



УДК 616.61-002.3-036.12-053.2:575

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GENETIC POLYMORPHISM OF TOLL-LIKE RECEPTOR 4 — PREDICTOR OF SUSCEPTIBILITY TO RECURRENT COURSE OF CHRONIC PYELONEPHRITIS IN CHILDREN

Summary. Objective of investigation: to study the role of Toll-like receptor 4 Asp299Gly gene polymorphism in the implementation of susceptibility to chronic pyelonephritis, to analyze its association with the major manifestations of the disease and to assess the impact on the synthesis of interleukin-6 in the blood of sick children.

Methods. We examined a group of children aged 1–15 years with chronic pyelonephritis in the active stage ($n = 60$). Control group consisted of 95 apparently healthy people.

Results. There were revealed significantly higher prevalence of Toll-like receptor 4 Asp299Gly gene polymorphism among children with chronic pyelonephritis ($n = 60$), identified a statistically significant correlation of mutant genotypes Asp299Gly, Gly299Gly with manifestation of the disease before the age of 3, and frequent episodes of acute respiratory viral infections, torpid urinary syndrome, unstable remission and increased secretion of interleukin-6 ($p < 0.001$). Thus, genetically determined Toll-like receptor 4 Asp299Gly gene polymorphism is a marker of high risk of early manifestation of chronic inflammation in the kidney interstitium and prolonged recurrent clinical course of the disease in children.

Key words: gene, polymorphism, Toll-like receptor 4, chronic pyelonephritis, children.

Pyelonephritis is a classic example of multifactorial pathology which is realized by the interaction of multiple environmental factors and hereditary predisposition [11, 12]. The reaction of the immune system in pyelonephritis begins from the moment of contact with the infectious agent antigen (acute inflammation) or after increasing the number of microbes higher «level breakthrough» (urine microbial count of higher than 100 000). Ability to recognize microorganisms, which are constantly in contact the human body, is a key starting mechanism that implements the deployment of adaptation and adaptive reactions of microorganism [3, 4, 8].

Determination of the important role of innate immunity came with the identification of distinctive pattern receptors (pattern recognition receptors — PRR), namely, evolutionary conserved family of receptors, known in modern scientific literature as family Toll (Toll-like receptors — TLR), which play decisive role in the early defense of the body from pathogens [1, 2, 5]. According to current knowledge, TLR4 is a central element of the structural and molecular multilevel system image of distinctive receptors as in charge the binding of molecular patterns of pathogens and the formation of a protective response in collaboration with lipopolysaccharide (LPS) — extracellular component of the outer membrane of gram-negative bacteria [2, 7].

The molecular structure of TLR4 characterized by variable extracellular N-terminal domain, which consists of

oligopeptide fragments with high leucine repeats, which determines the ability to interact with LPS [1, 7, 9]. TLR4 activation starts from the interaction of complex LPS/CD14/MD-2 [5, 6, 8]. The resulting complex is accompanied by its conformational changes and starts intracellular cascade of reactions at the final stage which is the initiation of transcription of genes that regulate the synthesis of proinflammatory cytokines [2]. Single nucleotide polymorphisms may interfere with the regulation of the innate immune response by the interaction of LPS, which is a key factor imbalance in the immune system. With the advent of molecular genetic studies in research laboratories in the United States for the first time shown that genetic defects are responsible for the synthesis of PRR, resulting in inadequate functioning of the database of molecular structures, accompanied by violation of signal transduction for nuclear factor (NF- κ B) and disordinated synthesis of pro-inflammatory and anti-inflammatory cytokines [10, 12, 13]. Therefore, polymorphisms of genes encoding TLR-4, can determine level of concentration of inflammatory and anti-inflammatory cytokines and thus determine degree of expressed inflammatory response [6, 8]. A similar mechanism may play a crucial role in the

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formation of chronic inflammation and act as a risk factor for chronic inflammation, such as chronic pyelonephritis (CP) [2].

Interleukin (IL)-6 is one of the mediators of sub acute and chronic inflammation [9, 11]. In recent studies analysis of cytokine uroepithelium potential and its realization is devoted to a series of works, mainly of experimental nature. For example, the experimentally induced pyelonephritis on mice demonstrated that cytokines are produced in response to bacterial infection by local cells, not just filtered. There are works in which the authors propose to use the definition of the level of IL-1 and IL-6 at children with acute pyelonephritis as an early test of nephrosclerosis, and to assess the dynamics of the inflammatory process, the adequacy of therapy and the need for continued uroepithelium therapy after discharge from hospital [14–16]. There are studies that indicate a compelling need to identify IL-6 and IL-8 among children with CP as laboratory criteria for the formation of fibrotic processes in the kidney and reflux nephropathy. Although the clinical significance of interleukin-6 is being finalized existing work make it possible to assume that the level of cytokines among patients with Parkinson's disease may be associated with the severity of local inflammation.

There are studies that suggest that the presence of mutant alleles of genes TLR4 Asp299Gly increased risk of acquiring uric genital infections for adults and lead to asymptomatic bacteriuria among children [2, 5, 6]. There is a proven correlation heterozygous (Arg753Gln) by the mutant allele genotype Toll-like receptor 2 with high sensitivity to *Escherichia coli* and increased sensitivity to key intracellular pathogens among children with chronic pyelonephritis [4]. Similar studies among children in our country have not been conducted. Therefore, a more detailed understanding of the genetic structure of susceptibility to chronic pyelonephritis is reasonable to study the prevalence of missense mutations among pediatric patients.

The aim of our research has also included studies the role of gene polymorphisms Asp299Gly TLR4 in the implementation of susceptibility to chronic pyelonephritis, analyze its association with major manifestations of the disease and to evaluate the impact on the synthesis of IL-6.

Material and Methods

A clinical and laboratory examination of 60 children aged between 1–15 years with chronic pyelonephritis who were hospitalized in the pediatric ward № 2, Poltava Regional Children Clinical Hospital. Survey was carried in the active stage of the disease. The diagnosis of chronic pyelonephritis installed in accordance with the recommended criteria on the basis of general clinical, laboratory and instrumental examination of children by order of the Ministry of Health from 11.03.2008 № 627 «On approval of the treatment protocol for children with urinary tract infections and tubulointerstitial nephritis» [12].

The control group amounted to 95 healthy individuals with genetic base of samples of Institute of Genetic and immune basics of pathology and pharmacokinetics of Ukrainian Medical Stomatological Academy. From all patients we received voluntary written consent to participate in research, which was conducted with the permission of the Commis-

sion on Bioethics by Ukrainian Medical Stomatological Academy.

Material for the study was peripheral venous blood. Genotyping polymorphic areas Asp299Gly conducted by polymerase chain reaction (PCR) using oligonucleotide primers. Amplification was performed on thermocyclers Tertsyk (DNA Technology, Moscow). Polymorphic area of the gene Asp299Gly Toll-like receptor 4 was amplified by PCR in a 25 ml reaction mixture. Determination of IL-6 in serum of children with CP was conducted by enzyme immune-assay (ELISA). The material research was peripheral venous blood sampling which was conducted cube vein in sterile vacuum containers. The study was conducted using a set of reagents for ELISA determination of the concentration of IL-6 in serum and urine of man interleukin-6 ELISA-Best (JSC Vector-Best, Russia). Research made on the Research Institute for Genetic and immunological basis of disease and pharmacogenetics by Ukrainian Medical Stomatological Academy (Poltava).

Mathematical treatment of the data carried out using the program Statistica for Windows 7.0 (StatSoft Inc) and spreadsheets MS Excel. The distribution of genotypes in the investigated polymorphic loci tested for compliance with Hardy-Weinberg equilibrium using the criterion χ^2 . To compare allele frequencies between the groups under study, using Pearson's χ^2 test corrected for Yates continuity when the number of degrees of freedom, which is equal to 1. Comparison of genotype frequencies between groups studied, performed by analysis of interface tables using Fisher's exact test. For comparison, the frequency of choices in unrelated groups calculated the odds ratio (OR) with 95% confidence interval (CI). For all types of analysis considered differences statistically significant at $p < 0.05$ at $0.05 < p \leq 0.1$ noted a trend towards differences.

Results

The analysis of the frequency distribution of genotypes in the studied gene TLR4 Asp299Gly in groups of observations is given in Table 1.

As shown in the table, «wild-type» of genotype TLR4 met in 96.84 % of healthy individuals, the frequency of heterozygous genotype was 3.16 %, GG mutant was not found. While among children with chronic pyelonephritis frequency of heterozygous genotype AG almost 3 times the performance of control group (11.67 % and 3.16 %, respectively), and one child was identified mutant genotype GG.

The study of the prevalence of normal and mutant alleles of the gene 299Gly Toll-like receptor 4 in the surveyed groups (Table 2) showed that the mutant allele Gly more than 4 times frequently detected among children with chronic pyelonephritis (7.5 %) compared with the control group (1.58 %) ($\chi^2 = 4.576$; OR = 1.059, CI = 0.9989–1.122, $p = 0.049$).

Genetic markers may determine susceptibility to disease in general, or to be associated with specific pathogenesis significant features. Therefore, as part of our work is an important step was to investigate the influence of single nucleotide polymorphisms Asp299Gly TLR4 gene on the course and characteristics of clinical manifestations of chronic pyelonephritis in children. To solve this problem, we formed two groups of children: one — patients with heterozygous and

homozygous for the mutant allele genotype (Asp/Gly, Gly/Gly), and the second included children with normal distribution allele (Asp/Asp). Analyzing the parameters of the disease, we found probable differences among patients with different variants of genotypes (Table 3).

Analyzing these data, we found that genotypes Asp/Gly and Gly/Gly in children with CP were associated with early manifestation of the disease up to 3 years of age ($\chi^2 = 12.31$; $p < 0.05$), whereas in pediatric patients with normal TLR4 allele distribution was observed later debut CP ($\chi^2 = 3.92$, $p < 0.001$). Comparing the clinical data, found that all children with mutant genotype dominated by frequent episodes of acute respiratory infections ($\chi^2 = 4.03$; $p < 0.05$), torpid urinary syndrome ($\chi^2 = 6.19$; $p < 0.001$) and non-remission ($\chi^2 = 7.98$; $p < 0.001$), while a «wild-type» was characterized debut CP after 5 years and a short bladder syndrome. It should be noted that children with genotype Asp/Asp and Asp/Gly was characterized by significantly more pronounced leucocyturia according to the analysis of urine by Nechyporenko during hospitalization and was 44156.25 ± 101.70 per 1 ml, in contrast to children with normal distribution of alleles where this figure is more than a factor of 2 was lower (17861.70 ± 89.69 per 1 ml, $p < 0.05$).

It should be noted that the output level of all pediatric patients IL-6 had a great range of values, and the average was 10.58 ± 2.40 pg/ml. Therefore, an important step was the analysis of IL-6, depending on the genotype of TLR 4. Index level of IL-6 healthy donors was taken by conventional norm for this population, and according to a set of instructions reagents averaged 2 pg/ml with a range of 0–10 pg/ml. Analyzing the results, it should be noted that children with genotype Asp/Asp and Asp/Gly output level IL-6 was 1.56 times higher than the normal of genotype carriers of TLR4 (12.70 ± 4.34 and 8.01 ± 1.44 , respectively, $p < 0.02$). Thus, the interaction with uropathogen strains epithelial cells of children with heterozygous and homozygous for the mutant allele genotypes correspond to faster production of pro-inflammatory cytokines, including IL-6.

Whereas the regulation of immune and inflammatory responses by using cytokines, which on the one hand, the protective function, on the other — are involved in the pathogenesis of the disease, increasing the value of IL-6 in the group of children from heterozygous and homozygous allele of genotype may reflect severity of microbial activity in tubules inflammatory processes, interstitial renal tissue. Proof of this appeared correlation was found between the concentration of

Table 1. The distribution of genotype frequencies TLR4 Asp299Gly gene polymorphism among healthy individuals and children with pyelonephritis, % (n)

Genotype	Control group, n = 95	Children with CP, n = 60	P*
AA	96.84 (92)	86.67 (52)	0.0385
AG	3.16 (3)	11.67 (7)	
GG	0 (0)	1.67 (1)	

Note: * — the level of significance, test the resulting χ^2 .

Table 2. The distribution of allele frequencies TLR4 Asp299Gly gene polymorphism among healthy individuals and children with CP

Allele	Frequency in control group	Frequency in children with CP	Pearson χ^2 df = 1	OR (95% CI)	P*
A	98.42 % (187)	92.5 % (111)	4.576	1.059 (0.9989–1.122)	0.049
G	1.58 % (3)	7.5 % (9)			

Note: * — the level of significance, test the resulting χ^2 .

Table 3. Analysis of correlation of genotypes with clinical manifestations of Parkinson's disease

Clinical manifestations of chronic pyelonephritis		Number of patients		Yates corrected χ^2 OR (95% CI)	P
		Asp/Gly, Gly/Gly (8 patients), n (%)	Asp/Asp (52 patients), n (%)		
Debut of CP till 3 years	Yes	6 (75)	9 (17.31)	12.31	< 0.05*
	No	2 (25)	43 (82.69)	14.33 (2.48–82.83)	
Debut of CP after 3 years	Yes	2 (25)	23 (44.23)	1.58	> 0.05
	No	6 (75)	29 (55.77)	0.47 (0.07–2.4)	
Debut of CP after 5 years	Yes	0 (0)	20 (38.46)	3.92	< 0.001*
	No	8 (100)	32 (61.54)	0.0001 (1.03–0.003)	
Torpid urinary syndrome	Yes	8 (100)	15 (28.85)	6.19	< 0.001*
	No	0 (0)	37 (71.15)	0.0001 (9.9–0.01)	
Frequent acute respiratory infections	Yes	7 (87.5)	17 (32.69)	4.03	< 0.05*
	No	1 (12.5)	35 (67.31)	9.87 (1.27–103.7)	
Unstable remission	Yes	7 (85.7)	11 (23.9)	7.98	< 0.001*
	No	1 (14.3)	35 (76.1)	13.9 (2.0–80.6)	

Note: * — the level of significance, test the resulting χ^2 .

IL-6 levels at leukocyturia ($r = 0.86$, $p < 0.05$) and relapse after treatment ($r = 0.59$, $p < 0.05$) in the data of patients.

Conclusions

Thus, the TLR4 gene polymorphism is misens-mutation that alters the extracellular domain of the receptor, resulting in the loss of opportunities to communicate with bacterial LPS, leads to disruption of transmission of the activation signal to NF κ B, followed by immune imbalance that covers all aspects of the immune system and is characterized by long-term recurrent chronic inflammatory process in the kidneys with increased synthesis of IL-6 levels.

Polymorphisms Asp299Gly TLR4, commits mechanisms regulating innate immune response, defines the changing nature of the course and severity of clinical manifestations of the disease. As a result of the research and analysis of the results, we can suppose the presence of at least one association of mutant genotypes (Asp/Gly, Gly/Gly) or allele (Gly) TLR4 gene with increased risk of chronic pyelonephritis among children.

References

1. Features treatment of pyelonephritis among children at the present stage // *Current Pediatrics*. — 2003. — Vol. 2, № 1. — P. 317.
2. Ignatov M.S. Solved and unsolved problems of childhood econephrology / M.S. Ignatov, E.A. Harina, V. Long // *Pediatrics*. — 1997. — № 6. — P. 54-57.
3. Malchenko L.A. The immune system in various forms of microbial and inflammatory diseases of the urinary tract of children / L.A. Malchenko, A.D. Petrushina, S.L. Morgunov // *II Congress of Pediatric Nephrology Russia: Proceedings of the Congress*. — Moscow, 2000. — P. 34.
4. Dasaeva L.A. The modern concept of the etiology and pathogenesis of urinary stones / L.A. Dasaeva, E.M. Shilov, S.N. Shatokhina // *Nephrology and Dialysis*. — 2003. — Vol. 5, № 2. — P. 128-133.
5. Izmaylova I.V. Researchment of polymorphisms genes TLR2 ARG753GLN that TLR4 ASP299GLy, THR399ILE in urogenital

infections / I.V. Izmaylova, O.A. Shlikova, I.P. Kaidashev // *Zh. Academy of Medical Sciences of Ukraine*. — 2010. — Vol. 16. — P. 72-73.

6. Izmaylova O.V. The role of Toll — polymorphisms like receptor 4 Asp-299Gly in the development of bacterial infections that are transmitted by sex pathways / O.V. Izmaylova, O. Shlikova, N.O. Bobrova, I.P. Kaidashev // *Problems of Ecology and Medicine*. — 2009. — Vol. 13, № 5-6. — P. 3-6.

7. Kryuchko T.A. Clinic-genetic aspects of chronic pyelonephritis among children: structure propensity and initial prognosis / T.A. Kryuchko, V.P. Ostapenko, T.V. Kushnereva // *Child Health*. — 2013. — № 3 (46). — P. 59-64.

8. Kryuchko T.O., Ostapenko V.P., Kajdashev I.P. New look at the etiology of chronic pyelonephritis among children with polymorphism of TLR2 gene / T.O. Kryuchko, V.P. Ostapenko, T.V., I.P. Kajdashev // *Pediatrics East. Europe*. — 2013. — № 3 (03). — C. 42-51.

9. Ferwerda B. Functional Consequences of Toll-like Receptor 4 Polymorphisms / B. Ferwerda, M.B. McCall, K. Verheijen, B.J. Kullberg // *Mol. Med*. — 2008. — 14 (5-6). — P. 346-352.

10. Garcia-Rodriguez C. Toll-like receptor 4-dependent pathways as sensors of endogenous «danger» signals. New evidences and potential therapeutic targets / C. Garcia-Rodriguez // *Immunology*. — 2007. — Vol. 26, № 4. — P. 210-215.

11. Hollingsworth J.W. TLR4 signaling attenuates ongoing allergic inflammation / J.W. Hollingsworth, G.S. Whitehead, K.L. Lin, H. Nakano, M.D. Gunn, D.A. Schwartz // *Immunology*. — 2006. — № 176. — P. 5856-62.

12. Holloway J.W. Genetics of allergic disease / J.W. Holloway, I.A. Yang, S.T. Holgate // *Allergy Clinical Immunology*. — 2010. — № 125. — P. 81-94.

13. Kim H.M. Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran / H.M. Kim et al. // *Cell*. — 2007. — 130. — P. 906-17.

14. Krishnan J. Toll-like receptor signal transduction / J. Krishnan, K. Selvarajoo, M. Tsuchiya, G. Lee, S. Choi // *Experimental Mol. Med*. — 2007. — 39. — 421-38.

15. Rallabhandi P. Analysis of TLR4 polymorphic variants: new insights into TLR4/MD-2/CD14 structure, and signaling / P. Rallabhandi, J. Bell, M.S. Boukhalvala // *J. Immunology*. — 2006. — Vol. 177. — P. 322-332.

16. Schroder N.W. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease / N.W. Schroder, R.R. Schumann // *Lancet Infect. Dis*. — 2005. — Vol. 5. — P. 156-164.

Получено 20.09.13 □

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ГЕНЕТИЧЕСКИЙ ПОЛИМОРФИЗМ TOLL-ПОДОБНОГО РЕЦЕПТОРА 4 — ПРЕДИКТОР СКЛОННОСТИ К РЕЦИДИВИРУЮЩЕМУ ТЕЧЕНИЮ ХРОНИЧЕСКОГО ПИЕЛОНЕФРИТА У ДЕТЕЙ

Резюме. Цель исследования: изучить роль полиморфизма Asp299Gly гена Toll-подобного рецептора 4 в реализации склонности к хроническому пиелонефриту, проанализировать его ассоциацию с основными проявлениями заболевания и оценить влияние на синтез интерлейкина-6 в крови больных детей.

Методы. Обследована группа детей с хроническим пиелонефритом в активной стадии в возрасте 1–15 лет ($n = 60$). Группу контроля составили 95 практически здоровых людей.

Результаты. Выявлена достоверно более высокая распространенность полиморфизма гена TLR4 Asp299Gly среди детей с хроническим пиелонефритом ($n = 60$), определена статистически значимая связь мутантных генотипов Asp299Gly, Gly299Gly с манифестацией заболевания до 3-летнего возраста, частыми эпизодами острой респираторной вирусной инфекции, торпидным мочевым синдромом, нестойкой ремиссией и повышением секреции интерлейкина-6 ($p < 0,001$). Таким образом, генетически детерминированный полиморфизм Asp299Gly Toll-подобного рецептора 4 является маркером, определяющим высокий риск ранней манифестации хронического воспалительного процесса в интерстиции почек и затяжное рецидивирующее течение заболевания у детей.

Ключевые слова: ген, полиморфизм, Toll-подобный рецептор 4, хронический пиелонефрит, дети.

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ГЕНЕТИЧНИЙ ПОЛІМОРФІЗМ TOLL-ПОДІБНОГО РЕЦЕПТОРА 4 — ПРЕДИКТОР СХИЛЬНОСТІ ДО РЕЦИДИВУЮЧОГО ПЕРЕБІГУ ХРОНІЧНОГО ПІЕЛОНЕФРИТУ В ДІТЕЙ

Резюме. Мета дослідження: вивчити роль поліморфізму Asp299Gly гена Toll-подібного рецептора 4 у реалізації схильності до хронічного піелонефриту, проаналізувати його асоціацію з основними проявами захворювання й оцінити вплив на синтез інтерлейкіну-6 у крові хворих дітей.

Методи. Обстежена група дітей із хронічним піелонефритом в активній стадії віком 1–15 років ($n = 60$). Групу контролю становили 95 практично здорових людей.

Результати. Виявлено вірогідно вищу поширеність поліморфізму гена TLR4 Asp299Gly серед дітей із хронічним піелонефритом ($n = 60$), визначено статистично значимий зв'язок мутантних генотипів Asp299Gly, Gly299Gly з манифестацією захворювання до 3-річного віку, частими епізодами гострої респіраторної вірусної інфекції, торпідним перебігом сечового синдрому, нестійкою ремісією і підвищенням секреції інтерлейкіну-6 ($p < 0,001$). Таким чином, генетично детермінований поліморфізм Asp299Gly Toll-подібного рецептора 4 є маркером високого ризику ранньої манифестації хронічного запального процесу в інтерстиції нирок і тривалого рецидивуючого перебігу захворювання в дітей.

Ключові слова: ген, поліморфізм, Toll-подібний рецептор 4, хронічний піелонефрит, діти.