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# Analysis of the IL-10, IL-12, and TNF-α Gene Polymorphisms in Patients With Vesicoureteral Reflux Among the Southeast Iranian Population

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**Abstract**

**Background:** Vesicoureteral reflux (VUR) is a common childhood disorder that is characterized by the abnormal movement of urine from the bladder into the ureters or kidneys.
**Objectives:** The aim of this study was to determine whether the genetic polymorphisms of the *IL-10*, *IL-12*, and *TNF-α* genes are involved in the development of VUR.
**Patients and Methods:** The tetra amplification mutation refractory system-polymerase chain reaction (Tetra-ARMS PCR) was applied to analyze the four polymorphic sites of the *IL-10AG-1082, IL-10CA597*, *IL-12CA1188*, and *TNF308GA* genes in 124 VUR children and 110 healthy controls.
**Results:** A significant, highly increased risk of VUR disease was found for the *CA*, *AA*, and combined genotypes of *IL-10CA597* (OR = 5.2, 95% CL: 1.80 - 18.25; P = 0.0006, OR = 9.1, 95% CL: 1.11 - 122.75; P = 0.02, OR = 5.3, 95% CL: 1.82 - 18.61; P = 0.00052, respectively); the *AG*, *GG*, and *AG* + *GG* genotypes of *IL-10AG-1082* (OR = 12.8, 95% CL; 2.9 - 113.9; P = 0.00003, OR = 12.62, 95% CL: 2.93 - 114.53; P = 0.00003, respectively); and the *AA* genotype of *IL-12* (*AA*, OR = 0.19, 95% CL: 0.5 - 0.55; P = 0.0006). The frequency of the *C* allele in both *IL-10CA* and *IL-12CA* was greater in patients with VUR than in the healthy controls. No association was found between *TNF308GA* and the risk of VUR.
**Conclusions:** The results demonstrated significant associations between the *IL-10* (*AG-1089*, *IL-10CA*) and *IL-12 (AA)* gene polymorphisms and a highly increased risk of VUR.

*Keywords:* VUR, Polymorphism, *IL-10*, *IL-12*, *TNF-α*

## 1. Background

Vesicoureteral reflux (VUR) is a heterogeneous disease in which a reverse flow of urine occurs from the bladder into the ureters and kidneys. The disease is more prevalent in newborn boys than in girls (1). VUR is generally classified as either primary or secondary reflux. Primary VUR is a congenital anomaly that occurs during embryonic growth (2). The cause of secondary VUR is an increased bladder outflow obstruction and the resultant high-pressure bladder situations (3). According to an international reflux study in children, VUR can be graded as I, II, III, IV, or V (4). The true prevalence of VUR is unknown in many populations, although it has been estimated that the prevalence of VUR is 0.4% - 1.8% in healthy children and 30% in children with urinary tract infections (UTI). Additionally, it has been reported that the prevalence of VUR is significantly higher in children whose patients have the disease (5-7). Linkage analysis has revealed some chromosomal regions that may contain the genes responsible for VUR, such as chromosomes 6p21, 10q26, and 19q13. Some families with VUR have been linked to the HLA locus on chromosome 6p21 (8, 9). The pattern of transmission of VUR can be multifactorial or autosomal-dominant inheritance with variable penetrance, autosomal recessive, and X-linked disease (10, 11). The effects of cytokine variation on the development of VUR have also been detected (12). Interleukin-10 (IL-10) is a cytokine with an anti-inflammatory activity that inhibits the production and function of TNF-α, IL-1, IL-6, IL-12, and IFN-γ, which has been located on chromosome 1 at 1q31-1q32 (13). IL-10 is a stimulatory factor for mast cells, B cells, and thymocytes, and it acts on many other cell types, including monocytes/macrophages, T cells, NK cells, neutrophils, endothelial cells, and PBM. It has been shown that several diseases are associated with the polymorphism of the IL-10 promoter region, and high IL-10 production is

associated with autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (14). IL-12 is a pro-inflammatory cytokine that stimulates the production of IFN-γ. IL-12 produces dendritic cells and phagocytes in response to pathogens during infection. It is involved in the differentiation of naive T cells into Th1 cells (15). Tumor necrosis factor α (TNF-α) is a cytokine that is involved in systemic inflammation. TNF-α is produced by activated macrophages, lymphocytes, and T helper cells, and it stimulates the synthesis of other growth factors and cytokines. Its gene is located on chromosome 6p21.3 (16). TNF-α is also produced by intrinsic kidney cells. Direct cytotoxicity occurs from this cytokine to the renal cells, which leads to direct renal injury, necrotic cell death, and apoptosis. It can also cause alterations of the intra glomerular blood flow and a decrease of glomerular filtration as a result of the disequilibrium between the factors promoting vasoconstriction and vasodilatation in order to change the function of endothelial cells (17).

## 2. Objectives

The purpose of this study was to assess the role of polymorphic variants of the *IL-10AG-1082*, *IL-10CA597*, *IL-12CA1188*, and *TNF308GA* genes in the development of VUR in samples of the Iranian population.

## 3. Patients and Methods

### 3.1. Subjects and Clinical Data

The study was conducted from September 2010 to September 2011 in Zahedan, Sistan and Baluchestan province, southeast Iran. The study involved 124 VUR patients (73 females and 51 males) with different grades of progression (I = 5, II = 19, III = 40, IV = 14, and V = 6). The VUR patients had a mean age of 2.51 years (± 2.89) and a mean weight of 11.51 kg (± 6). The study also involved 110 healthy controls (66 females and 44 males) with a mean age of 2.79 years (± 5.9) and a mean weight of 11.23 kg (± 5.9) (Table 1). Informed consent was obtained from all subjects, and the study was approved by the Zahedan University of Medical Sciences ethical committee. The diagnosis of primary VUR cases who had been referred to the pediatric unit at Ali Asghar hospital (Zahedan, Iran) was made by voiding cystourethrogram (VCUG) according to the international reflux classification, and the cases were classified as grades I - V. The clinical data included patient histories from the antenatal period, physical examinations, laboratory analysis (including urinalysis, urine culture, BUN, and creatinine), and radiological investigations, including VCUG, renal ultrasonography, intravenous pyelography (IVP), renal nuclear scan (DMSA scintigraphy and diethylenetriaminepenta acetic acid (DTPA) renogram), and magnetic resonance imaging (MRI). The exclusion criterion for the study was reflux secondary to another condition (e.g., neurogenic bladder, non-urological disorders, syndromes, or chromosomal disorders).

### 3.2. DNA Extraction and PCR

Blood samples were collected from all participants via venipuncture and then kept in EDTA-coated tubes to be used for DNA extraction at the time of clinical examination. The control samples were obtained from individuals without any history of renal disease or inflammation disturbance. Single nucleotide polymorphisms (SNPs) located in and around the *IL-10*, *IL-12*, and *TNF-α* genes were detected by PCR using the tetra amplification refractory mutation system (ARMS) designed for the detection of various alleles and genotypes. Two outer and two inner primers (forward and reverse) were used to detect variations in the *IL-10*, *IL-12*, and *TNF-α* genes, as previously reported (18).

### 3.3. Statistical Analyses

SPSS version 10.0 (SPSS, Chicago, IL) and epical info version 7 were used for all statistical analyses. The associations of polymorphisms in the *IL-10AG-1082*, *IL-10CA597*, *IL-12CA1188*, and *TNF308GA* genes with the risk of developing VUR were identified by estimating the odds ratios (OR) and 95% confidence intervals (95% CL) using epical info version 7. A significant P value was held to be less than 0.05. Pearson's $\chi^2$ test was used to analyze the categorical variables.

## 4. Results

As presented in Table 2, the *CA*, *AA*, and combined *CA* + *AA* genotypes of *IL-10 (CA)* significantly increased the risk of VUR (OR = 5.2, 95% CL: 1.80 - 18.25; P = 0.0006; OR = 5.3, 95% CL: 1.82 - 18.61; P = 0.00052, respectively). All of the genotype forms (*AG*, *GG*, and *AG* + *GG*) of *IL-10AG* highly increased the risk of the disease (OR = 12.6, 95% CL: 2.9 - 113.9; P = 0.00003; OR = 15, 95% CL: 0.6 - 117.31; P = 0.06; OR = 12.62, 95% CL: 2.93 - 114.53; P = 0.00003, respectively).

The allele frequency for the C of the *IL-10CA597* and *IL-12CA1188* genes showed statistically significant differences between the VUR cases and the controls (P = 0.001 and P = 0.025, respectively) (Table 3).

As shown in Table 4, the gene-gene interaction analysis of *IL-12CA* and *IL-10AG* showed a significant correlation between the *CC/AG* (OR = 9.43, 95% CL: 1.15 - 438.76; P = 0.022) and *CA/AG* (OR = 8.47, 95% CL: 0.89 - 417.57; P = 0.034) genotypes and the risk of developing VUR. This analysis also showed significance for *IL-12CA/IL-10CA* in terms of the *CC/AA* and *CA/AA* genotypes (P = 0.000015 and P = 0.024, respectively). In addition, it was observed that the gene-gene interaction of *IL-10AG* and *IL-10CA* (*AG/CA* and *AG/AA*) was significantly associated with an increased risk of VUR (P = 0.0020 and P = 0.03, respectively).

**Table 1.** Demographic Characteristics of the Patients With VUR and the Healthy Controls[a]

| Variation | VUR Cases | Controls |
|---|---|---|
| **Sex** | | |
| Male | 51 | 44 |
| Female | 73 | 66 |
| **Weight, kg** | 11.5 ± 6 | 11.23 ± 5.9 |
| **Age, y** | 2.51 ± 2.89 | 2.79 ± 5.9 |
| **Grade** | | |
| I | 5 | |
| II | 19 | |
| III | 40 | |
| IV | 14 | |
| V | 6 | |

[a]Values are expressed as No. or mean ± SD.

**Table 2.** Number and Frequency of the *IL10AG-1082*, *IL10CA597*, *IL12CA1188*, and *TNF308GA* Genotypes in VUR Patients and Healthy Controls[a]

| SNPs | VUR Cases, n = 124 | Controls, n = 110 | OR | CI | P Value |
|---|---|---|---|---|---|
| ***IL10CA597*** | | | | | |
| *CC* | 5 (4.03) | 20 (18.18) | Reference | | |
| *CA* | 114 (91.93) | 88 (80) | 5.2 | 1.80 - 18.25 | 0.0006 |
| *AA* | 5 (4.03) | 2 (1.82) | 9.1 | 1.11 - 122.75 | 0.02 |
| *CA + AA* | 119 (95.97) | 90 (81.82) | 5.3 | 1.82 - 18.61 | 0.00052 |
| ***IL10AG-1082*** | | | | | |
| *AA* | 2 (1.61) | 19 (17.27) | Reference | | |
| *AG* | 120 (96.77) | 90 (81.82) | 12.6 | 2.9 - 113.9 | 0.00003 |
| *GG* | 2 (1.61) | 1 (0.91) | 15 | 0.6 - 1176.31 | 0.06 |
| *AG + GG* | 122 (98.39) | 91 (82.73) | 12.62 | *AG + GG* | 0.00003 |
| ***IL12CA1188*** | | | | | |
| *CC* | 65 (52.42) | 53 (48.18) | Reference | | |
| *CA* | 54 (43.55) | 35 (31.82) | 1.26 | 0.69 - 2.29 | 0.48 |
| *CA + AA* | 59 (47.58) | 57 (51.82) | 0.84 | 0.49 - 1.46 | 0.6 |
| ***TNF308GA*** | | | | | |
| *GG* | 115 (92.74) | 105 (95.45) | Reference | | |
| *GA* | 8 (6.45) | 5 (4.55) | 1.46 | 0.41 - 5.8 | 0.58 |
| *GA + AA* | 9 (7.26) | 5 (4.55) | 1.64 | 0.48 - 6.44 | 0.42 |

[a]Values are expressed as No. (%).

**Table 3.** Allele Frequency (%) Among Individual VUR Cases and Healthy Controls[a]

| Genes | VUR Cases | Controls | P Value |
|---|---|---|---|
| ***IL10CA597*** | | | |
| *C* | 124 (50) | 128 (58.18) | |
| *A* | 124 (50) | 92 (41.82) | 0.001 |
| ***IL10AG-1082*** | | | |
| *A* | 124 (50) | 128 (58.18) | |
| *G* | 124 (50) | 91 (41.36) | 0.09 |
| ***IL12CA*** | | | |
| *C* | 184 (74.19) | 141 (64.09) | |
| *A* | 64 (25.81) | 79 (35.91) | 0.025 |
| ***TNF308GA*** | | | |
| *G* | 238 (95.97) | 215 (97.73) | |
| A | 10 (4.03) | 5 (2.27) | 0.306 |

[a]Values are expressed as No. (%).

**Table 4.** Gene Combinations of the *IL12CA*, *IL10AG*, *Il-10AG*, *Il-10 AC*, and *TNF308GA* Genotypes in the VUR Cases and Healthy Controls

| Genes | VUR Cases | Controls | OR | CI | P Value |
|---|---|---|---|---|---|
| ***IL12CA/IL10AG*** | | | | | |
| *CC/AA* | 1 | 7 | Reference | | |
| *CC/AG* | 63 | 46 | 9.43 | 1.15 - 438.76 | 0.022 |
| *CA/AA* | 1 | 5 | Reference | | |
| *CA/AG* | 52 | 30 | 8.47 | 0.89 - 417.57 | 0.034 |
| ***IL12CA/IL10CA*** | | | | | |
| *CC/CC* | 2 | 7 | Reference | | |
| *CC/CA* | 6 | 45 | 0.47 | 0.064 - 5.71 | 0.6 |
| *CC/AA* | 30 | 1 | 78.55 | 6.43 - 4870.85 | 0.000015 |
| *CA/CC* | 3 | 8 | Reference | | |
| *CA/CA* | 49 | 27 | 4.75 | 1.03 - 30.09 | 0.000015 |
| ***IL12CA/TNF308GA*** | | | | | |
| ***IL10AG/IL10CA*** | | | | | |
| *AG/CC* | 5 | 16 | Reference | | |
| *AG/CA* | 111 | 73 | 4.82 | 1.60 - 17.60 | 0.0020 |
| *AG/AA* | 4 | 1 | 11.35 | 0.87 - 668.31 | 0.034 |
| ***IL10AG/TNF308GA*** | | | | | |
| *AG/GG* | 113 | 86 | Reference | | |
| *AG/GA* | 7 | 4 | 1.33 | 0.33 - 6.40 | 0.76 |
| ***IL10CA/TNF308GA*** | | | | | |
| *CA/GG* | 105 | 85 | Reference | | |
| *CA/GA* | 8 | 3 | 2.15 | 0.50 - 12.97 | 0.35 |

## 5. Discussion

Anti-inflammatory and pro-inflammatory cytokines play an important role in the regulation of the immune system in response to various microorganisms (19). It has been confirmed that *IL-10* genes increase the risk of UTIs. TNF-α, IL-1, and IL-6 synthesized by renal cells performing in autocrine and paracrine styles may provoke a variety of effects on different renal structures, and they play a major role in the expansion and progression of some renal disorders. The renal effects of inflammatory cytokines are linked to the expression of different molecules, alteration of the extracellular matrix, intra glomerular hemodynamic abnormalities, glomerular basement membranes, necrosis, apoptosis, oxidative stress, and endothelial permeability (17). In this study, a significant association was found between the genotypic frequency of *IL-10CA* and *IL-10AG* and an increased risk of VUR. Fidan et al. (20) reported that the *IL-10* gene polymorphisms are linked to the development of reflux nephropathy in patients with primary VUR, and it was also noted that the *GCC/GCC* and *ACC/ACC* haplotypes for the three *IL-10* promoter loci were associated with an increased risk of renal scarring. Finally, they pointed out that certain genotypes of cytokines' gene polymorphisms may be associated with increased or decreased susceptibility to reflux nephropathy, with varying trends seen for patients with high-grade and low-grade VUR. Manchanda et al. (21, 22) demonstrated that the *AA* genotype of *IL-10-1082 G/A* and both variants of *TNF-α* ( − 308 and + 488) have a significant association with an increased susceptibility to end-stage renal disease. Bantis et al. (23, 24) showed that the *IL-10* gene's *G-1082A* polymorphism plays an important role in the development of nephropathy and focal segmental glomerulosclerosis (FSGS). Some studies have reported that the *TNF-α AA* genotype is not associated with reflux nephropathy and renal scarring, although the *TNF-α-308A* allele could be connected to a higher susceptibility to VUR (25, 26). In addition, Bienias et al. (27) demonstrated that there were no significant differences between children with unilateral and bilateral vesicoureteral reflux in terms of the serum level of TNF-α. Shu et al. (28) showed the effect of *TNF-α* polymorphism on the susceptibility to IgA nephropathy. Pro-inflammatory cytokines such as TNF-α initiate the parenchymal damage that leads to renal scarring. Other studies have shown increased levels of this cytokine (TNF-α) in embryonic dysplastic kidneys (29).

### 5.1. Conclusion

The results of the present study demonstrated significant associations between *IL-10 (AG-1089*, *IL-10CA)* and *IL-12*

*(AA)* gene polymorphism and a highly increased risk of VUR. Ultimately, more studies involving a large sample size in various genetic populations are recommended for the verification of the present data and in order to elucidate the results.

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