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GENETIC POLYMORPHISM ARG753GLN OF TLR-2, LEU412PHE OF TLR-3, ASP299GLY OF TLR-4 IN PATIENTS WITH INFLUENZA AND INFLUENZA-ASSOCIATED PNEUMONIA

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ABSTRACT

The aim of the research is to study the prevalence and to determine the prognostic significance of polymorphism ARG753GLN of the TLR-2 gene, Leu412Phe of TLR-3, Asp299Gly of TLR-4 in influenza.

Materials and methods: 112 patients with influenza were examined (63 patients with uncomplicated course and 49 with influenza-associated pneumonia). The genotyping of the polymorphic site of ARG753GLN of the TLR-2 gene, Asp299Gly of the TLR-4 gene, and Leu412Phe of the TLR-3 gene was carried out by polymerase chain reaction using oligonucleotide primers.

Results: It has found that the prevalence of the mutant allele 299Gly of TLR-4 in patients with uncomplicated influenza is 6.4 %, with influenza- associated pneumonia – 7.1 %, which exceeds the population control indicators by 3.8-4.3 times (1.7 %, $p<0.05$). Mutant allele 412Phe of TLR-3 is significantly more common in patients with influenza-associated pneumonia (42.9%), as compared with uncomplicated influenza (24.6%, $p<0.01$) and healthy people (30.0%, $p<0.05$). The increased risk of influenza development is associated with the Asp/Gly genotype of TLR-4 (OR=4.22) and combination of mutant genotypes Leu/Phe and Phe/Phe of TLR-3 with Asp/Gly of TLR-4 and Arg/Gln of TLR-2 (OR=15.0); influenza-associated pneumonia – with genotype Phe/Phe of TLR-3 (OR=4.5).

Conclusions: It has been found out that among patients with influenza and influenza-associated pneumonia, the mutant allele 299Gly of TLR-4 and combinations of polymorphisms Arg753Gln of TLR-2, Leu412Phe of TLR-3, Asp299Gly of TLR-4 are detected reliably more often. The frequency of the mutant allele 412Phe of TLR-3 is higher among patients with influenza-associated pneumonia. Markers of increased risk of influenza are 299Gly allele and genotype Asp/Gly of TLR-4 and the combination of mutant genotypes Leu/Phe and Phe/Phe of TLR-3 with Asp/Gly of TLR-4 and Arg/Gln of TLR-2; for influenza-associated pneumonia – allele 412Phe and genotype Phe/Phe of TLR-3.

KEY WORDS: influenza, influenza-associated pneumonia, Arg753Gln polymorphism of the TLR-2 gene, Asp299Gly polymorphism of the TLR-4 gene, Leu412Phe polymorphism of the TLR-3 gene, genotype, allele

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INTRODUCTION

Influenza and other acute respiratory infections are the most large-scale diseases that occupy the leading place in the structure of infectious diseases and constitute up to 80-90% of all cases of infectious pathology [1]. According to WHO estimates, each year, influenza globally afflicts up to 500 million people, 2 million of whom die. The highest number of fatal cases in influenza is due to complications, the leading place (80-90%) among which belongs to pneumonia [2], that develops mainly in patients at risk (pregnant women, patients with diabetes, obesity, chronic diseases of the lungs and heart, people of senior age groups, etc.) [3, 4]. However, according to WHO, 30% of patients who were previously considered healthy may also have severe and complicated influenza, which necessitates further study of factors affecting the course and sequelae of the disease [4].

It is known that the individual susceptibility of the body to infections is determined by pathogenicity of a microorganism, factors of the environment and the state of the immune system. It is the innate immune system that plays a crucial role in protecting the body from pathogens whose recognition is based on the Toll-like (TLR) receptor family. Excitation of TLR during the infection of the respiratory tract leads to the activation of genes involved in the regulation of the inflammatory process, the innate mechanisms of protection against infectious agents, acquired immunity [5]. The genes that control the type of immune response, the sensitivity/resistance to the influenza, the propensity to various forms of the course and development of complications, include primarily the TLR-2, TLR-3, and TLR-4 genes [6]. These receptors are involved in the recognition of the viral structural proteins and ligands of gram-positive and gram-negative bacteria (TLR-2 and TLR-4), as well as of dlRNA, which is the product of replication and transcription of RNA- and DNA-genomic viruses (TLR-3) [7-9].

Recent studies have shown that the TLR dysfunction, associated with the polymorphism of their genes, leads to the disturbance in the recognition of pathogens and imbalance in the functioning of the congenital immunity system, thus increasing the sensitivity to infections and determining the

severity of the course of the infectious process, which acquires the nature of the systemic inflammatory response [10].

Taking into account the data of scientific literature, which indicates that susceptibility to infectious agents is genetically determined, the search for markers associated with the development of influenza and its complications among alleles of the TLR genes is a relevant task, the solution of which will allow us to predict the severity of the course and consequences of this pathology.

THE AIM

The aim of the research is to examine the prevalence and determine the prognostic significance of polymorphism Arg753Gln of the TLR-2 gene, Leu412Phe of the TLR-3 gene, Asp299Gly of the TLR-4 gene in influenza.

MATERIALS AND METHODS

In order to achieve this aim, 112 patients were examined, out of them – 63 with uncomplicated course of the disease and 49 with influenza-associated pneumonia. The patients were treated at the regional clinical infectious disease hospital (Poltava, Ukraine), in the epidemiologic season of 2009-2014, among them 55 women (49.1%), 57 men (50.9%), aged from 17 to 61 years (mean age 34.4 ± 1.38). Most of the examined patients (76.8%) are young and middle-aged people. The population control group to study the prevalence of the Arg753Gln polymorphism of the TLR-2 gene and Asp299Gly of TLR-4 was 90, for the Leu412Phe of TLR-3 – 80 apparently healthy residents of Poltava region.

The study included patients of both sexes, aged ≥18 years with laboratory- diagnosed influenza, who did not belong to the risk groups of the complications of this disease (pregnant women, patients with diabetes mellitus, obesity, chronic diseases of the lungs and heart, liver, kidneys, as well as persons of elderly age groups, etc.). Exclusion criteria were age <18 years, negative results of laboratory tests (serological and molecular biology) for influenza, the presence of risk factors for the development of complications.

All studies were conducted after the signing of the informed consent by patients. The study was approved by the Commission on Ethical Issues and Bioethics of the Higher State Educational Establishment of Ukraine «Ukrainian Medical Stomatological Academy» (Approval No. 350).

Influenza was diagnosed on the basis of characteristic clinical and epidemiological data and confirmed by the results of laboratory tests (serological and molecular biology). The A/H1N1 virus has been isolated in 39.3%, A/H3N2 – in 35.7%, A/H2N2 – in 0.9%, B – in 21.4%. Mixed-forms were represented by the combination of antigenic variants of influenza A viruses (H1N1 + H3N2) (1.8%), A/H1N1 and B (0.9%) viruses that were registered in patients with influenza-associated pneumonia.

The diagnosis of pneumonia was verified according to the recommendations of the European Respiratory Society (ERS, 2011) and the Order of Ministry of Public Health of Ukraine No. 128 as of March 19, 2007 «On Approval of Clinical Protocols for the Provision of Medical Service in the Specialty «Pulmonology».

To determine polymorphism ARG753GLN of TLR-2, Asp299Gly of TLR-4, and Leu412Phe of TLR-3, we collected the samples of patients peripheral blood (2 ml) in the vials with ethylene diamine tetraacetic acid (EDTA) which were stored at -20°C and transported for testing in the laboratory (Research Institute of Genetic and Immunological Foundations of Pathology and Pharmacogenetics, «Ukrainian Medical Stomatological Academy» Poltava, Ukraine). Genomic DNA was isolated using the «Kit for DNA/RNA isolation from serum or plasma» (LitTech, Russia).

Polymorphic area Arg753Gln of the TLR-2 gene and Asp299Gly of TLR-4 were amplified at the «Tertsik» amplifier («DNA-technology», Russia), by PCR using specific oligonucleotide primers for the TLR-2 gene: 753TLR2F, 5'-GAGTGGTGCAAGTATGAACTGGA-3'; and 753TLR2R, 5'-TCCCAACTAGACAAAGACTGGTCT-3', for TLR-4: 299TLR4F, 5'-GATTAGCATACTTAGACTACTACCTCCATG-3'; and 299TLR4R, 5'-GATCAACTTCTGAAAAAGCATTCCCAC-3'.

The amplification programs for the TLR-4 and TLR-2 genes included: initial denaturation at 95°C for 5 minutes, 32 cycles: 95°C for 30 seconds, ignition at 58°C, 60 seconds, chain elongation at 72°C, 60 seconds, the program was completed with final elongation at 72°C, 3 min.

To identify the alleles of the TLR-4 gene, we applied restriction analysis of amplicons using restriction endonuclease Bsp19 (SibEnzim, Russia), for TLR-2 – endonuclease restriction Pst I (SibEnzim, Russia) at 37°C. As a result of the restriction, fragments of 263 bp and 222 bp were obtained for the Asp299Gly polymorphism and 300 bp for Arg753Gln.

To determine the alleles of the Leu412Phe polymorphic site of the TLR-3, genomic DNA gene was isolated from peripheral blood leukocytes using the reagent kit of «DNA-EXPRESS-blood» (LitTech, Russia). The amplification program for the TLR-3 gene included initial denaturation at 93°C for 60 seconds, 35 cycles: 93°10 seconds, ignition at a specific temperature of 64°C for each pair of primers, 10 seconds, chain elongation at 72°C, 20 seconds, final elongation at 72°C, 60 seconds.

The breakdown products of the polymorphic site of the TLR-4, TLR-2 genes were detected by electrophoresis in the 3% agarose gel in 1 TBE (50 mM tris-H_3PO and 2 mM EDTA, PH = 8.0), for 2 hours at a voltage of 2 V 1 cm of gel; TLR-3 – in 1 x tris-acetate (TAE) buffer, prepared from 50 x TAE buffer (0.04M tris-acetate, 0.002M EDTA, pH = 8.3) at a voltage of 10-15 V per 1 cm of gel. Gels were stained with 1% solution of ethidium bromide with subsequent visualization of the results in UV light.

In mathematical processing of the data, we used the software «Statistica for Windows 7.0» (StatSoft Inc, USA) and MS Excel. The distribution of investigated polymorphic genotypes was checked for compliance with the Hardy-Weinberg equilibrium using the χ^2 criterion. Comparison of the frequencies of genotypes and alleles between the studied groups was conducted using Fisher's exact test. The differences were considered reliable at $p<0.05$. The relative risk of disease and complications were evaluated using OR

Table 1. Distribution in frequencies of genotypes and alleles of the Leu412Phe polymorphism of the TLR-3 gene among patients with influenza, influenza-associated pneumonia and healthy subjects, abs. number (%)

Genotype and alleles	Healthy subjects n = 80	Groups of patients		F p≤	OR (95 % CI)
		uncomplicated influenza n = 63	influenza-associated pneumonia n = 49		
Leu/Leu	36 (45.0)	35 (55.5)	16 (32.7)	0.2403 a 0.1973 b 0.0216 d	1.53 (0.79-2.97) a 0.59 (0.28-1.24) b 0.39 (0.18-0.84) d
Leu/Phe	40 (50.0)	25 (39.7)	24 (48.9)	0.2399 a 0.9999 b 0.3437 d	0.66 (0.34-1.28) a 0.96 (0.47-1.95) b 1.46 (0.69-3.1) d
Phe/Phe	4 (5.0)	3 (4.8)	9 (18.4)	1.00 a 0.0309 b* 0.0299 d*	0.95 (0.2-4.41) a 4.28 (1.24-14.75) b 4.5 (1.15-17.65) d
Leu	112 (70.0)	95 (75.4)	56 (57.1)	0.3519 a 0.0434 b 0.0042 d	1.31 (0.77-2.23) a 0.57 (0.34-0.96) b 0.44 (0.25-0.77) d
Phe	48 (30.0)	31 (24.6)	42 (42.9)	0.3519 a 0.0434 b* 0.0042 d*	0.76 (0.45 – 1.29) a 1.75 (1.04-2.95) b 2.3 (1.3-4.06) d

Note: here and in Table 1, 2, p is the level of significance obtained by Fischer's exact test for differences in the frequencies of genotypes and alleles between: a – healthy subjects and patients with uncomplicated influenza; b – healthy subjects and patients with influenza-associated pneumonia; d – patients with uncomplicated influenza and influenza-associated pneumonia.

rate with defining 95% confidence interval (CI). The indicator OR=1 was considered as a lack of association; OR>1 – as a positive association ("predisposition"), OR<1 – as a negative association of allele or genotype with the disease.

RESULTS AND DISCUSSION

As a result of the molecular-genetic examination of 112 patients with influenza, the following genotypes of the studied TLRs were obtained: TLR-2 – Gln753Gln, Arg753Gln; TLR-3 – Leu412Leu, Leu412Phe, Phe412Phe; TLR-4 – Asp299Asp, Asp299Gly. The distribution of genotypes corresponded to the expected Hardy-Weinberg equilibrium in the groups of patients with influenza, influenza-associated pneumonia and healthy people for all investigated polymorphic loci.

In analyzing the results of the study, it was found that among the patients with influenza, the Leu412Phe polymorphism of the TLR-3 gene, Asp299Gly of TLR-4, as well as their combination with Arg753Gln of TLR-2 were determined more often, as compared with apparently healthy ones. Distribution in frequencies of genotypes and alleles of the Leu412Phe polymorphism of TLR-3 among the examined groups are presented in Table 1.

As can be observed from the data presented in Table 1, the prevalence of the mutant homozygous genotype Phe/Phe of TLR-3 and allele 412Phe was significantly higher among patients with influenza-associated pneumonia by 3.7 and 1.4 times (p=0.03 and p=0.04), as compared with healthy subjects and by 3.8 and 1.7 times (p=0.02 and p=0.004) – as compared to patients with influenza. The presence of the 412Phe mutant allele in the genome of patients with influenza and homozygous genotype Phe/Phe by 2.3 and 4.5 times increases the risk of influenza-associated pneumonia (OR=2.3; 95% CI: 1.3-4.06 and OR=4.5; 95% CI: 1.15-17, 65, respectively).

A comparative analysis of the frequencies in genotypes and alleles of the Asp299Gly polymorphism of the TLR-4 gene between the examined groups of patients and the healthy subjects is presented in Table 2.

Regarding the Asp299Gly polymorphism of the TLR-4 gene, it has been found that frequency of the heterozygous genotype Asp/Gly and the mutant allele 299Gly is higher among patients with influenza by 3.8 (p<0.05), with influenza-associated pneumonia by 4.3 times (p=0.03), as compared to healthy subjects. In carriers of the 299Gly allele of the TLR-4 gene, there was a higher risk of influenza by 4.0 times (OR=4.0; 95% CI: 1.04-15.39) than in those with the Asp299Asp genotype.

When comparing the frequencies of genotype and alleles of the Arg753Gln polymorphism of TLR-2, there were no statistically significant differences between the examined groups. Frequency of the heterozygous genotype Arg/Gln of TLR-2 among the patients with influenza was 4.8%, influenza-associated pneumonia – 6.1%, healthy – 3.3% (p>0.05), and the 753Arg alleles – 2.4%, 3.1% and 1.7% (p>0.05) respectively.

Table 2. Distribution of genotypes and alleles of the Asp299Gly polymorphism of the TLR-4 gene among patients with influenza, influenza-associated pneumonia and healthy subjects, abs. number (%)

Genotype and alleles	Healthy subjects n=90	Groups of patients		p (F)	OR (95 % CI)
		uncomplicated influenza n=63	influenza-associated pneumonia n=49		
Asp/Asp	87 (96.7)	55 (87.3)	42 (85.7)	0.0516 a 0.0334 b 0.9999 d	0.24 (0.06-0.93) a 0.21 (0.05-0.84) b 0.87 (0.29-2.6) d
Asp/Gly	3 (3.3)	8 (12.7)	7 (14.3)	0.0516 a* 0.0324 b* 0.9999 d	4.22 (1.07-16.59) a 4.95 (1.22-20.13) b 1.15 (0.38-3.41) d
Gly/Gly	0 (0.0)	0 (0.0)	0 (0.0)	0	0
Asp	177 (98.3)	118 (93.6)	91 (92.9)	0.0561 a 0.0367 b 0.9999 d	0.25 (0.06-0.96) a 0.22 (0.06-0.87) b 0.88 (0.31-2.52) d
Gly	3 (1.7)	8 (6.4)	7 (7.1)	0.0561 a* 0.0367 b* 0.9999 d	4 (1.04-15.39) a 4.54 (1.15-17.97) b 1.13 (0.4-3.24) d

The absence of mutant homozygous genotypes Arg753Arg of TLR-2 and Gly299Gly of TLR-4 was noted for both patients with influenza and healthy subjects, which corresponds to the data of scientific literature about the low frequency of their prevalence in the population [11, 12].

Combinations of mutant genotypes of TLR-2, TLR-3, TLR-4 was only registered in patients with influenza (11.1%, p<0.01) and influenza-associated pneumonia (14.3%, p<0.003), and it was not detected in healthy subjects. Most (80.0%) combinations included polymorphically modified genotypes of TLR-3. The most common, both among patients with influenza and influenza-associated pneumonia, was the combination of the heterozygous genotype Leu/Phe of TLR-3 with Asp/Gly of TLR-4 (6.3% and 10.2% respectively).

It has been established that the presence of mutant genotypes of TLR-3 in the genome in combination with TLR-2 and TLR-4 by 15.0 times (OR=15.0; 95% CI: 1.83-286.93) increases the risk of influenza development in the carriers of these mutations.

Thus, we have proved that the presence of PheLeu412 polymorphisms of the TLR-3 gene, Asp299Gly of TLR 4 and their combinations with Arg753Gln of TLR-2 make it possible to predict the risk for development of influenza and influenza-associated pneumonia. Previous studies have shown that polymorphism of TLR genes causes the predisposition to a variety of diseases, as well as the severity of their course. Hence, at present, the Asp299Gly polymorphism of the TLR-4 gene is associated with the development of hematogenous osteomyelitis, systemic candidiasis, bronchial asthma, sepsis, caused by gram-negative bacteria, respiratory viral infections in children [13-17].

The association between Arg753Gln of TLR-2 has been established with increased risk for development of tuberculosis, acute rheumatic fever in children, septic shock, caused by gram-positive bacteria, CMV-infections in patients after liver transplantation [18-21]. In the study by Nachtigall I. et al. [22], the connection between the Arg753Gln polymorphism of the TLR-2 gene and the Asp299Gly of the TLR-4 gene with rapid progression and severe sepsis has been proven.

A number of scientific studies link the SNP Leu412Phe polymorphism of TLR-3 with the development of subacute sclerosing panencephalitis in the cortex, myocarditis and dilated cardiomyopathy in enterovirus infection, severe course of atypical pneumonia with the development of GERD, induced by the coronavirus [23-25].

Thus, active research of genetic variability of TLR in the last decade produces evidence that polymorphism of single nucleotides through the formation of specific gene alleles makes an important contribution to the individual features of development of protective reactions, as well as susceptibility to a variety of diseases.

CONCLUSIONS

1. The frequency of the Asp/Gly heterozygous genotype of TLR-4 in patients with influenza constituted 12.7%, with influenza-associated pneumonia – 14.3%, which exceeded the indicators of population control by 3.8-4.3 times (3.3 %, p<0.05).
2. The homozygous genotype Phe/Phe of TLR-3 in patients with influenza-associated pneumonia was determined with the frequency of 18.4 %, which exceeded the rates of patients with uncomplicated influenza (4.8%, p=0.02) and healthy subjects (5.0%, p=0.03).
3. The combination of mutant genotypes of TLR-2, TLR-3, TLR-4 was not detected in healthy subjects and was determined in patients with influenza and influenza-associated pneumonia with the frequency from 11.1% to 14.3% (p<0.05).
4. The presence of polymorphically modified genotypes of TLR-4, TLR-3 and their combinations with TLR-2 allows

us to predict the risk for development of influenza and influenza-associated pneumonia. Markers of increased risk of influenza are the 299Gly allele and the Asp/Gly genotype of TLR-4 (OR=4.0 and OR=4.22, respectively) and the combination of mutant genotypes Leu/Phe and Phe/Phe of TLR-3 with Asp/Gly of TLR-4 and Arg/Gln of TLR-2 (OR=15.0); influenza-associated pneumonia – the 412Phe allele and the Phe/Phe genotype of TLR-3 (OR=2.3 and OR=4.5, respectively).

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