorphism Detection

Genotyping of urokinase gene polymorphism was done by AS-PCR. AS-PCR is an application of the polymerase chain reaction (PCR) that permits the direct detection of any known point mutation in human DNA. AS-PCR, also called "PCR Allele-Specific amplification" (PASA) or "amplification refractory mutation", allows SNP to be detected in minimally equipped laboratories. This method relies on obtaining a PCR product specific to the SNP polymorphism using AS primers that have the 3'end base complementary to the SNP site. The advantage of the basic AS-PCR is the economical and rapid detection of the amplification products on agarose gels [21.23]. Nevertheless, two reactions are needed, one for each SNP allele. In this study mutated allele was inserted at the 3' of the reverse primer. PCR of the polymorphisms was carried in a total volume of 25 ml containing 12.5 µl Master mix (Cina gene; 1.5 mM $MgCl_2$, 0.08 unit/µl Ampli Taq DNA polymerase and 0.4 mM dNTPs), genomic DNA and 0.1-1 µM primers. The primers for the urokinase gene polymorphisms were designed by the online primer3 plus software. Then primers were analyzed by oligoanalyzer software and UCSC. Primer sequences were as follows:

F: 5'-CTT TGA CTG GGA AAC TCT TC-3'

R1: 5'-GTT AAA GCT ATT GTC GTT CG-3'

R2: 5'-GGT AAA GCT ATT GTC GTT CA-3'

PCR analysis of urokinase gene polymorphism was carried out in a total volume of 25 ml. Thermal cycling parameters consisted of thirty PCR cycles of 30 s at 94 °C, 30 s at 56.7 °C and 45 s at 72 °C. The cycle program was preceded by incubation at 94 °C for 5 min and was followed by a final extension at 72 °C for 5min. The PCR products were visible in a 0.5% agarose gel, stained with ethidium bromide. For urokinase 3'-UTR polymorphism, PCR products were 443 bp.

Statistical Analysis

Statistical Analysis was performed using the computer

software SPSS (ver.16.0). Allele frequencies of patients and controls were compared using a 2x2 contingency table and Fisher's exact test. Differences were considered significant statistically at $p < 0.05$. HWE analysis was performed for two groups using the chi-square test. If the disease-causing alleles were related with the disease in population, the patient group does not follow the Hardy Weinberg equilibrium [20]. Odds ratio (OR) with 95% confidence intervals (CI) were calculated for individual alleles of the urokinase gene in association with the stone disease. Moreover, effect modification was examined for both sexes and different age groups.

RESULTS

Genotype Frequency

The results of electrophoresis of PCR products for each case were found with normal forward and reverse primers. This result also was detected for normal forward primers and mutated reverse primers for urokinase gene polymorphism (Figs. 1 and 2). According to the figures, individuals 2, 6, 8, 9 and 10 had a normal genotype, 3 and 7 had a heterozygous genotype, 1, 4 and 5 had a homozygous genotype for 3'-UTR urokinase gene.

No individual with a TT genotype was observed in the control group. Among the cases, 23 patients were found with genotype TT. The odds ratio for the risk of the "T" allele in patients with stones was 1.7. ($P < 0.001$; OR = 1.7; 95% CI = 0.2988-1.158) (Table 1).

Allele Frequency

T Allele frequency in patients and the control groups were 41% and 18%, respectively. This difference was statistically significant (Table 2).

TESTING FOR HARDY–WEINBERG EQUILIBRIUM (HWE) AND GENETIC MODELS

Control genotypes should be in HWE when they are selected in large size and they have random mating. If the SNP has a true genetic effect that is not controlled by a multiplicative model, the cases will not be in HWE [23].

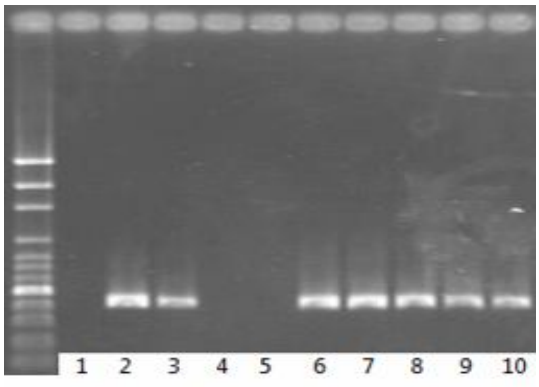

Fig. 1. Electrophoresis of PCR products with normal forward and reverse primers for 10 patients.

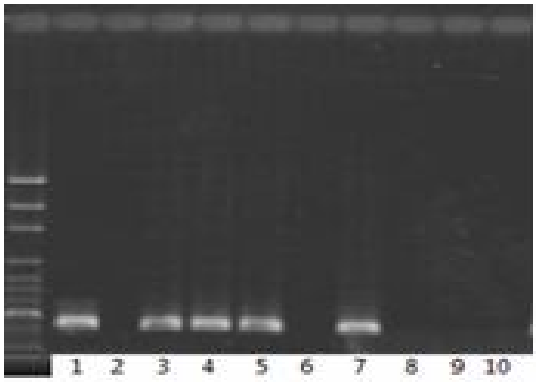

Fig. 2. Electrophoresis of PCR products with normal forward primer and mutated reverse primer with T/C polymorphism on the 3' first nucleotide for 10 patients.

$$\chi^2 = n\sum_{i=1}^{k}\frac{(p_{ii}-p_{i2})^2}{p_{i2}} + n\sum_{i=1}^{k-1}\sum_{j=i+1}^{k}\frac{(p_{ij}-2p_ip_j)^2}{2p_ip_j} = \sum_{i=1}^{k}\frac{(n_{ii}-np_{i2})^2}{np_{i2}} + \sum_{i=1}^{k-1}\sum_{j=i+1}^{k}\frac{(n_{ij}-2np_ip_j)^2}{2np_ip_j}$$

$$df = k\,(k\text{-}1)/2$$

$\chi^2 = 0.01$(Control group), $\chi^2 = 37.22$ (Case group)

In this study, the chi-square test showed that the control population is in HWE, and as expected, the patient group

Table 1. Genotype Frequency of 3'-UTR Urokinase Gene Polymorphism in Patients and Control Groups

Groups		TT	C/T	CC	p-value
Case	M	11 (31%)	7 (20%)	17 (49%)	0/016
	F	12 (34%)	4 (12%)	19 (54%)	0/005
	T	23 (33%)	11 (16%)	36 (51%)	0/0001
Control	M	1 (3%)	14 (40%)	20 (57%)	
	F	1 (3%)	9 (26%)	25 (71%)	
	T	2 (3%)	23 (33%)	45 (64%)	
F: Female		M: Male			T: Total

Table 2. The Allele Frequency of 3'UTR T/C Polymorphism in Patient and Control Groups

Groups		T allele frequency	C allele frequency	P-value
Case	M	29 (41%)	41 (59%)	0/0002
	F	28 (40%)	42 (60%)	
	T	57 (41%)	83 (59%)	
Control	M	16 (23%)	54 (77%)	
	F	11 (16%)	59 (84%)	
	T	27 (19%)	113 (81%)	

does not follow HWE. The sample was then examined by different genetic models, and it was revealed that the sample is significant at 95% CI for a recessive genetic model. This means that two copies of the defective gene is necessary for disease development.

Effect Modification

Effect modification, also known as interaction, means that the degree of association between an exposure and an outcome differs in different subgroups of the population. We analyzed T allele distribution among both sexes (Tables 3 and 4).

$$Q = \sum \frac{ad}{n} = [24\times1\ /70] + [23\times1\ /\ 70] = 0.67$$

$$R = \sum \frac{cb}{n} = [34\times11 / 70] + [34\times12 / 70] = 11.17$$

$$OR = \frac{Q}{R} = 0.05$$

$$v = \frac{ghef}{n^2 (n-1)} = [58\times12\times35\times35 / 70^2 \times (70 - 1)] + [57\times13\times35\times35 / 70^2 \times (70 - 1)] = 5.2$$

$$\chi2 = \sum \frac{(ad - OR\times cb)^2}{OR\times V\times n^2}, \text{df} = \text{c-1}$$

Male group $\chi2 = 0.04$ Female group $\chi2 = 0.01$
Total $\chi2 = 0.05$
Since there are two groups, there is one degree of freedom (2-1 = 1). The chi-square statistic is 0.05 at the $p < 0.05$ level with 1 degree of freedom Therefore there is no heterogeneity in odds ratios across the two groups (Male and Female groups).

Table 3. Genotype Distribution between Males

Male	CG CG CG TA	TA TA	Total
Case	24	11	35
Control	34	1	35
Total	58	12	70

Table 4. Genotype Distribution between Females

Female	CG CG CG TA	TA TA	Total
Case	23	12	35
Control	34	1	35
Total	57	13	70

DISCUSSIONS

In this study, the effect of 3'-UTR urokinase gene polymorphism was studied in association with kidney stones in Tehran province using AS-PCR. We found that the frequency of T allele (disease-causing allele) on the nucleotide +4065 of 3'-UTR urokinase gene was more in patients with calcium kidney stones compared with control subjects. There is evidence of a possible association between T/C urokinase gene polymorphism and formation of kidney stones in Tehran population.

Since 95% CI = 0.2988-1.158A includes 1, it is not statistically significant. Small association does not mean that there is not a causal effect, though the larger the association, the more likely that it is causal.

Some of the studies have introduced this polymorphism as a risk factor for kidney stones [16,17]; while others have rejected the relationship between this polymorphism and kidney stones [18,19]. This difference may be due to differences in the criteria for selection of patients and controls.

Li *et al*. demonstrated that the presence of the T/C polymorphism in the 3'-UTR of urokinase gene increases the risk of calcium kidney stones [1]. This polymorphism is associated with increased kidney stone especially in the Asian regions. This study was performed by RFLP-PCR (Restriction Fragment Length Polymorphism). Although AS-PCR is one rapid and inexpensive method, RFLP-PCR method is a good alternative to prevent the possible mismatches. Li Considered that the dysfunction of urokinase gene and formation of calcium kidney stones are results of this polymorphism in the 3'-UTR region of urokinase gene. This is because the 3-'UTR sequence plays an important role in regulating gene expression. Regulatory regions within the 3'-untranslated region can influence polyadenylation, translation efficiency, localization, and stability of the mRNA [1,2]. The 3'-UTR contains both binding sites for regulatory proteins as well as micro RNAs. One feasible model is that 3′-UTR contains binding sites for regulatory proteins. Sequence alterations in the 3′-UTR may

change the binding patterns of regulatory proteins and then the stability of mRNA [1].

In another study by Kim *et al*. using RFLP-PCR, no significant association was found between polymorphism in the 3'-UTR of urokinase gene and the formation of calcium oxalate stones. In this study, T allele frequency in cases was 71.3% and it was 68.9% in the control group. C allele frequency was 28.7% in patients and it was 31.1% in the control subjects. They suggested that this result is due to racial differences among different populations [18].

It should be noted that the association between a disease and genetic factors need more investigations in different populations. On the other hand, one of the important limitations of the current study was the lack of investigation on the relationship between other pathology metabolic parameters and the desired polymorphism. Clinical outcome can be thought of as a synthesis of many risk factors, with intermediate phenotypes as subtotals. Genetic factors contributing to intermediate phenotypes will generally be uncomplicated to identify because of the improved signal-to-noise ratio in the fraction of variance explained by any single factor. Therefore, it is critical to realize how the definition of a phenotype can affect the prospects of an association analysis. Studies using a single clinical endpoint are similar to attempting the near-impossible with only one chance of success, whereas studies that collect multiple phenotypes are more likely to help us to understand the contribution of genetic factors to components of the disease, regardless of whether a significant effect can be established on the clinical endpoint.

Accordingly, polymorphism in the 3'-UTR of urokinase gene should be used as a marker for identifying people who are prone to kidney stones.

CONFLICT OF INTEREST OF STATEMENT

We declare that we have no conflict of interest.

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