Role of Genetic Mutations in Development of Immunological and Clinical Disorders in Children with Chronic Pyelonephritis

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Summary. At the present time, the study of mechanisms of recognition of foreign agents, which is realized by means of Toll-like receptors (TLR) of the innate immune system, has become one of the main tasks of clinical immunology.

The aim of our study was to investigate the prevalence of polymorphism of Toll-like receptor 4 (Asp299Gly, Gly299Gly) among children with chronic pyelonephritis (CP) and determine the association of this TLR4 polymorphism with phenotypic features of chronic pyelonephritis and level of interleukin-6 (IL-6).

Significantly higher frequency of the mutant allele 299Gly among children with CP was revealed. Significant correlation between the presence of 299Gly TLR4, association of U. urealyticum and M.hominis in lower sections of urinary tract and highest levels of IL-6 concentration was reflected. Sick children with polymorphous locus of TLR4 gene had higher risk of CP early manifestation and formation of its recurrent course with protracted urinary syndrome and unstable remission in comparison with the carriers of “wild” genotype.

Obtained results prove the important role of TLR4 in the realization of innate immune response in children with CP and allow considering the TLR4 polymorphism as an additional prognostic indicator in this category of patients.

Keywords: innate immunity, polymorphism, Toll-like receptors, interleukin-6, intracellular pathogens.

Introduction. The issues connected with the studies of risk factors, features of clinics and early diagnostics of infectious inflammatory diseases of urinary systems (IUS) in children have particular significance for pediatricians due to high level of their prevalence and not always favorable prognosis. Pyelonephritis (PN) refers to the most common IUS, which stipulates high levels of acute disease incidence and stable prolonged kidney damage [1, 2, 15]. Therefore, it requires timely diagnostics and adequate treatment, because in case of involvement of kidney interstitium into inflammatory process there is high risk of nephrosclerosis. And the risk of scar formation in kidneys depends on the number of events of urinary tract infections. The process of scar formation is accompanied by irreversible injury of kidney parenchyma, which causes the reduced partial kidney function and early disablement of young people [3, 4, 6, 7, 17].

It should be noted that besides the pathogen characteristic the development and progress of PN is determined by the individual properties of master macroorganism (firstly, adequate immune response), which are the reflection of its genetic structure. Therefore, one of modern approaches in the prevention of development of any pathology includes the genetic prognosis of its formation risk. It has become possible only in recent years, when the biomedical investigations aimed at the development of the methods of detection of molecular peculiarities of multifactor diseases started. Currently, more and more factors, which indicate the genetic susceptibility to the development of many kidney diseases, are being revealed [6, 7, 8, 9, 21, 22].

Immune system response in case of PN starts from the moment of contact with infectious agent-antigen (in case of acute inflammation) or from the moment of increase of the number of microbial bodies above the “breakthrough level” (microbial number of urine is higher than 100 000). The ability to recognize the microorganisms, with which the human organism is in constant contact, is the key start mechanism, which implements the deployment of adaptive and adjustment responses

of macroorganism [8, 9, 12]. The relevant system of microorganism detection is essential for human being because it provides the initiation of “danger reaction” in case of ingress of antigenic structures in organism internal environment. Lipopolysaccharide (LPS), which is detected in gram-negative bacteria, has become one of the first structural components of microorganisms, which is capable to activate the cascade of defensive and adjustment responses of macroorganism. And therapeutic effect of pyrogenal and prodigiosan, which were used in clinical practice for the treatment of sick people with lingering and chronic course of infectious diseases for a long period of time, was based on such mechanism. LPS is capable to activate the innate and acquired systems of organism defense. Using the method of positional cloning of the gene, which is responsible for the recognition of bacterial lipopolysaccharide, the role of Toll-like receptors (TLR) in immune organism defense was confirmed. Then, it was given the number 4 (TLR4) [18, 19, 21, 22].

Taking into account the significant role of TLR4 in the implementation of defense mechanism of innate immune system, the scientists concentrated their attention in the search of causes of function impairment. Our attention was drawn to the polymorphism of TLR4 Asp299Gly, because there is a number of studies, which indicate that the presence of these gene allele causes the growth of risk of urinogenital infections such as Chlamydia, mycoplasmosis, ureaplasmosis, gardnerellosis, trichomoniasis [10, 11]. Gene polymorphism suggests that several structurally diverse copies of the same protein can be made from the same gene, and at the same time, part of copied variants does not have activity or might have opposite function. Since the differences in genes, which control the defensive reactions of organism, can provide different character of the course of inflammatory response and specific immunological responses in case of introduction of foreign agents, the study of prevalence of single nucleotide replacements in children with chronic PN is of special interest [15, 16, 21].

Research objective: to study the prevalence of polymorphism of Toll-like receptor 4 (Asp299Gly, Gly299Gly) among the sick children who have chronic pyelonephritis using the population genetic research and to determine the association of this polymorphism of TLR4 with phenotypic features of chronic pyelonephritis and level of interleukin-6.

Materials and methods of study. The clinical and laboratory examination of 60 children with chronic pyelonephritis during the stage of exacerbation, who were under inpatient treatment at the pediatric department No. 2 (nephrological beds) of Children's Regional Clinical Hospital in Poltava, was performed.

Selection of biological samples from sick children who were observed was performed under the condition of absence of infectious diseases. Peripheral venous blood, sampling of which was performed from cubital vein into sterile vacutainer with stabilizer (DTA) with the further introduction into eppendorf with the reagent "Dna-express", was used as the study material. Genetic typing of polymorphous section of Asp299Gly was performed by the method of polymerase chain reaction (PCR) using oligonucleotide primers. Amplification was performed using the amplifier "Tertsyk" ("DNA-Technology", Moscow).

Polymorphous section Asp299Gly of the gene of Toll-like receptor 4 was amplified using PCR; 25 μl of reaction mixture contained: 2.5 μl of 10 x Buf for amplification; 2 mM of magnesium chloride; 0.2 mM of every dNTP; 2.5 units of DNA polymerase Tag; 20-50 ng of genomic DNA; 66 ng of primers for Asp299Gly.

Determination of IL-6 content in blood serum of children with CP was performed using enzyme immunoassay (ELISA) with the set of reagents "Interleukin-6-ELISA-BEST" (CJSC "Vector-Best", Russian Federation). Peripheral venous blood, sampling of which was performed from cubital vein into sterile vacutainers, was used as the study material. IL-6 concentration was analyzed twice – before treatment and at the end of treatment. Children with CP exacerbation were

under inpatient treatment. They received the diet No. 5, antibacterial therapy and other types of pharmacological therapy according with the treatment protocol for this disease [13, 14].

General clinical, instrumental, clinical and laboratory and biochemical studies in sick children were performed using the conventional methods. Glomerular filtration rate (GFR) was determined on the basis of creatinine using the formula of G.J. Schvartz: GFR = 48.4 x height (cm) / content of creatinine in blood (μmol/l) with the correction for age and sex coefficients [5].

Bacteriological study of urine with the detection of microbial agent and its susceptibility to antibacterial and chemotherapeutic agents and molecular-biological study (polymerase chain reaction for detection of Chlamidia trachomatis, Ureaplasma urealyticum, Mycoplasma hominis, study material – scrape of epithelial cells from urethra) in 86.7% of sick children were performed prior to the prescription of antibacterial therapy.

Control group consisted of 95 healthy children. Written consent for participation in the scientific study was obtained from all patients; the studies were carried out on the basis of approval of bioethics commission of the High State Educational Establishment “Ukrainian Medical Stomatological Academy”.

Mathematical processing of obtained data was performed using the program “STATISTICA for Windows 7.0” (StatSoft Inc) and spreadsheets MS Excel. The distribution of genotypes by studied polymorphous locuses was checked with regard to the compliance with Hardy-Weinberg balance using the criterion $\chi 2$. In order to compare the frequencies of alleles among the studied groups, Pearson criterion $\chi 2$ with Yates correction for continuity was used for the number of degrees of freedom equal to 1. Comparison of genotype frequencies among the studied groups was performed on the basis of analysis of contingency tables using Fischer’s exact test. In order to compare the frequencies of variants in unconnected groups the odds ratio (OR) was calculated with the determination of 95% confidence interval (CI). For all types of analysis, the differences with $p<0.05$

were considered to be statistically significant; in case if $p \leq 0.1$ the tendency to difference was observed.

Obtained results.

At the first stage of our study, the fraction of mutant genotype TLR4 in examined children was determined. In majority of practically healthy children (96.8%), the normal distribution of alleles AA of TLR4 gene was detected ("wild type" of genotype). Frequency of heterozygous genotype AG was insignificant (3.2%), and mutant genotype GG was not detected at all. The frequency of heterozygous and mutant genotypes AG and GG in children with CP was 86.7% and 11.7% respectively, and it reliably exceeded the parameters of control group ($\chi 2=5.78$; OR=4.72; CI=1.2-8.56; $p=0.023$). Reliably higher frequency of mutant allele 299Gly was detected among children with CP, which was observed more frequently by 4.4 times in the group of sick children with CP compared to healthy children ($\chi 2=5.78$; $p=0.015$). Sick children with CP were characterized by the average level of actual heterozygosis by Asp299Gly (AG). The lowest number (1.6%) of sick children was homozygotes Gly299Gly (GG), and this fact was reflected during the calculation of relative risk.

The special attention was drawn to the analysis of mutation Asp299Gly of TLR4 with the basic disease manifestations. Therefore, at the second stage of our study, the differential analysis of peculiarities of clinical and laboratory manifestations of CP in children who have polymorphism of Asp299Gly, Gly299Gly types and children with normal distribution of alleles of TLR4 gene was performed. First of all, it was detected that sick children with Gly/Gly or Asp/Gly genotype of polymorphous locus of TLR4 gene had higher risk of early manifestation of CP ($\chi 2=15.2$; $p<0.001$) and formation of its recurrent course with protracted urinary syndrome ($\chi 2=4.0$; $p<0.001$) and unstable remission ($\chi 2=8.0$; $p<0.001$) in comparison with the carriers of "wild" genotype (Fig. 1).

Analysis of the frequency of CP exacerbation depending on TLR4 genotype showed that the average frequency of recurrences in the carriers of mutant gene 299Gly was 3.8±0.2 times per year as opposed to the children with “wild genotype” who had the number of recurrences by 3 times lower and equal to 1.1±0.2 times per year. Clinical manifestations during the exacerbation in children with different distribution of alleles of this gene also differed; thus, sick children-carriers of mutant genotypes were characterized by lower pronouncement of general inflammatory reaction of organism during CP exacerbation, and the parameters of erythrocyte sedimentation rate and banded neutrophils in blood (by 1.6 and 2 times ($p<0.05$) respectively) indicated this fact in comparison with these parameters in children with normal distribution of gene alleles. In case of the presence of mutant genes TLR4 in children, more pronounced leukocyturia ($\chi2=14.9$; $p<0.001$) and hyposthenuria ($\chi2=14.7$; $p<0.001$) were observed (Fig. 1).

In children with Gly/Gly or Asp/Gly genotype of polymorphous locus of TLR5 gene, the number of leukocytes in urine (by Nechiporenko) exceeded the parameters in children without polymorphism of these genes by almost 30 times and was higher than the norm at the moment of discharge from the hospital ($p<0.05$). This category of patients also had more pronounced impairments of concentration and excretion functions of kidneys during CP exacerbation, in particular, reduction of diurnal dieresis ($\chi2=14.7$; $p<0.001$), decrease of relative urine density ($\chi2=3.5$; $p<0.001$) and glomerular filtration rate (GFR) ($\chi2=15.1$; $p<0.001$).

It was established that there are associations of genotypes of TLR4 polymorphous locuses not only with clinical and laboratory parameters of children with CP but with microflora character. In particular, the significant correlations between the presence of 299Gly TLR4 and association of U. urealyticum and M.hominis ($\tau=0.58$) were revealed; the frequency of U. urealyticum and M. Hominis detection is reliably higher by 11.7 and 5.9 times among the patients-homozygotes

Gly/Gly and heterozygotes Asp/Gly of polymorphous locus of TLR4 gene as compared to children with “wild” genotype ($p<0.005$).

Within the framework of our paper, we studied the influence of mutant alleles of TLR4 gene on the synthesis of pro-inflammatory cytokine IL-6 in children with chronic pyelonephritis. It should be noted that in sick children the initial level of IL-6 had wide range of values, from 2.49 ± 0.11 to 33.35 ± 7.7 pg/ml at the average, and it depended on the activity extent of PN. It was shown that the average values of IL-6 in sick children with CP were 10.58±2.40 pg/ml as opposed to 3.43±0.21 pg/ml in practically healthy children, which exceeded the control by more than 3.1 times ($p<0.005$). However, among the groups of sick children with different variants of genotypes of TLR4 receptor the level of IL-6 by genotype AG, GG TLR4 was lower by 2.2 times in comparison with the same parameter in patients with normal distribution of alleles of TLR4 gene but it remained high even during the remission period ($p<0.01$). Direct correlative relationship with the higher level of IL-6 in patients during the remission period with mutant distribution of TLR4 alleles ($\tau=0.74$; $p<0.05$), frequent disease recurrences ($\tau=0.86$; $p<0.05$) and protracted urinary syndrome ($\tau=0.76$; $p<0.05$) was revealed.

At the same time, more significant differences in the reaction of IL-6 were detected depending on the type of pathogen (Table 1). Results visually demonstrate that the simultaneous detection of U. urealyticum and M. hominis in lower sections of urinary system of sick child were accompanied by the highest concentration of pro-inflammatory cytokine, which exceeded the control parameters by 6.3 times ($p<0.05$). Bacterial concurrent infection and association of colon bacillus with U. urealyticum and M. hominis were characterized by the increase of parameters of IL-6 content by 4.1 and 4.6 times in comparison with control group ($p<0.05$).

Discharge of monocultures E. coli, U. urealyticum, M. hominis from sick children was characterized by slightly lower increase of the level of studied cytokine but the value of IL-6 concentration also reliably differed from control ($p<0.05$). When analyzing the level of IL-6 in

children with CP caused by other pathogens, there was no reliable difference found in comparison with IL-6 concentration in children from control group.

Therefore, the study results demonstrate some inadequacy of immune responses in sick children with CP with the detected associations of moleculites in lower sections of urinary system – in case of the absence of pronounced clinical activity, presence of the highest parameters of IL-6 products, on the contrary. In our opinion, this fact indicates that the defensive system of master organism does not always perceive the micoplasms as foreign agents, since the capability of some micoplasms to synthesize the factor, which is identical to IL-6, is proved fact, and the researchers explain it on the basis of exchange of genetic material as a result of prolonged evolutionary coexistence with the master [20].

Conclusions:

It was shown that the frequency of heterozygous and homozygous mutant genotypes AG and GG of polymorphous locus of TLR4 in children with CP exceeded this parameter in healthy children ($\chi 2=5.78$; $p=0.023$). The frequency, with which the polymorphous alleles of genes of Toll-like receptors are observed in sick children with chronic pyelonephritis, was determined: mutant alleles 299Gly of TLR4 gene were more frequent among children with chronic pyelonephritis by 4.4 times in comparison with population control.

The presence of mutant alleles 299Gly of TLR4 gene in children with pyelonephritis increases the risk of manifestation of this pathology up to 3-years age, and it is associated with recurrent course of disease, unstable remission, lower expression of general inflammatory reaction of organism, protracted urinary syndrome and more pronounced impairments of concentration and excretion functions of kidneys during CP exacerbation in comparison with the carriers of “wild” genotype ($p<0.001$). Polymorphism of genes Asp299Gly, Gly299Gly TLR4 in sick children with chronic pyelonephritis is associated with certain microbial agents; in particular, the frequency of U. urealyticum and M. Hominis detection is reliably higher by 11.7 and 5.9 times among the patients-

homozygotes Gly/Gly and heterozygotes Asp/Gly of polymorphous locus of TLR4 gene as compared to children with “wild” genotype ($p<0.005$). Protracted hyperproduction of inteleukin-6 in sick children with chronic pyelonephritis reflects the stable activity of inflammatory process in case of the presence of certain genetic prerequisites, which is proved by direct correlative relationship between the presence of mutant alleles 299Gly of TLR4 in genome and increased level of interleukin-6 in sick children during the period of exacerbation and remission ($p<0.01$). At the same time, the detection of U. urealyticum and M. Hominis in lower sections of urinary system of sick child was accompanied by the highest concentration of pro-inflammatory cytokine, which exceeded the parameters of control by 6.2 times ($p<0.05$).

Given facts allow considering the gene polymorphism Asp299Gly, Gly299Gly of TLR4 as the cause of dysfunction of this receptor, which disturbs the process of recognition of certain pathogens and in such manner assists in the formation of chronic inflammatory process in kidneys with the inclination to recurrent disease course; this statement allows viewing this mutation as the additional prognostic parameter in children with chronic pyelonephritis.

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