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ORIGINAL ARTICLE

INVESTIGATION OF TLR4 POLYMORPHISM IN CHILDREN WITH VESICOURETERAL REFLUX AND RENAL SCARRING

Sav NM[1*], Eroz R[2], Kalay Duran N[3], Kilicaslan O[4], Erisen Karaca S[5]

[*]**Corresponding Author:** Nadide Melike SAV, Address: Duzce Universitesi Araştırma Uygulama Hastanesi, Pediatrik Nefroloji B.D, Merkez, DUZCE, TURKEY; Phone: +905378683281; Fax:+903805421390; e-mail address: savmelike@gmail.com

ABSTRACT

Vesicoureteral reflux (VUR) is an important factor in the etiology of recurrent urinary tract infections (UTIs). Permanent kidney damage may develop in children with high-grade VUR in the long term. This damage may progress with the development of scar tissue in some patients. The *TLR4* gene is an important resistance mechanism, especially against UTIs. *TL*R4 gene polymorphism is associated with recurrent UTIs and kidney scar development in the long term. This study aimed to examine the relationship between scar development and *TLR4* gene polymorphism in children with VUR. This cross-sectional study included 49 patients with recurrent UTIs and primary vesicoureteral reflux. Patients were divided into two groups (26 patients with the scar, and 23 patients without scar) according to the presence of scar tissue. *TLR4* gene polymorphisms of the patients were evaluated by Next Generation Sequencing. The *TLR4* gene polymorphism was significantly higher in the compound heterozygous group with scarring than in the group without scarring (p=0.03). Gene polymorphisms, c.958T>C, c.942A>G, c.776A>G, c.1076C>T, c.896A<G, c.1196C>T, c.1078C>T were presented more commonly in the group with scarring. Moreover, gene polymorphisms c.942A>G and c.776A>G were defined for the first time in this study among patients with scar tissue. The higher incidence of some *TLR4* gene polymorphisms in patients with scarring suggested that these variations might cause permanent kidney damage. In addition to genetic predisposition, environmental factors such as untreated UTIs might also contribute to scar formation.

Keywords: Next Generation Sequencing; Toll-Like Receptor 4; Urinary tract infection; Vesico-ureteral reflux

INTRODUCTION

Urinary tract infection (UTI) is an important health problem that is commonly seen in children and has short and long-term complications. One of the common causes of the disease is vesicoureteral reflux. Vesicoureteral reflux (VUR) is a pathology characterized by reflux of urine accumulated in the bladder from one or both ureters to the kidney as a result of anatomical disorders [1]. Primary VUR occurs due to poor development and dysfunction of the congenital ureterovesical junction whereas secondary VUR occurs due to increased intravesical pressure [2].

Primary VUR is seen in 1-2% of the pediatric population; however, this rate rises to 30-40% in the presence of urinary tract infections, and recurrent UTI is an important cause of renal parenchymal damage [3]. Bacterial infection in the renal parenchyma causes an acute inflammatory reaction, accelerating scar formation and progression to chronic kidney disease (CKD) [4].

Many studies reported that resistance to bacterial UTIs is controlled by some genes. Among these genes, toll-like receptors (TLR) are transmembrane proteins involved in the innate immune response. Single gene defects or variations in genes encoding TLR, chemokines, and chemokine receptors alter the susceptibility of the host to urinary pathogen invasion [5]. *TLR4* is the first toll-like receptor identified in humans and is expressed in monocytes and dendritic cells. *TLR4* recognizes bacterial

[1] Department of Pediatric Nephrology, Duzce University, Duzce, Turkey
[2] Department of Medical Genetics, Aksaray University, Aksaray, Turkey
[3] Department of Pediatrics, Duzce Atatürk Devlet Hastanesi, Duzce, Turkey
[4] Department of Pediatric Infection, Prof. Dr. Cemil Tascioglu City Hospital, Istanbul, Turkey
[5] Department of Pediatric Endocrinology, S.B. Keçiören Eğitim ve Araştırma Hastanesi, Ankara, Turkey

lipopolysaccharides and contributes to host defense against Gram-negative bacteria [6].

Different polymorphisms in the *TLR4* gene cause UTI facilitates the emergence of the disease, and may pave the way for CKD in the long term. Identification of these polymorphisms will enable accurate genetic counselling and rapid screening of individuals with risk factors. This study aimed at analyzing *TLR4* gene polymorphisms in pediatric patients with recurrent UTI and VUR using Next Generation Sequencing (NGS).

MATERIALS AND METHOD

This cross-sectional study was carried out with 49 patients who were followed up due to primary vesicoureteral reflux at Duzce University, Faculty of Medicine, Department of Pediatric Nephrology, Duzce, Turkey.

Patients under 18 years of age with VUR and recurrent UTIs were included in the study. Oral and written informed consent was obtained from all individual participants and their families included in the study. Those who did not give consent and those who had additional renal or other system anomalies and patients with stage 5 CKD were excluded. The study protocol was approved by the Institutional Ethics Committee of Duzce University School of Medicine (Ethics No: 2019/285). The study was conducted by the ethical principles set forth in the Declaration of Helsinki. This project was supported by the Scientific Research Project Department of Duzce University (Grant number: 2021.04.03.1194).

Diagnosis of UTI was made based on history and exam findings and confirmed with appropriately collected urine. The presence of VUR was confirmed by voiding cystourethrography (VCUG) and the severity of VUR was graded according to the International Reflux Study in Children (IRSC) (I-V) [7]. A DMSA scan was performed 6 months after the last UTI. Patients were divided into two groups according to the presence of any kidney scars determined in the DMSA scan. Office blood pressure was measured by the auscultation method. Before starting blood pressure measurements, the patient rested in a sitting position for at least 3-5 minutes, relaxed and rested. The arm was outstretched, in line with the mid-sternum and supported. An appropriately sized cuff was wrapped around the upper arm and connected to a manometer and blood pressure was measured.

Genomic DNA was isolated from 200 µl peripheral leukocytes of the cases using DNA isolation kits (Anatolia Diagnostics and Biotechnology Products Inc., Istanbul, Turkey). Polymerase chain reaction (PCR) pools generated before the NGS reaction were purified by the NucleoFast 96 PCR (MACHEREY-NAGEL GmbH) kit. Then the quantification of the PCR products was standardized on NanoDrop 1000 (Thermo Fisher Scientific Inc.) and the *TLR4* gene was sequenced by NGS (MISEQ-Illumina). Serum and urine biochemical parameters were also recorded.

Statistical Analysis

The data were analyzed via IBM SPSS Statistics 22.0 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). The Shapiro-Wilk test was performed to examine the distribution of data. All quantitative variables were reported by mean±standard deviation (SD) and median (interquartile range: IQR), as categorical variables were summarized by frequency and %. The Mann-Whitney U test was performed to compare the patients with and without scares with respect to the quantitative variables. Pearson chi-square test, Fisher's exact test and Fisher-Freeman-Halton test were used to reveal the differences between two groups for categorical variables. A p-value≤0.05 was considered statistically significant.

RESULTS

A total of 49 individuals, 26 (53.1%) of them with kidney scars and 23 (46.9%) without scars were included in the current study. Both groups were similar in terms of age. The distribution of cases with scars was as follows: Seven (14.3%) with bilateral multiple scars, four (8.2%) with one scar on the right, eight (16.3%) with multiple scars on the right, one (2%) with multiple scars on the right and renal atrophy on the right, three (6.1%) with one scar on the left, and three (6.1%) with multiple scars on the left. The age of patients with kidney scars was found to be significantly higher than that of patients without kidney scars ($p<0.001$). Two groups were similar with respect to the gender distribution and the level of serum urea ($p=0.786$ and $p=0.667$, respectively). The levels of systolic and diastolic blood pressures and serum creatinine were significantly higher in patients with scars compared to those without any scar (for all, $p<0.001$). However, although the estimated glomerular filtration rate was lower in the group with scars, no statistically significant difference was observed between the groups ($p>0.05$), (Table 1).

The distribution of VUR severity within each group is presented in Figure 1. There was a significant difference between the two groups with respect to the severity of VUR ($p<0.001$). The patients with grade 4 VUR were significantly more frequent in the group with scars, while the patients with grade 1 VUR were significantly more frequent in the group without any scar ($p<0.05$).

Compound heterozygous variations were more common in patients with kidney scarring ($p<0.05$) (Table 2).

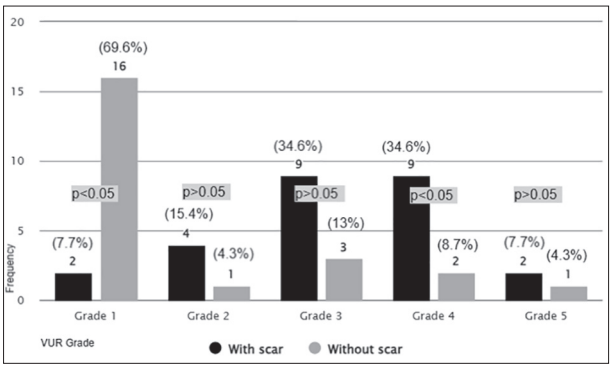

Figure 1. The distribution of VUR severity within groups (VUR: Vesicoureteral reflux)

Furthermore, although not statistically significant, the variations, heterozygous c.942A>G p.Lys314 in Ex4 rs56070048, heterozygous c.896A<G p.Asp299Gly in Ex3 rs4986790, heterozygous c.1196C>T p.Thr399Ile rs4986791 in EX3 and heterozygous c.1078C>T p.Ser360Pro in Ex3 were found more commonly in patients with scarring compared to those without scarring (p>0.05) (Table 2).

DISCUSSION

One of the mechanisms in the body that resist disease agents and develop resistance is the recognition and identification of the pathogen. TLRs play an important role in the healthy progress of this process. The coordinated activity of TLRs on the cell surface or inside the phagosomes enables the release of cytokines, recruitment of neutrophils, and release of free radicals and phagocytosis in the immune system, [8]. These responses determine the severity of the disease. Signaling disorders in the immune system resulting from polymorphisms in receptors and cytokines affect the susceptibility to infectious pathogens and the development of complications [9]. In this study, it has been shown that VUR increases the susceptibility to scar formation with the effect of *TLR4* gene variations. These variations were determined by the NGS method [10]. To our knowledge, this is the first study in which the associations of *TLR4* gene polymorphisms with UTI were investigated by NGS screening.

Pyelonephritis is an important risk factor for CKD in children. Although anatomical anomalies such as VUR are associated with recurrent UTIs in the majority of patients,

Table 1. Comparison of anthropometric characteristics and blood values of cases with and without scarring

	Renal scar (+) (n=26)	**Renal scar (-) (n= 23)**	p
Age, year (Mean±SD)	10.5±4.37	9.91±3.43	0.3
Gender (boy/girl) (n)	8/18	6/17	0,9
Systolic blood pressure, mmHg (Mean±SD)	114.84±12.05	93.91±6.74	**0.000**
Diastolic blood pressure, mmHg (Mean±SD)	68.88±8.86	54.26±6.29	**0.000**
Serum urea (mg/dL) (Mean±SD)	25.33±6.87	24.22±6.46	0.667
Serum creatinine (mg/dL) (Mean±SD)	0.56±0.19	0.37±0.13	**0.000**
e-GFR (mL/min/1.73m^2) (Mean±SD)	108.72±23.54	121.53±32.35	0.11

e-GFR: estimated glomerular filtration rate

Table 2. TLR4 gene variation distribution status in cases with and without scarring

Variation Status in *TLR4* gene	**Renal scar (+), n(%)**	**Renal scar (-), n(%)**	**p**
Heterozygous	17 (65.4)	20 (87)	>0.05
Compound heterozygous	9 (34.6)	2 (8.7)	**<0.05**
Normal	0 (0)	1 (3)	>0.05
c.958T>C p.Ser320Pro in Ex4	25 (96.2)	22 (95.7)	>0.05
c.942A>G p.Lys314 in Ex4 (rs56070048)	2 (7.7)	0 (0)	>0.05
c.776A>G p.Asp259Gly in Ex4 (rs4986790)	2 (7.7)	1 (4.3)	>0.05
c.1076C>T p.Thr359Ile in Ex4 (rs4986791)	4 (15.4)	1 (4.3)	>0.05
c.896A<G p.Asp299Gly in Ex3 (rs4986790)	2 (7.7)	0 (0)	>0.05
c.1196C>T p.Thr399Ile rs4986791 in EX3	1 (4.3)	0 (0)	>0.05
c.315C>T p.Pro105 rs5030711 in Ex4	0 (0)	1 (4.3)	>0.05
c.1078C>T p.Ser360Pro in Ex3	1 (4.3)	0 (0)	>0.05

the urinary system is usually normal both anatomically and functionally in patients with recurrent UTIs [11]. This suggests that some other factors related to host defense may also be involved in pyelonephritis and scar formation [12]. Successful defense against bacterial infection requires coordinated work of the innate and adaptive immune responses. TLRs are important for the recognition of microorganisms by the innate immune system as well as for laying a bridge between innate and adaptive immune responses [8]. These receptors act as critical sensors of microbial attack and also serve as effectors of the innate defense that ensures the elimination of pathogens [13]. It was suggested that TLR gene polymorphisms may affect an individual's ability to respond to TLR ligands, resulting in altered susceptibility to infections or inflammation [14]. This altered susceptibility may clinically emerge as decreased inflammatory response, protection against pyelonephritis, as in asymptomatic bacteriuria, or, conversely, recurrent UTIs [9, 12].

On the other hand, the effects of the *TLR4* gene on kidney damage were addressed through different mechanisms. It is thought that endogenous molecules that accumulate at non-physiological amounts or sites during cellular damage can bind to *TLR4* and trigger inflammation [15]. It was reported that there is a relationship between the *TLR4* expression and the degree of kidney damage in progressive CKD due to inflammation-induced fibrosis [6]. Cellular debris which is a product of the degradation of extracellular matrix as a result of cellular damage and increased matrix cycle and endogenous ligands such as heat shock proteins could be activated *TLR4* [16]. Endogenous *TLR4* ligands such as fibrinogens, heparan sulfate, hyaluronan, and fibronectins are overproduced during progressive renal fibrosis and tubulointerstitial damage and bind to *TLR4* on macrophages. Then, with the activation of antigen-producing cells, NF-κB dependent gene expression occurs [17]. Interstitial inflammation and fibrosis occur continuously in the process [6].

In this study, carriage of compound heterozygous variation in the *TLR4* gene was much more common in the group with kidney scarring than in the group without scarring. Some studies showed that *TLR4* gene polymorphism affects cellular immune response and cytokine production in vitro and that this paves the way for the deterioration of resistance against microorganisms. It was also noted that each polymorphism has different effects in defense against different microorganisms [18]. In a study conducted by Svanborg et al., it was found that C3H/Hej mice inoculated with virulent Escherichia coli strains could not eliminate Escherichia coli infection and developed UTI. The researchers stated that the response of these animals to bacteria was weakened and the resistance to infection decreased because of impaired neutrophil migration due to the defective *TLR4* gene [19]. In another study, it was shown that some *TLR4* gene polymorphisms increased the prevalence of Gram-negative infections and that these polymorphisms facilitated the progression to sepsis and septic shock [20]. The fact that *TLR4* polymorphism decreases resistance, especially against Gram-negative bacteria and that UTIs occur frequently due to Gram-negative microorganisms may explain the increase in the frequency of kidney scar tissue development in these patients. Scar development is more common in the presence of frequent and complicated UTIs. Therefore, the higher incidence of *TLR4* polymorphism in patients with scar tissue suggests that this group of patients more frequently have complicated UTIs.

One of the variations detected in the study group was c.942A>G. This variation was not detected in the group without scarring whereas it was determined at a rate of 6.9% in the group with scarring. Torices et al. reported that this variation can be seen at a low rate in patients with rheumatoid arthritis; however, there is no information about its clinical significance in the literature [21]. On the other hand, the relationship between kidney scar development and the same variation has not yet been defined. The absence of this variation in the control group was suggested that c.942A>G variation may be a factor that increases the susceptibility to scar development. Furthermore, another variation found at a rate of 10.3% in the patient group, but not seen in the control group, was the c.776A>G variation. It was reported that this variation reduces *TLR4* response to lipopolysaccharides and leads to less inflammatory cytokine production. As a result, it was stated that an adequate inflammatory response could not be given and that the resistance to infections decreased in the presence of this variation [22]. It could be concluded that the risk of permanent damage increases with the decreased inflammatory response and insufficient clearance of infectious agents from the environment. However, why this condition resulted in scarring in some patients has not been clarified yet. Perhaps, the infection may be difficult to eliminate and scarring may be easier due to multiple polymorphisms. However, some inflammatory cytokines are known to be associated with the development of scar tissue and resistance to infections. One of the most important cytokines is TNF-alpha. It was reported that TNF-alpha causes tissue damage. This damage could also be seen in the kidneys; however, *TLR4*-mediated blockade of TNF-alpha production is also associated with improvement in kidney functions in experimental models [23]. On the other hand, it is known that different doses of cytokines have different effects. Although the release of low-dose cytokine is an important factor in resistance to infections, high-dose releases can cause kidney damage. Therefore, the variations found in this study may trigger the development of

scar tissue by reducing the block in TNF-alpha production. However, we thought that further detailed experimental studies are required to reveal the effects of cytokines on permanent kidney damage since they are released locally as well as systemically.

Some genetic polymorphisms identified might be directly associated with the increased frequency of certain infections in the body. In particular, the decrease in the resistance of the urinary system to infections was associated with the presence of these polymorphisms. One of the reasons for the susceptibility to UTIs might be c.896A<G variation. In the study conducted by Agnese et al., it was pointed out that the risk of Gram-negative bacterial infection increased in people with *TLR4* c.896A<G polymorphism (ASP299Gly) [24]. Similarly, the c.1196C>T variation detected in this study was shown to increase susceptibility to invasive Gram-negative bacterial infections [25]. Karoly et al. reported the frequency of *TLR4* c.896A<G polymorphism as 13% with VUR and 2% without VUR in patients with UTI. The authors stated that this allele is a risk factor for recurrent UTIs independent of urinary anomalies [26]. In another study, the frequency of *TLR4* c.896A<G polymorphism was found to be 12.5% in patients with UTIs. The researchers also reported that this variation was more common in children with scar-positive pyelonephritis than in children with scar-negative pyelonephritis [12]. Another variation, which was more common in patients with scar tissue, was c.1076C>T. The rate of this variation was 13.8% in the patient group and 5% in the control group. Although some studies in the literature showed that this variation increased the susceptibility to UTI, there were no data regarding its effects on scar tissue development. In a meta-analysis conducted by Huang et al., many data were evaluated showing that the rs4986791 variation increases the susceptibility to UTI. The researchers stated that this variation is unlikely to be associated with the frequency of UTIs since the current studies have been carried out with a small number of patients [27]. Although there is no clear information about this variation, we thought that it may possibly increase the development of scar tissue in the presence of recurrent UTIs.

The c.958T>T gene encodes the T6SS protein. T6SS protein is one of the main contact-dependent delivery system proteins responsible for interactions between bacterial cells. At least one type of this protein was found in gram negative bacteria [28]. Gene mutations are also thought to play a role in the etiology of chronic damage due to infectious agents. Therefore, c.958T>T polymorphism may contribute to the development of scar tissue. However, the fact that this mutation was demonstrated in both groups in our study suggests that gram-negative infections, although frequent, do not contribute to the development of scar tissue.

c.315C>T is a polymorphism shown in some parasitic infections and some bacterial infections that might be associated with cancer [29, 30]. However, there is no data in the literature related with scar tissue development. We think that it is coincidental that this polymorphism was found in only one patient in our study.

Moalem et al. identified a mutation, suggesting that the c.1078>T polymorphism may be associated with ciliary dysgenesis [31]. Ciliary functions play an important role in the elimination of infectious agents. Disruption of these functions may trigger scar development secondary to infections. In our study, this polymorphism was detected in only one patient who developed scar tissue. Therefore, based on this result, it is difficult to claim that c.1078>T polymorphism may be associated with scar tissue.

It should not be ignored that genetic predisposition combined with environmental effects is an important factor in the development of scar tissue. It could not be stated that scar tissue develops based on genetic factors alone. This may explain the lack of scarring in some patients, even in the presence of genetic variation. Additionally, kidney scar tissue could develop without genetic variation. On the other hand, vesicoureteral reflux could also be genetically transmitted on its own. The prevalence of VUR has been reported as 27-51% in siblings, 80-100% in monozygotic twins, and 35-50% in dizygotic twins [32]. In a study, it was reported that the rate of VUR development in children whose parents had VUR was 66% [33]. Therefore, it could be said that genetic variations also contribute to scar development significantly. On the other hand, there may be an association between the increase in the degree of VUR and the development of scar tissue. It has been reported that scar tissue develops in 89% of children with high-grade VUR after an episode of pyelonephritis[4]. In our study, there was no relationship between genetic mutation and the degree of VUR.

There were some limitations in this study. Compound heterozygous mutation was found to be significantly higher in patients with scarring, but the study sample size was small. To confirm these results, the relationship between genetic and clinical findings can be clearly demonstrated by studies including a larger number of patients. Another limitation of the study is the lack of a healthy control group or a group with UTI but not VUR.

CONCLUSION

It is known that *TLR4* gene variations increase the frequency of infection and consequently the susceptibility to the development of scar tissue. This is the first study investigating *TLR4* gene variations by NGS method. Variations of c.958T>C, c.776A>G, c.1076C>T, c.896A<G,

c.1196C>T, c.315C>T, c.1078C>T were found at high rates in patients with kidney scarring. Studies with many patients are needed for revealing the effects of both genetic and environmental factors on the development of kidney scarring.

STATEMENTS AND DECLARATIONS

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Competing interests

The authors declare no competing interests and have no financial interests to declare

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