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Original Article

Relationship between the Polymorphism I/D of Gene ACE and Polymorphism – 308A/G gene TNF-α with Vesicoureteral reflux (VUR) in the Children of Kerman Province

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Abstract

The present study was done between children of Kerman Province with the aims of considering the relationship ofACE and TNF-α genes with aVesicoureteral reflux disease which had been the most common hereditary diseases of the genitourinary tract and is caused to increase the possibility of making to urinary tract Infection at infant children. In this study, polymorphism I/D gene ACE analyzed through PCR and polymorphism -308 A/G gene TNF-α through PCR-RFLP. In this study, 100 children were evaluated in two groups of people with vesicoureteral refluxand control children. Studies showed that 84 percent of control children and 44 percent of sick children had G allele and genotype GG. Also, 56 percent of sick children and 16 percent of control children in the genomic position of –308 A/G gene TNF-α had A allele and genotype AA. Statistical tests showed that there is a significant difference between the frequency of alleles A and G and also genotype frequencies (AA, GG) between the population of sick and control children in the possibility level of 1 percent and also there is a correlation between polymorphism gene TNF-α intheposition of-308 A/G and the possibility of Vesicoureteral reflux. Foundation of this study showed that allele D at 60 and 77 percent of sick and control children in the gene ACE. It is that 40 percent of sick children and 23 percent of control children had allele I. also, studying on the genotype frequencies of gene ACE showed that 38 percent of sick children and 76 percent of control children was the carrier of genotype DD and genotype II was seen in 18 percent of sick children and 22 percent of healthy children and 44 percent of sick children and just 2 percent of control children had genotype ID. Statistical evaluations showed in general that there is a relationship between the genotype frequencies and the possibility of Vesicoureteral reflux in studied population for polymorphism I/D gene ACE, but this difference was not between homozygote genotypes (II, DD) and the factor of difference has been heterozygote genotypes (ID). According to these results, polymorphisms I/D gene ACE and -308 A/G gene TNF-α is used as a marker to recognize children who have more talent to affect Vesicoureteral reflux.

Key words: Vesicoureteral reflux, Polymorphism I/D gene ACE, Polymorphism -308 A/G gene TNF-α

INTRODUCTION

Vesicoureteral reflux means abnormal return of urine from urinary bladder to ureter and pelvis and it was one of the most common hereditary diseases of the genitourinary tract that is caused to increase the possibility of making to urinary tract Infection at infant children and may observed in two forms like primary and secondary (1, 2 and 16). Primitive reflux which is common among girls is omlur in almost 1 percent of children and is recognized in 30 percent of children with urinary infection. This illness is generated due to the inexpressive valve in the joint position of the ureter to the urinary bladder (15). Secondary Vesicoureteral reflux is created after inflammation process and or urologic surgery (6 and 10). Also, the secondary position can omlur by the factors of high pressure in the urinary bladder like neurogenesis urinary bladder. On the other hand, block factors are cause to facilitate urinary reflux from inexpressive sphincter and resonate the reflux degree (9 and 13). The factor of TNF-α (Tumor Necrosis Factor Alpha) is a homotrimeric protein that plays role in creating

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the inflammation. Tumor necrosis factor is creating by macrophages, but a part of this Cytokine is creating in the other cells. Codding gene TNF-α has set in the short arm chromosome number 6 (7). Observations have shown that the presence of G nucleotide in the position of -308 is caused to increase the level of the TNF-α statement and different studies have proved the relationship of this polymorphism with the possibility of affection to many illnesses (8). Gene ACE which code converter enzyme Angiotensin in human is set on chromosome number 17 and polymorphism in its intron 16 leads to create three type of genotype insertion/insertion (II), insertion/Deletion (ID) and Deletion/Deletion (DD) (5). Polymorphism I/D is known as a genetic factor in some illnesses due to being or loss a slice of 287 pairs game in intron 16 gene ACE (3). According to the importance of genes TNF-α and ACE, it is possible that polymorphisms of these genes can affect in recognizing unknown factors of Vesicoureteral reflux. So, the present study was done with the aim to determining the relationship of polymorphism I/D gene ACE and polymorphism -308A/G gene TNF-α with Vesicoureteral reflux (VUR) in children of Kerman Province. Methods of Voiding Cystourethrogram (VCUG) and Direct Radionuclide Cystography (DRC) are the most important methods of recognizing Vesicoureteral reflux (10).

Ohtomo et al (2001) study the relationship polymorphism I/D gene ACE with Vesicoureteral reflux in 78 people with this illness. The results of this study showed that existence of allele D in this gene is in relation to increasing the possibility of Vesicoureteral reflux. Yoneda et al (2001) evaluated the possibility of the relation of polymorphism gene ACE and Vesicoureteral reflux in families with this illness and healthy families who hadn't observed any history from Vesicoureteral reflux. Blood controls of 86 families are tested by the history of 2 to 3 cases with Vesicoureteral reflux. In this study, polymorphism I/D gene ACE, 407 people were considered through PCR. The frequency of genotypes DD, ID and II in healthy people was 23, 56 and 11 percent. It is that the frequency of these genotypes in ill families was 28, 47 and 25 percent. The results of this study showed that there were not any statistical relationships between polymorphism I/D gene ACE and the possibility of Vesicoureteral reflux in families with this illness and healthy families. Fidan et al (2013) evaluated the relationship of polymorphism of cytokinin genes including TNF-alpha (region -308), TGF-beta (regions +869 and +915), interleukin 6 (region -174), interleukin 10 (regions -1082, -819 and -592) and IFN-gamma (region +874) by Vesicoureteral reflux. The results of this study showed that genotype GG in the position of -308 TNF-alpha was in relation to increasing the risk of VUR.

MATERIALS AND METHODS

In this study, 50 children with Vesicoureteral reflux were studied as illness group and 50 healthy children as control group. All of experiments of this study were done at laboratory of Medical Genetics of Dr Mohammad Reza Bazrafshani (Kerman). At first, 1 ml EDTA 10% was added to 10 ml of bloodto prevent from coagulation. In the next step, 9 ml of Cell Lysis Buffer solution was discarded in a test tube to lubricate cells and 3 ml bloods were added to it. After that, samples were centrifugedat 2500 rpm speed and for 10 min. then, the supernatant was discarded and the remaining sediment in the bottom of the tube was kept. Then, it was added 5 ml of TKM1solution on the remaining amount of sediment solution to the core wall of lymphocytes was torn and release its contents to extract DNA. This tube was centrifugedat 2500 rpm speed for 10 minutes and the supernatant was discarded. In this step of test, a white sediment was remaining at the bottom of tube that amount of 1.5 ml TKM2 was added to it along with 100 µl SDS and tube was set within device bath at 30 min at 65 °C. In this step, purer DNA was obtained from test by deleting proteins from environment. After that the solution into the tube was clear, it was added 6 M to 570 µlNaCl and it was centrifugedat 2900 rpm round for 10 minutes. In the next step, first it was discarded 4 ml cold alcohol 96 percent in a sterile test tube. Upon completion of centrifugation, the supernatant was added to a tube containing 96% alcohol.The tube door was blocked with Teflon and the tube was shaken gently to clear up the DNA cloud in it. Then, it was poured 1 ml of 70% alcohol in the 1.5 ml micro tube and formed DNA transferred gently in tubes with pipette tip to micro tubes. The micro tubes were centrifuged for 5 minutes at 12000 rpm speed. After the end of the centrifuge, white sediment was seen at the end of the micro tubes. Supernatant was discarded and micro tubes with open for 10 to 15 minutes were in the area to dry. Next, it was added rate of 300 µl of TE on the deposition that is preservatives liquid of DNA and finally, micro tubes set in incubator overnight at 37 ° C to DNA deposit is dissolved completely. The next day, DNA was ready for PCR steps.TNF-α gene polymorphism was analyzed by PCR-RFLP method.In this experiment, three responses PCR (to repeat) were performed using specific primers and with a thermo cycler instruments for each sample. The features of primers used are given in Table 1 to identify the polymorphism -308 A/G TNF-α gene. All the polymorphism TNF-α was performed including primer design, primer binding temperature, $MgCl_2$ concentration and temperature cycles set of PCR on the basis of Article Savvidou et al (14). Each of PCR reaction was by final volum 12.5 µl including 300 ng ofgenomic DNA, 300 nmol from each one of the primers, 100 mM dNTPs, 1.5 mM of

Table 1: Sequence and joint temperature of used primers to recognize polymorphism genes TNF-α and ACE

Gene	Primers sequence	Joint temperature
TNF-α (–308) A/G	Sense: 5'-AGGCAATAGGTTTTGAGGGCCAT-3' Antisense: 5'-TCCTCCCTGCTCCGATTCCG-3'	57°C
ACE I/D (first step)	Sense: 5'-CTGGAGACCACTCCCATCCTTTCT-3' Antisense: 5'-GATGTGGCCATCACATTCGTCAGAT-3'	58°C
ACE I/D (second step)	Sense: 5'-TGGGACCACAGCGCCCGCCACTAC-3' Antisense: 5'-TCGCCAGCCCTCCCATGCCCATAA-3'	67°C

$MgCl_2$, 0.5 unit DNA polymeraz, 1.25 µl buffer 10x PCR which was by distilled water reached to the final volume.

Program of PCR device for gene TNF-α was including denaturation for 3 minutes at 95 °C and then 33 cycles for 30 second at 94 °C, 45 seconds at 57 °C, 45 seconds at 72 °C and finally 72 ° C for 5 minutes. PCR productions digest by limited enzyme NcoI which their length was 108 pairs. Activity temperature of enzyme NcoI was 37 °C.

It was added 10 µl amount of PCR product to 3µl of buffer enzyme and 10 units of the enzyme (approximately 0.5µl) and 17.5µl of distilled water and was placed for one day in a water bath at 37 ° C. Then it was electrophoresis by disabling the enzyme at 60 ° C, digested products on 2% agarose gel. Edging parts of 108 pair game indicated the presence of the A allele. However, the two pieces 20 and 88 pair's game indicated the presence of G allele in the genome position. It was used from PCR method to identify the polymorphism I/D ACE gene. In this experiment, it was performed three responses PCR using specific primers for each sample (to repeat). The features of primers used are given in Table 1 to identify the polymorphism I/D ACE gene. Each PCR reaction with a final volume of 12.5µlcontains 300 ng of genomic DNA, 300 nmol of each primer, 100 mM mixed dNTPs, 1.5mMof $MgCl_2$, 0.5 unit of DNA polymerase, 1.25µlbuffer 10x PCR which would be discussed with distilled water is reached to final volume. The program of PCR device for gene ACE was as denaturation for 10 min at 94 ° C and then 32 cycles of 30 seconds at 94 ° C, 50 seconds at 58 ° C, 60 seconds at 72 ° C and finally 72 ° C for 5 minutes. In the case of polymorphism ACE I/D should be noted that in the polymerase chain reaction when the allele Insertion will be behind Deletion allele, the amplification of Deletion allele is dominant (70). As a result, in order to ensure the amluracy of genotypes, DD should do the second PCR. For this purpose, in the ACE gene, some samples which had genotype DD, became PCR by primers which had brought in Table 1. In this step, the program of PCR was as denaturation for 10 minutes at 94 °C and then 30 cycles for 30 seconds at 94 °C, 30 seconds at 67 °C, and 30 seconds at 72 °C for 5 minutes. PCR production of ACE gene was electrophoresis in 2 percent agarose gel. In the first stage PCR, the presence of 490 bp band is indicating I allele and the presence of 190 bp band is indicated D allele. However, the presence of open 335 bp band was indicated I allele in the second step. After the end of electrophoresis, the agarose gel was moved to the vessel containing 0.5µg/ml ethidium bromide and was remaining in it for 15 to 30 minutes. To view the banding patterns of DNA were done shooting by gel documenter gels. Then the photos transfer to a computer connected to the device and the results were evaluated.

After data collection, the analysis was performed using SPSS v.19 software.Genotypic and allelic frequencies distribution compared was performed using the chi-square test. In all tests, the possibility level of 5% was considered as significant level.

RESULTS

Genotype children with VUR and control children assessed for TNF-α gene in District -308 is shown in Table 2. Some children with VUR and children's DNA pattern control for the genomic position are shown in Figures 1 and 2. In this study, in the presence of the A allele in a genomic region -308 TNF-α, restriction enzyme NcoI could not digest products of polymerase chain reaction and just was seen a piece of DNA, 108 bp from the electrophoretic pattern. It was while in the presence of allele G, restriction enzyme NcoI, and digested PCR DNA fragment derived from two pieces 88 and 20 bp, respectively. Based on these results in homozygous genotype GG, two bands of 88 and 20 bp was seen and a genotype homozygous AA, a band of 108 bp was seen respectively. Following in people with heterozygous AG genotype were to see three bands 20, 88 and 108 bp. Results showed that 42 (84%) healthy children had GG genotype, and only 16 percent of these children were carrying the AA genotype. However, the GG genotype patients were observed in the pediatric population in 22 patients (44%) and AA genotype in 28 patients (56 percent) of children in the population.

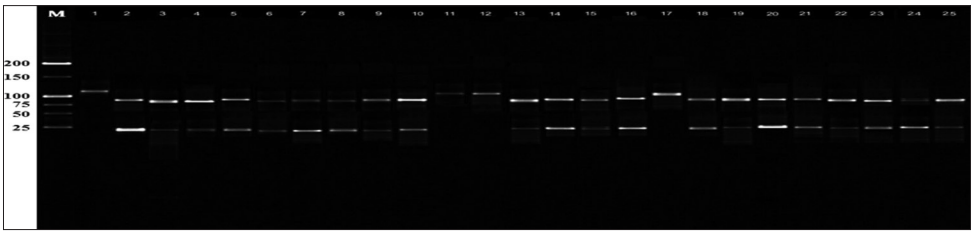

Figure 1: DNA pattern of children with VUR for TNF-α gene in zone -308 A/G

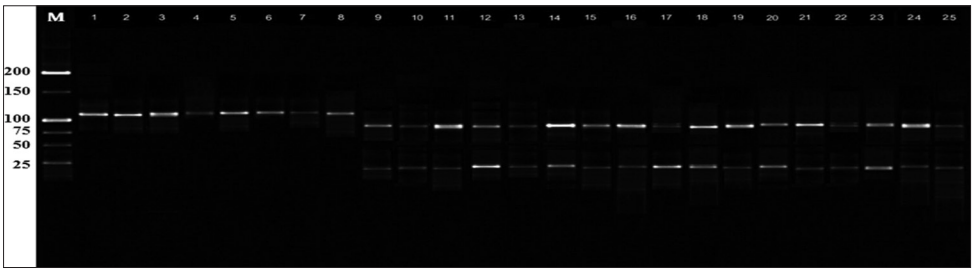

Figure 2: DNA pattern of control children for TNF-α gene in zone -308 A/G

Table 2: Genotype children with VUR and control children assessed for TNF-α gene position in zone -308 A/G

Children with VUR				Control children			
1	AA	26	GG	1	AA	26	GG
2	GG	27	AA	2	AA	27	GG
3	GG	28	AA	3	AA	28	GG
4	GG	29	AA	4	AA	29	GG
5	GG	30	AA	5	AA	30	GG
6	GG	31	AA	6	AA	31	GG
7	GG	32	AA	7	AA	32	GG
8	GG	33	AA	8	AA	33	GG
9	GG	34	AA	9	GG	34	GG
10	GG	35	AA	10	GG	35	GG
11	AA	36	AA	11	GG	36	GG
12	AA	37	AA	12	GG	37	GG
13	GG	38	AA	13	GG	38	GG
14	GG	39	AA	14	GG	39	GG
15	GG	40	AA	15	GG	40	GG
16	GG	41	AA	16	GG	41	GG
17	AA	42	AA	17	GG	42	GG
18	GG	43	AA	18	GG	43	GG
19	GG	44	AA	19	GG	44	GG
20	GG	45	AA	20	GG	45	GG
21	GG	46	AA	21	GG	46	GG
22	GG	47	AA	22	GG	47	GG
23	GG	48	AA	23	GG	48	GG
24	GG	49	AA	24	GG	49	GG
25	GG	50	AA	25	GG	50	GG

TNF-α gene in DNA pattern in area -308 A/G GG genotype was characterized with two bands of 88 and 20 bp genotypes AA, with a band of 108 bp and AG genotypes with three bands 20, 88 and 108 bp the game.

On the other hand, 56 percent of children with VUR were in TNF-α gene allele A -308 genomic position that the allele in a population of healthy children were seen only in 16 percent of patients. The genotypes study showed that none of the sick and healthy children tested were not heterozygous (AG) for gene loci -308 TNF-α. Results showed that 42 (84%) healthy children were with genotype GG and only 16 percent of the children carrying the genotype AA and in the pediatric population of genotype GG was observed in 22 patients (44%) and genotype AA was observed in 28 patients (56%) of children in the population.

Statistical results showed that there was a significant difference for TNF-α gene loci -308 between A and G allele frequencies in the population of children with VUR and children witnessed the 1% level. The genotype of children with VUR and investigated control children is shown in Table 3 for polymorphism I/D ACE gene. DNA pattern of some children with VUR and control children is shown in Figures 3, 4, 5 and 6 for polymorphism I/D ACE gene.

In the DNA template for the position of I/D ACE gene in the first step of PCR, the presence of a band of 490 bp is indicated I allele and the presence of 190 bp band is indicated D allele.

In the DNA template for the position of I/D ACE gene in the second stage of PCR, the presence of a band of 335 bp was indicated I allele.

In the DNA template for the position of I/D ACE gene in the first stage of PCR, the presence of a band of 490 bp was indicated I allele and the presence of a band of 190 bp was indicated D allele.

In the DNA template for the position of I/D ACE gene in the second stage of PCR, the presence of a band of 335 bp was indicated I allele.

The results showed that allele D respectively was observed in 60 and 77 percent of pediatric patients and control population. This is while 40 percent of patients and 23% of healthy children had I alleles. Statistical tests showed that there was no communication between patients and control population allele frequency in the population and risk of VUR at the 5% level of possibility. But calculated number was 16/3 in chi-square that the result of this test showed that the amount was significant at the level of 7%. In other words, in the probability of 93%, there is a significant relationship between the frequencies of alleles of polymorphism (I/D) ACE gene and risk of VUR. The frequency of genotypes showed that 19 patients (38%) of pediatric patients tested, 38 patients (76%) of the control population was carrying the DD genotype and genotype II was seen in polymorphism I/D gene ACE and 18 percent of children the patient in 22 percent of healthy children. But it was important to note that 44 percent (22 people) of the population of children with VUR have had ID genotype and Genotype frequency of heterozygotes was only 2% in the pediatric population control children and just one healthy child had this genotype.

Table 3: Genotype of children with VUR and investigated control children for the position of I/D ACE gene

Children with VUR				Control children			
1	DD	26	DD	1	DD	26	DD
2	ID	27	DD	2	DD	27	DD
3	ID	28	ID	3	DD	28	DD
4	II	29	DD	4	DD	29	DD
5	ID	30	DD	5	II	30	DD
6	II	31	ID	6	II	31	DD
7	ID	32	II	7	II	32	II
8	ID	33	ID	8	ID	33	DD
9	DD	34	ID	9	II	34	DD
10	ID	35	DD	10	DD	35	DD
11	ID	36	ID	11	DD	36	DD
12	ID	37	ID	12	DD	37	DD
13	DD	38	ID	13	DD	38	DD
14	DD	39	ID	14	DD	39	DD
15	ID	40	II	15	DD	40	DD
16	DD	41	II	16	II	41	DD
17	ID	42	DD	17	II	42	II
18	ID	43	ID	18	DD	43	DD
19	II	44	DD	19	II	44	DD
20	ID	45	DD	20	DD	45	DD
21	ID	46	DD	21	DD	46	DD
22	DD	47	II	22	DD	47	II
23	II	48	II	23	DD	48	II
24	DD	49	DD	24	DD	49	DD
25	DD	50	DD	25	DD	50	DD

The results of different combinations showed that there was a difference between the combination of DD, ID, II and combination of DD, II + ID at the 1% level. However, in the compounds II, DD and II, DD + ID genotype frequencies there were no statistical difference between the two populations. In general, it can be said the statistical evaluation showed that there was a correlation in the population studied between genotype frequencies and the possibility of VUR, but this difference was not between homozygous genotype (II, DD) and the factor of genotype differences were being heterozygotes (ID).

DISCUSSION

It was cleared by studying on genotype frequencies that there weren't a heterozygote person (AG) for genomic

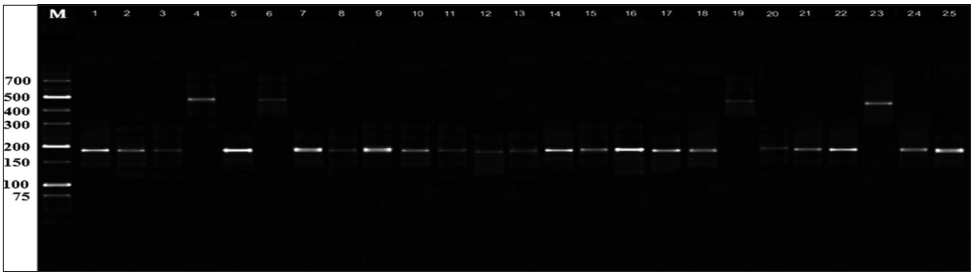

Figure 3: DNA pattern of children with VUR for the position of I/D ACE gene in the first stage of PCR

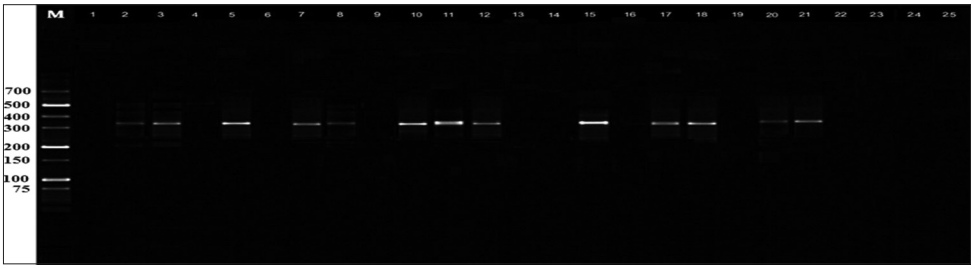

Figure 4: DNA pattern of children with VUR for the position of I/D ACE gene in the second stage of PCR

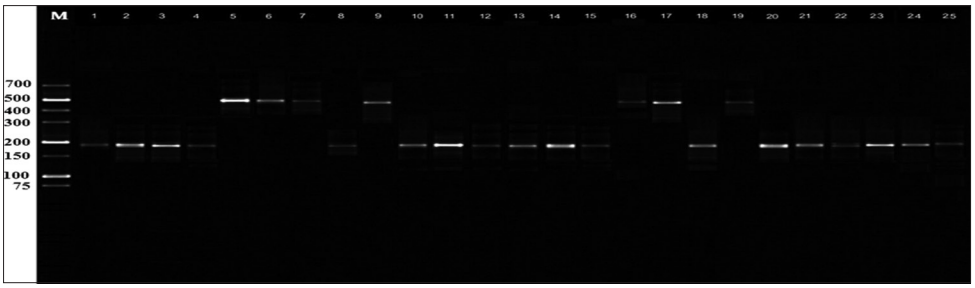

Figure 5: DNA pattern of some of the control children to the position of I/D ACE gene in the first stage of PCR

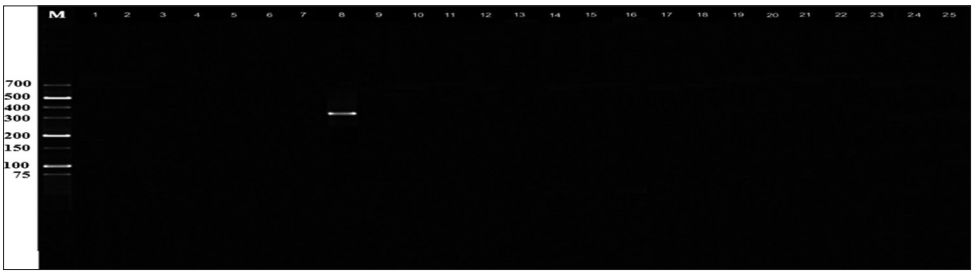

Figure 6: DNA pattern of some of the control children to the position of I/D ACE gene in the second stage of PCR

position -308 A/G gene TNF-α in the studied population of the patient and healthy children. The results showed that the frequency of allele A and G are not the same in place of genomic -308 A/G TNF-α gene, so that most of the patients were carrying allele A and allele G were seen less among patients population. The survey also showed that in a population of children with VUR were 28 patients with genotype AA. However, this genotype was observed only in 8 out of control children. In contrast, 42 of the 50 people were control population having GG genotype that this genotype was seen at 22 patients' children. Statistical tests showed that there was a significant difference between genotype frequencies for these loci in the population of children with VUR and control children. These results showed that children in the population of the province is a strong correlation between gene polymorphisms of TNF-α in zone -308 A/G with VUR disease. As a result, this region of the genome can be used as a marker (marker) suitable for identifying children who have more talent to the disease VUR. To explain the physiological role of polymorphisms Area -308 A/G in TNF-α gene can be argued that the polymorphism by the impact on the expression of this gene induces different immune responses in different people and this susceptibility has effects on vesicoureteral reflux disease. But studies on the role of TNF-α gene polymorphism in zone -308 A/G with vesicoureteral reflux and other diseases susceptibility to reflect the results have been inconsistent.Fidan and colleagues in 2013, was evaluated the relationship between polymorphism of cytokine including TNF-α (Area -308 A/G), TGF-beta1 (869+ and 915+ areas), interleukin-6 (District 174), interleukin-10 (in 1082 -, 819- and 592-) and IFN -gamma (District +874) and the incidence of VUR. They also reported similar results about the association between TNF-α gene in zone -308 A/G the risk of VUR. Liu et al. have provided conflicting results (2003) in gene polymorphism of TNF-α (areas of T/C 1031-, C/A 863-, C/T 857-, -308 A/G and G/A 238) with disease VUR in children Taiwan. Their findings showed that the frequencies of alleles were different in the areas of T/C 1031- and C/A 863- among the population of children with VUR and control children and as a result, polymorphism of stated genomic regions is in relation with VUR. The results of the present study showed that it see no difference of gene ACE of D and I allele in two populations of children with VUR and control children at the 5% level, there was relationship between the frequencies of allele D and I of this gene in the level possibility of 7 percent and the possibility of VUR. But, the study involves genotypic frequencies had more facts. The test results of genotypic frequencies showed that if we consider only homozygous individuals (DD and II) to determine the relationship between polymorphism I/D polymorphism and ACE at risk of VUR, this test shows that there is no relationship between both of them. However, the relationship is statistically significant at the 1% level, if we insert both homozygous (DD and II) and heterozygote information (ID) into our test. According to the results of these tests indicate that heterozygous are the causative agent population difference between sick and healthy children. As mentioned earlier, 44% of the population of pediatric patients was genotype ID,however, the genotype frequency of heterozygotes was only 2 percent in the pediatric population control. In other words, children homozygous (DD and II) for ACE gene are less prone to VUR and in turn risk children heterozygous (ID) of the disease is very high. Liu et al. 2003 ACE gene

polymorphism and the risk associated with VUR evaluated in the pediatric population in Taiwan. The results showed that the ACE gene polymorphism in healthy children and patients was statistically different among the population. This result suggests that there is a relationship between polymorphism I/D ACE gene and the risk of VUR. In another study Zhou et al 2012 study of polymorphisms of I/D polymorphism and ACE disease VUR in the population of children Caucasus, Turkey and Central Asia showed that it was not observed the relationship between polymorphism I/D ACE gene and the VUR disease in a population of Caucasian and East Asian. However, in the pediatric population of Turkey children, allele D, and DD genotype was associated with increased risk of VUR. By comparing the results of this study with other researchers' foundations, it can be said that we cannot deny or amlept the relationship of polymorphism I/D gene ACE with VUR. Probably, this relationship with alleles' frequencies is in relation with different societies. Also, there is a possible that the base genetic with UVR is not dependent on polymorphism I/D gene ACE and a set of different genes with complex contrast effects is responsible for this disease.

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