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Toll-like receptor (TLR2, TLR4) polymorphisms and their influence on the incidence of urinary tract infections in children with and without urinary tract malformation

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Abstract

Introduction: *Toll-like receptors (TLRs) contribute to the innate immune system. They are an element of non-specific immunity, which enables organisms to react quickly to foreign antigens, without being previously exposed to them. TLRs are pattern recognition receptors. TLR gene polymorphisms are widely investigated in connection with various infections. The aims of the study were: to investigate the role of TLR2 and TLR4 polymorphisms in the course of urinary tract infections (UTIs); to test for differences in distribution of these polymorphisms between children with urinary tract malformations suffering from recurrent UTI (rUTI), children with malformations but without rUTI and healthy controls; to determine whether these polymorphisms predispose to rUTI; and to analyse how polymorphisms and urine neutrophil gelatinase-associated lipocalin (NGAL) and interleukin 8 (IL-8) concentrations affect one another.*

Material and methods: *The group consisted of 133 children (1-18 years old), 68 female and 65 male. The group was divided into 4 subgroups: A (rUTI with urinary tract malformations), B (urinary tract malformations without rUTI), C (rUTI) and D (healthy controls). Polymorphisms were analysed using PCR-RFLP. IL-8 and NGAL urine concentrations were established using immunoenzymatic methods.*

Results: *TLR2 Arg753Gln and TLR4 Arg299Gly appeared significantly more often among children with rUTI. No correlation between urine IL-8 and urine NGAL and polymorphisms was found. Urine NGAL concentration was significantly higher among children with urinary tract malformations.*

Conclusions: *TLR2 Arg753Gln and TLR4 Asp299Gly may predispose to rUTI. Urine NGAL concentration suggests the presence of kidney tissue injury, of varying degrees, among children with urinary tract malformations.*

Key words: *children, polymorphism, TLR4, TLR2, urinary tract infections.*

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Introduction

Urinary tract infections (UTIs) are among the most common infections among children. They affect 2-20% of children, and 30% of children who suffer from UTI will develop another episode [1, 2]. Urinary tract infection (according to Polish guidelines) is defined as the presence of uropathogenic bacteria in the amount > 10^5 CFU/ml in midstream samples of urine [3]. UTIs can spread via the ascending route, which is the most common way. Spreading by blood-borne and lymphatic pathways is rare. *Escherichia coli* (uropathogenic *E. coli* – UPEC) is responsible for almost 90% of UTIs. Other common aetiological factors include *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Proteus mirabilis* [4]. UTIs can be defined according to their location: lower or upper urinary tract infection. From the clinical point of view, it is important to divide UTIs into complicated and uncomplicated, which indicates the presence of additional risk factors and takes

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into account the bacteriological profile of the pathogen. The bacterial flora in complicated infections is more diverse, although still about 50% of these infections are caused by *E. coli*. The problem is the antibiotic resistance of the flora. Taking into account the frequency of UTIs, recurrent infections can be defined [4]. The diagnosis of a UTI is established on the basis of a general and microbiological urine examination. Due to the complex symptomatology of UTI in young children, urinalysis should be performed in every child up to 24 months of age in the case of fever of unclear origin [5]. A quick diagnosis of UTIs is very important because post-inflammatory scarring is a direct consequence of the inflammatory process in the renal parenchyma [6]. Scars cause a loss of active kidney parenchyma and thus can lead to chronic kidney disease with all its consequences. Various mechanisms protect humans from UTIs, including the unidirectional flow of urine provided by the peristalsis of the ureters and the mechanisms that prevent the backflow of urine from the bladder. The Tamm-Horsfall protein prevents bacteria from adhering to the epithelium of the urinary tract, lactoferrin has an antibacterial effect, and lipocalin associated with neutrophil gelatinase (NGAL) prevents bacteria from accessing iron. In the male sex it is the length of the urethra and its greater distance from the anus. Situations where the risk of developing UTIs is higher include urinary tract malformations (e.g. vesicoureteral reflux), conditions with abnormal urine flow (e.g. posterior urethral valves or urolithiasis), and bladder and bowel disfunction syndrome (BBD). Certain comorbidities may contribute to urinary tract infections: immune disorders, diabetes or diseases requiring the use of immunosuppressants [7, 8]. Treatment of a UTI depends on the patient's age and the severity of the infection. The selection of an empirical drug depends on local microbiological data; the treatment is verified on the basis of the urine culture result [3, 4].

Toll-like receptors

Toll-like receptors (TLRs) are a part of innate immunity. They belong to the group of pattern recognition receptors (PRR) that recognize constant elements of the microbial structure (pathogen-associated molecular patterns – PAMP). They are found on macrophages, dendritic cells, lymphocytes and neutrophils. They are present not only on the immune cells, but also on endothelial cells, cardiomyocytes, and fibroblasts. Structures to which TLRs react include lipopolysaccharide, glycoproteins, lipoproteins, heat shock proteins, fusion proteins, and the genetic material of microorganisms [9]. The TLR activation pathway is complex. It leads to activation of the transcription factor NF-κB, which controls gene expression for proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin 2 (IL-2). TLR2 recognizes e.g. triacyl lipopeptides and diacyl lipopeptides; it can also recognize lipopolysaccharide (LPS; different from LPS recognized by TLR4). TLR4 is a receptor whose main function is to recognize LPS.

Polymorphism of TLR genes

The TLR receptors whose gene polymorphisms are most often analysed are TLR2 and TLR4. The studied polymorphism for TLR2 was *+2258 G>A* (*Arg753Gln*; *R753Q*; *rs5743708*), for TLR4: *+896 A>G* (*Asp299Gly*; *D299G*; *rs4986790*). TLR2 polymorphism (*rs5743708*) is characterized by a guanine to adenine replacement at nucleotide 2258 (*+2258 G>A*). This changes the amino acid at position 753 from arginine to glutamine (*Arg753Gln*). TLR4 polymorphism (*rs4986790*) changes adenine to guanine at nucleotide 896 (*+896 A>G*). The effect of this is the change of the amino acid from aspartic acid to glycine at position 299 (*Asp299Gly*) [10, 11].

Aims of the study: 1. Investigate the role of TLR2 and TLR4 polymorphisms in the clinical course of UTI in children. Does the prevalence of the studied *Arg753Gln* and *Asp299Gly* polymorphisms differ among children with urinary tract defect suffering from recurrent UTIs, compared to children with the defect without concomitant recurrent UTIs (rUTIs), and to healthy children? 2. Assessment of whether TLR2 and TLR4 polymorphisms may predispose to recurrent UTIs. 3. Analysis of the relationship between selected indicators of kidney damage (NGAL) and inflammation (interleukin 8 – IL-8), and TLR 2 and TLR 4 polymorphisms and the clinical course of recurrent infections.

Material and methods

The study was conducted as a cross-sectional clinical trial with the assessment of the clinical data, gene polymorphisms and selected biochemical markers in urine (NGAL and IL-8) and in the blood (creatinine). The study group consisted of 133 patients aged 1 to 18 years, including 68 girls and 65 boys. The group was divided into four subgroups (A, B, C, D); subgroup D was the control group. The selection of patients for each group took place on the basis of clinical and diagnostic criteria, using a questionnaire concerning the medical history. All legal guardians gave their consent to participate in the study. The consent of the Bioethics Committee of the Medical University of Lodz was also obtained (consent number: RNN/50/11/KB of January 18, 2011). The division of the study group is as follows: Group A – children with urinary tract malformations and recurrent UTIs. The group consisted of 31 people aged 1 to 16 years; Group B – children with urinary tract malformation, but no recurrent UTIs. The group consisted of 31 people aged 1 to 18 years; Group C – children without urinary tract malformation but with recurrent UTIs. It consisted of 38 patients aged 1 to 17 years. Group D – control group, healthy children, no defect of the urinary system and no UTIs – 33 people aged 1 to 18 years.

Among urinary tract malformations, vesicoureteral reflux (VUR) was predominant (71%); others were hypospadias, uretero-pelvic junction obstruction, neurogenic bladder, and dysplastic kidney.

Analysis of polymorphisms

Blood samples used for DNA extraction were collected in sodium citrate tubes and stored at –80°C. DNA was extracted with the GeneMATRIX Quick Blood Purification Kit (EURx Ltd, Poland) according to the manufacturer's protocol. Polymorphisms of *TLR2* and *TLR4* genes were determined using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) analysis. A C1000TM thermal cycler (Bio-Rad) was used [10, 11].

Biochemical analysis

The analysis were carried out in the laboratory of the Polish Mother's Memorial Hospital in Lodz. Creatinine concentration was measured in the blood. It was determined by the enzymatic method using Vitros biochemical analysers. The glomerular filtration rate was then calculated, based on the Schwartz formula: estimated glomerular filtration rate (eGFR) [ml/min/1.73 m^2] = 0.413 × body height [cm]/SCr [mg/dl], where SCr is the serum creatinine concentration [12]. The concentrations of NGAL and IL-8 in urine were assessed after collecting the entire study group (urine samples were centrifuged and the supernatant was frozen at –40°C until analysis) using the enzyme immunoassay method (ELISA kit, Biorbyt Ltd, United Kingdom), according to the protocol attached to the kits. The concentration of the tested samples was determined in duplicate, and the results where the coefficient of variation was > 10% were repeated. The test properties were as follows: NGAL – assay range 78-5000 pg/ml, sensitivity 40 pg/ml; IL-8 – assay range 15.6-1000 pg/ml, sensitivity 1 pg/ml.

Statistical analysis

The statistical analysis comprised basic measures of the structure description: arithmetic mean and standard deviation. The analysed parameters were tested for differences between the groups by comparing them with each other, between all test groups together and the control group and/or between each test group and the control group. These differences were checked for statistical significance. The value of $p < 0.05$ was adopted as the threshold of statistical significance. Statistical analysis was performed using the Statistica 13 (StatSoft) package.

Results

For the *Arg753Gln* (*R753Q*) polymorphism in the TLR2 receptor gene, the prevalence of RR homozygotes was observed in each group. The numbers are as follows: in the control group (group D) – 97% (33 patients); in group A – 90% (28), in group B – 84% (26), in group C – 97% (37). The differences in the occurrence of this polymorphism between the study groups and the control group were not statistically significant. In the study group, no patient was homozygous for the presence of QQ genotype. It was also analysed whether a given genotype was more common in a given sex. Regardless of the sex, the RR genotype was dominant. The prevalence of homo- and heterozygotes (RR, RQ) in the groups of children suffering from recurrent UTI was also compared. No significant correlation was found between the prevalence of a specific genotype and rUTI in children. Taking into account the presence of the urinary tract malformation accompanying rUTI, the differences in the prevalence of homo- and heterozygotes were also not statistically significant. The statistical evaluation of the advantage of the RR genotype in the group of sick children (A, B, C) compared to the control group (D) was also performed. It was found that the advantage of the RR genotype in the control group in relation to all other groups was not statistically significant if we calculate for three groups of sick children together (group D vs. groups A + B + C), but when calculating for each group separately (i.e. comparing the control group with each of the other groups), statistical significance was obtained for group D vs. C – rUTI patients without concomitant urinary tract malformation. For the TLR4 *Asp299Gly* (*D299G*) polymorphism, predominance of DD genotype (i.e. majority allele homozygosity) was observed in each group. The figures were as follows: in the control group (D) – 100% (34), in group A – 90% (28), in group B – 87% (27), in group C – 87% (33). In the study group, no patient was homozygous for the polymorphism (i.e. GG). It was analysed whether a specific genotype was more common in a given sex. However, no statistically significant correlations were obtained. No statistically significant correlation was also found between the studied genotype and recurrence of UTI in children. However, a statistically significant advantage of the DD vs. DG genotype was demonstrated among children from the control group (without infections and without urinary tract defects) compared to the group of children suffering from urinary tract infections, but without the malformation. In the group of children suffering from recurrent infections, despite the lack of the malformation, almost 87% had the DD genotype, compared to 100% in the control group. The statistical evaluation of the prevalence of DD homozygotes in the group of sick children (A, B, C) compared to the control group (D) was also performed. The advantage of the DD genotype in the control group in relation to all other groups was found to be statistically significant, both in total (group D vs. groups A + B + C) and for each group separately (i.e. group D vs. A, D vs. B, D vs. C) (Table 1).

When assessing the concentration of IL-8 in urine, the highest values were observed in group A (74.6 pg/ml).

Table 1. Predominance of DD genotype in healthy controls (group D) in comparison to group C (children with recurrent UTI without urinary tract malformation); predominance of DD genotype in healthy controls in comparison to all of the other groups (as a total and in comparison to each group separately)

		Group C		Group D		*p*
		n	***%***	***n***	***%***	
TLR4	DD	33	86.84	34	100	0.0283
	DG	5	13.60	0	0	

Group D		Group	Number of DD	Percent of DD	
Number of DD	Percent of DD				
34	100	A (n = 31)	28	90.32	0.05
		B (n = 31)	27	87.10	0.03
		C (n = 38)	33	86.84	0.03
Group D		Group A + B + C (N = 100)			
Number of DD	Percent of DD	Number of DD	Percent of DD		
34	100	88	88		0.03

However, no statistically significant difference between the groups was found. Also, no effect of recurrent UTIs on higher urinary concentrations of this pro-inflammatory cytokine was found. IL-8 levels were also compared in children with and without urinary tract malformations; there was no statistically significant difference.

Urine NGAL analysis revealed a statistically significant difference between the groups. The lowest concentration was in the control group (group D – 3475.2 pg/ml). Taking into account the presence of the malformation, a significant correlation was observed: higher NGAL concentrations are present among children with a urinary tract malformation, regardless of whether they have recurrent UTIs or not – groups: A and B (37775.23 pg/ml) compared to children without the malformation (both in the control group and in the group of children suffering from recurrent UTI but without the malformation – groups C and D; 19866.1 pg/ml). There were no statistically significant differences in the concentration of NGAL in urine depending on the presence or absence of recurrent UTI. The analysis of the relationship between eGFR and the presence of the defect showed that it is statistically significant. Children without a urinary tract malformation (groups C and D) had significantly higher eGFR (mean value 116.81 ml/min/1.73 m^2) than children with the defect – groups A and B (mean value 101.25 ml/min/1.73 m^2). Moreover, a negative correlation was observed between eGFR and NGAL in the entire study group, and the relationship was statistically significant. The correlation coefficient r was –0.22. The lower eGFR value correlated with the higher urinary NGAL concentration. In our study group there were no children with eGFR below 90 ml/min/1.73 m^2.

It was also analysed whether the above biochemical parameters – urinary concentration of IL-8, NGAL and glomerular filtration – were related to the presence of a specific TLR2, TLR4 polymorphism or a combination of both. No significant relationships were observed in any of these combinations.

Gender distribution

Among children with a urinary tract malformations and rUTIs, girls were dominant, and among children with a urinary tract malformation who did not suffer from recurrent UTIs, boys predominated. In the group of children without a malformation, but still suffering from rUTIs, girls dominated. Taking into account the age of the first UTI episode, in children with urinary tract malformation, it was statistically significantly lower than in the group of children without the defect.

It was analysed whether polymorphisms are important for the course of urinary tract infection. No statistically significant relationship was found.

Discussion

TLR gene polymorphisms are of interest to researchers in the context of individual susceptibility to infection. In this study we selected polymorphisms of TLR2 and TLR4 genes due to their importance in UTI in children with and without urinary tract malformation. It is important to look for new risk factors for UTI recurrence in children; the well-known ones do not always reflect the actual infectious status in a child. Establishing a prognosis, in a non-invasive manner, as to the frequency of recurrent infections in the future, it could allow one to properly plan the diagnostic and therapeutic process for a patient.

TLR2 polymorphism

The study by Chatzi *et al*. analysed the relationship between TLR polymorphisms (including TLR2 *Asp753Gln*) and susceptibility to infections (including UTIs). TLR2 *Asp753Gln* polymorphism was present in 2.2% of the subjects. No relationship was found between the occurrence of this polymorphism and susceptibility to infections. However, in our study a significant correlation was observed, because the TLR2 polymorphism was significantly less frequent in the group of healthy children compared to the group of children suffering from recurrent UTI (without urinary tract defects). In the whole study group, it was identified in 6.7% of children. The study groups in our study were different compared to Chatzi, i.e. children vs. adults; however, age should not influence the occurrence of polymorphism [13]. Tabel *et al*. assessed TLR2 *Arg753Gln* polymorphism in children suffering from UTI (without concomitant diseases, including urinary tract malformations). The frequency of polymorphism was higher in this group as compared to the healthy control group. This polymorphism predisposed to the appearance of more than 2 urinary tract infections, and also favoured asymptomatic UTI. In our study, a similar, significant relationship was observed. Additionally, a noticeable similarity between the studies was the predominance of girls in the group of children suffering from recurrent UTIs [14]. Kutukculer *et al*. also analysed the above TLR2 polymorphism. The results showed that the variant without polymorphism, *Arg753Arg*, is significantly less common in the study group (with recurrent febrile infections) compared to the healthy control group. Children with relapses were more likely to have the *Arg753Gln* polymorphism allele. This is in line with the results of our study [15].

TLR4 polymorphisms are generally associated with different susceptibility to infections with Gram negative bacteria and with the tendency to a septic course of these infections. Karoly *et al*. was the first to examine TLR4 *Asp299Gly* polymorphism and its impact on UTI. The study group consisted of 103 patients (81 girls and 22 boys, mean age 7.3 years) with recurrent UTIs. They were children with and without urinary tract malformation. The results of this study are consistent with the results of our study. TLR4 polymorphism was more common in the group of children with recurrent UTI without a urinary tract defect [16]. The previously cited study by Chatzi *et al*. also analysed the relationship between TLR 4 *Asp299Gly* polymorphism and susceptibility to infections (including UTIs). These polymorphisms were found to be associated with increased susceptibility to infections and longer hospitalization [13]. In our study, TLR4 *Asp299Gly* polymorphism was significantly less frequent in the group of healthy children, compared to children suffering from rUTI. Hussein *et al*. draw attention to a significantly higher risk of UTI with renal parenchyma involvement in patients with TLR4 *Asp299Gly* polymorphism. 380 paediatric patients were analysed; 98 had pyelonephritis and 282 had lower urinary tract infections. The third group was a group of healthy children. *Asp299Gly* polymorphism was significantly more frequent in the group with pyelonephritis compared to the group of healthy children. However, this was not associated with an increased incidence of post-inflammatory scars in the cited study. In our study, a similar conclusion was drawn, because the *Asp299Asp* variant was significantly more frequent among healthy children who did not have UTI recurrence. As for the course of infections (febrile or non-febrile), we found no significant differences [17]. The work of Karananou *et al*. investigated the *Asp299Gly* polymorphism in a group of 150 children [18], of whom 109 (mean age 6.65 years) were healthy controls, and the remaining 51 were children with a history of at least one UTI episode. In the studied group, UTI was more common in girls, and in girls also more often than in boys, it took the form of infection in the upper urinary tract. *E. coli* was responsible for all cases of UTI. Interestingly, among the patients, 13 out of 51 had abnormalities in the ultrasound of the urinary system (it was not specified of what kind). *Asp299Gly* polymorphism was found in 27.5% of children in the study group compared to 10.1% in the control group; this difference was significant. The results of this study are consistent with the results of our study. The work of Yin *et al*. also found higher frequency of the *Asp299Gly* polymorphism in the UTI group compared to the healthy control group (which is also consistent with the results of our study) [19]. The study by Ertan *et al*. analysed the *Asp299Gly* polymorphism among 30 children with recurrent UTIs and 30 healthy children. The frequency of this polymorphism did not differ between groups [20]. In the study of Akil *et al*. 112 patients with a first-time UTI episode (mean age 8.1 years) and 93 healthy children (mean age 9.2 years) were examined. There was higher frequency of *Asp299Gly* among patients with pyelonephritis as compared to a lower urinary tract infection. The percentage of children with polymorphism was also twice as high in patients with post-inflammatory kidney scars [21]. In our study, no statistically significant differences were found in the incidence of polymorphism depending on the course of UTI (nonfebrile vs. febrile). Bayram *et al*. analysed two TLR4 polymorphisms (Asp299Gly and Thr399Ile) as well as TLR4 monocyte and neutrophil expression among patients with rUTI (with and without renal scarring). However, no significant results considering Asp299Gly were found; only Thr399Ile polymorphism appeared to influence receptor expression and renal scarring [22].

Urine interleukin 8

One of the first studies on the concentration of IL-8 in urine was the work of Ko *et al*. The importance of IL-8 compared to leukocyturia during UTI was assessed.

The concentration of IL-8 in urine correlated positively with the amount of leukocytes in urine; it was estimated in an *in vitro* study that binding IL-8 with an antibody caused a decrease by about half of the chemotactic activity of urine [23]. In our study, a similar trend can be observed (higher values of IL-8 concentrations in children with recurrent UTIs), but the concentrations observed in this study were lower. This may be related to urine testing not in the acute phase of urinary tract infection. Higher concentrations in children with recurrent UTIs, even in the absence of current infection, may be explained by the fact that the inflammation in this group of patients has a chronic character. In the study of Galanakis *et al*. a significantly higher concentration of IL-8 was found in the group of children with VUR, compared to the group without this defect, suffering from UTI, and the group of healthy children. This may be due to the ongoing inflammatory process in the urinary tract in children with the defect, despite the lack of current infection. Similarly, in our study, the highest levels of IL-8 were found in the urine of children with the defect who had recurrent UTIs, but these relationships did not meet the criteria of statistical significance ($p = 0.49$) [24]. In our study, we can only talk about certain trends in IL-8 concentrations due to the lack of statistical significance. Nevertheless, these trends are in line with those in the literature. In the work of Tramma *et al*., IL-8 in the urine of children without active UTI was studied. IL-8 was undetectable in the urine of these patients, regardless of the presence of scars or vesicoureteral reflux [25]. In our study, the percentage of patients with undetectable levels of IL-8 was different in the study groups; the highest was found in the group with VUR, not suffering from urinary tract infections.

Urine NGAL

In the study of Cost *et al*., concentration of NGAL was assessed in children with ureteropelvic junction obstruction. The concentration of NGAL correlated inversely with the function of the kidney on the side of obstruction [26]. In our study, significant differences in study groups were also observed, with the highest NGAL concentration in the group of children with urinary tract malformations who did not suffer from rUTI. The group of children with the highest urine concentration of NGAL had the lowest eGFR. In the control group, where the concentration of NGAL was the lowest, eGFR was higher than in the groups of children with urinary tract defects. A study carried by Gupta *et al*. aimed to differentiate UTIs from asymptomatic bacteriuria in children with neurogenic bladder (NGB). Urinary NGAL concentration was found to be significantly higher in the group of patients with NGB with UTI. Moreover, the concentrations of NGAL in the urine of children with NGB (both with and without a UTI) were significantly higher than in healthy children in the control group [27]. In our study, the presence of the urinary tract malformation also translated into a higher concentration of NGAL. The value of NGAL in predicting the risk of inflammatory scarring in the kidney was the subject of studies by Ghasemi *et al*. It was found here that NGAL is not sensitive enough as a marker of renal interstitial tissue involvement during UTIs [28]. In our study, NGAL during acute infection was not investigated; however, children with recurrent UTIs were the group with the second highest urinary NGAL concentration. In the study of Lubell *et al*. 210 feverish patients were examined. In 35 cases, UTI was found to be the cause of the fever. Comparing the urinary NGAL concentration of these patients, promising results were obtained, showing that urinary NGAL concentration is more sensitive than the routinely assessed presence of urinary esterase and nitrates [29]. In our study, the study groups with recurrent UTIs presented significantly higher concentrations of NGAL than the control group, so it can be suspected that if the concentration was tested in an acute state of infection, these values would be even higher.

Conclusions

Analysed TLR2 and TLR4 polymorphisms may predispose to rUTI. In particular, in the TLR4 polymorphism, DG genotype was significantly less frequent than DD in the group of healthy children, and DD genotype was predominant in healthy children vs. all others. TLR2 and TLR4 polymorphisms do not play a role in the course of urinary tract infections. Higher urinary NGAL concentration in children with urinary tract malformation may suggest kidney tissue damage in this group of patients.

The authors declare no conflict of interest.

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