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**POLYMORPHISM PREVALENCE OF TLR 9
GENE IN PATIENTS WITH INFECTIOUS
MONONUCLEOSIS CAUSED BY EPSTEIN-BARR
VIRUS**

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Introduction

Today it is known that immune reply at infectious pathology develops due to congenital immunity factors being the hereditary defense system from pathogens which provides recognition and elimination of infectious agents in the first hours of their intrusion and signals production determining specific immune reply formation. [1]. Toll-like receptors (Toll-like receptors, TLR) are the main signal receptors expressing in the middle of the cells and on their surface: neutrophils, macrophages, dendritic cells, endothelial and epithelial cells as well as natural killers (NK) [2-4].

First the gene encoding TLR was described in 1985 by Cristian Nusline-Folhardt in fruit-flies [5]. The author found out that one and the same gene encodes the formation of dorsoventral polarity in embryogenesis and resistance to fungal infection in fruit flies, the researcher call it amazing (Germ. «toll») thus calling the whole group TLR. Human gemological gene encoding TLR 4 was first described by R. Medzhitov and C. Janeway in 1997 [1, 3-5].

Modern studies allowed the establishment of one of the main reasons which can influence on the TLR immune reply in infectious pathology, the genes polymorphism encoding them is meant. More data are accumulated which prove that the polymorphism of single nucleotides (single-nucleotide polymorphism SNP) at the expense of specific allele genes formation makes significant contribution in phenotypes differences among people including individual peculiarities of defensive reactions development as well as susceptibility to a number of diseases [4-6].

Differences in genes controlling the organism defense reactions can define various characters of inflammatory reply course and specific immune reactions in contact with alien structures. First of all it concerns the regulatory molecules genes providing the initial stages of the inflammatory reaction development: the pathogen recognition, the passage of intracellular activation signal and mediators of the inflammatory reaction synthesis [7-9].

The genes polymorphism assumes that some variants structurally differing from the copy of one and the same protein can be copied from one and the same gene, part of the copied variants from them is either non-active or can have adverse function [7, 10-12]. As for TLR-polymorphism it is stated that it can cause recognition of infectious agents violation and congenital immunity

system functioning misbalance and manifest in sensitization to infections and the development of chronic inflammatory processes [8, 10, 11, 13, 14]. Other studies prove that TLR genes polymorphism at the expense of expressed immune reply violations can stipulate the severity of infectious process course which regenerates to system inflammatory reply as well as tanatogenesis development [10, 15, 16]. Though the studies concerning prevalence of various types of genes polymorphism in patients with EBV-infection in modern scientific literature was carried out insufficiently.

Aim of the study

Investigate -1486T/C polymorphism frequency of the gene TLR-9 in patients with infectious mononucleosis caused by Epstein-Barr virus.

Materials and methods

The work was carried out at the department of general and clinical immunology and allergology of the medical faculty of V.N.Karazin Kharkov National University and clinical bases of the department, namely Kharkov Regional clinical infectious hospital in 2009-2016 within the framework of scientific-research theme “The study of immune, autoimmune and metabolic violations role in pathogenesis and consequences of the infectious process caused by herpes-viruses” the № of state registration is №0112U005911.

The studies on -1486T/C polymorphism definition of the gene TLR-9 was carried out for 52 patients with infectious mononucleosis (IM). Among them women – 31 (59,6%), men – 21 (40,4%) in the age from 18 to 34 years old. The control group for -1486T/C polymorphism prevalence study of the gene TLR-9 consisted of 40 healthy donors. Average age comprised 24,2±2,4 years old at a range from 18 to 44.

Allotment of the patients and healthy persons according to the age and sex are presented in table 1.

As it is seen from table 1, people of the young age prevailed in the study groups (89,1% and 10,9% consequently).

During the study the provision of Helsinki Declaration of the World Medical Association, ethical code of the Ukrainian doctor, informing of the patients about the character of the study were adhered. According to the International statistic classification of the diseases of the tenth revision (version 2007) clinical diagnosis in patients included into the research was defined as B27. In patients over 18 years old the verification of IM clinical diagnosis was carried out according to Zh.I.Vosianova and coauthors (2001) recommendations. Clinical examination of the patients with IM assumed the study of the complaints, epidemiologic anamnesis, disease and life anamnesis, objective examination of the patients, general clinical analyses of blood and urine in dynamics.

All the patients were examined paying attention on the state of periphery lymphatic nodes, chest and abdomen organs as well as cardiovascular system indices (pulse, arterial pressure, heart auscultation), thermometry, ultrasound research and radiography were also done.

Table 1. Allotment of the examined according to the age and sex, abs. number, (%)

Age, year	Patients with IM (n=52)				Control (n=40)			
	men		women		men		women	
	abs	%	abs	%	abs	%	abs	%
18-24	13	61,9	23	74,2	10	45,4	9	50
25-34	8	38,1	9	25,8	6	27,3	5	27,8
35-44	-	-	-	-	6	27,3	4	22,2
Total:	21	40,4	31	59,6	22	55	18	45

All the patients were carried out the accepted and special biochemical studies: clinical analysis of blood, urine, biochemical studies in the disease dynamics.

The patients with EBV-infection serum received during the exacerbation of the disease served the material of the study. Blood samples (10 ml) for the study were drawn on an empty stomach from ulnar vein into the sterile test tube of "Eppendorf" type.

For EBV DNA excretion by PCR method with the reverse transcription with hybridization-fluorescent detection of amplification products reagent kit «Amplisense» (Russia) was used. DNA excretion from the samples was carried out with the help of the kit for DNA revealing of the «Miniprep» (Silex M, Russia) firm using DNA sorption method on the sorbent according to Boom R. and coauthors., 1990. DNA amplification was carried out using the kit «DNA amplification» (Silex-M, Moscow) on the BIC amplifier.

The genomic DNA was excreted with the help of the "Set for DNA/RNA excretion from blood serum or plasm" (Lit Tech, Russia).

Polymorph section -1486 T/C, rs187084 of the gene TLR9 was carried out with the help of amplification by PCR method in real time regime in the way of restriction fragments length definition (RFLP)-PCR using NcoI restrictase and further oligonucleotide primers: 5'-GAGGACAACGAAATCCTGTTGGGCA-3', 5'-GTCGACGGTGGAGATACTGCTGAGG-3'.

DNA primers to genes-targets were selected using the GeneRunner v.3.0 program and synthesized by the firm «Litex» (Russia).

Electrophoresis separation of amplicons was carried out by the method of horizontal electrophoresis in the direction from cathode (-) to anode (+) in 3% agarose gel under 10-15 V tension on 1 sm of gel. 1x tris-acetate (TAE) buffer was used for electrophoresis which was prepared from 50x TAE buffer (0,04M tris-acetate, 0,002M EDTA, pH=8,3). Gels were stained by 1% ethidium bromide solution. DNA fragments analyzed came out in the way of red strips upon irradiation by UV-light with 310 nm wavelength.

The results of the study were processed by the method of variation and correlation statistics using «Statistica 10.0 for Windows» program. Arithmetic

averages (M), mean square deviation (σ), mean error of the arithmetic mean (m) were calculated for each variation series. Methods of parametric and non-parametric statistics were also used. Quantitative and qualitative analysis of intrasystem and intersystem correlation connections were carried out with the use of correlation structures method and Vald sequential analysis.

The allotment of the genotypes was defined using Hardy-Vineberg law, the law of population genetics, allowing to estimate the population risk of genetically-determined diseases as each population had its own set of allele fund and, consequently, different frequency of unfavorable alleles.

The allotment of polymorphic genotypes under study was verified on the conformity to Hardy-Vainberg balance with the help of χ^2 criterion.

The comparison of alleles frequencies and genotypes among the groups under study was carried out by the analysis of linking tables 3 and 2 with the help of Fisher test. Odds ratio (OR) was considered for the comparison of variants frequencies in non-connected groups with the definition of 95% of confidence interval (CI). Relevant risk of the disease and complications development was estimated with the help of OR index. The OR meaning and 95% of confidence interval were considered using Odds ratio calculator program (http://www.medcalc.org/calc/odds_ratio.php). The OR=1 index was considered as association absence; OR>1 – as positive association («tendency»), OR<1 – as negative allele association or genotype with the disease.

Results

The following -1486T/C genotypes of the gene TLR-9 – TT, TC, CC were received as a result of molecular-genetic examination of 51 patients with IM.

The allotment frequency of the discovered -1486T/C SNP genotypes of the gene TLR-9 in patients with IM was the following: TT genotype – 17 % (9 patients), TC – 46 % (24 patients) and CC – 37 % (19 patients). In patients of the control group wild type of TT genotype was found in 40,0% (16 patients), heterozygous TC genotype - in 45,7% (18 patients), while homozygous CC genotype was found in 14,3% (6 patients).

The occurrence frequency of -1486T/C genotype of the gene TLR-9 - TT, TC, CC in percentage (P) \pm standard deviation in the percentage (SdP – for binomial distribution) and the results of Student t-test are presented in table 2.

Table 2. 1486 T/C genotype occurrence frequency of the gene TLR-9 – TT, TC, CC, P (%) \pm SDp

TLR-9 rs187084 C/T	Patients with IM (n=52)	Control (n=40)
TT	17 \pm 37 ¹	40 \pm 49
TC	46 \pm 50	45 \pm 50
CC	37 \pm 48 ¹	15 \pm 36

Note: ¹ Reliable probability from the control group on the level p<0,05.

As it is clear from the table the occurrence frequency of TT genotype TT -1486T/C of the gene TLR-9 differed by statistical probability in comparison with analogue data of the control group and comprised 17±37 versus 40±49 ($p<0,05$). The occurrence frequency of TC genotype – 1486T/C of the gene TLR-9 did not differ by statistical probability from the control group indices – 46±50 versus 45±50 ($p>0,05$). Though the given index sufficiently differed statistically from the indices of the control group for CC genotype and comprised 37±48 versus 15±36 ($p<0,05$).

The allotment of occurrence frequencies of the genotypes for the patients with IM and the patients of the control group according to the results of the statistical analysis are presented in table 3.

Table 3. 1486 T/C genotype occurrence frequency of the gene TLR-9 in patients with IM

TLR-9 rs187084 C/T	Patients with IM (n=52)	Control (n=40)	Fisher criterion	OR (odds ratio)	95 % CI
TT	9 (17%)	16 (40%)	$P<0,05$	0,31	0,12 - 0,82
TC	24 (46%)	18 (45%)	$P>0,1$	1,05	0,46 - 2,4
CC	19 (37%)	6 (15%)	$P<0,05$	3,26	1,16 - 9,2

Analyzing the occurrence frequencies allotment of -1486 T/C genotypes of the gene TLR-9 in patients with IM statistically significant differences of the level $p<0,05$ were stated for TT and TC genotypes in the group of the patients with IM and the control group. Thus for homozygous TT genotype this index comprised 17% versus 40% ($p<0,05$), for CC genotype - 37% versus 15% ($p<0,05$), while for heterozygous TC genotype the allotment of frequencies had no statistically significant difference in comparison with the control group indices and was found with the same frequency in the groups of the patients under study 46% versus 45% ($p>0,1$).

According to the calculated index of the odds ratio presence of homozygous CC genotype in the genome of the patients with IM is specific for the patients with IM (CI:1,16-9,2 i OR=3,26, consequently) allowing to estimate it as a positive association in comparison with the received indices for homozygous TT genotype (CI:0,12-0,82 i OR=0,31, consequently) and heterozygous TC genotype (CI:0,46-2,4 i OR=1,05), which are estimated as a negative TT genotype association with IM and absence of associations with IM for TC genotype.

Discussion

Thus, the analysis of the received results of -1486T/C polymorphism of the gene TLR-9 allowed the detection of three main genotypes – TT, TC, CC. The study of the occurrence frequency of separate genotypes revealed the dominance of CC and TT genotypes in comparison with heterozygous TC genotype. The study of occurrence

frequency allotment of -1486T/C polymorphism of the gene TLR-9 for various genotypes demonstrated the specificity of changes for CC genotype in patients with IM and absence of such ones for TT and TC genotypes.

Our research as for 1486 TLR-9 C/C polymorphism detection connected with the diseases with IM confirms an important role of TLR-mediated signal in pathogenesis of EBV-infection. The study of polymorphism among receptors taking part in virus recognition will be necessary for genetic background detection connected with the risk of contamination, the disease course and possible IM consequences. It will make the detection of risk group possible among the patients and to conduct apropos therapy.

Conclusions

The following conclusions can be done according to the results of the statistical analysis:

1. It was proved that -1486T/C polymorphism of the gene TLR-9 is found more often in patients with IM.
2. The allotment of occurrence frequencies of -1486T/C polymorphism of the gene TLR-9 allowed the establishment of CC genotype association with manifest forms of IM.

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PREVALENCE OF POLYMORPHISM OF THE TLR 9 TYPE GENE IN PATIENTS WITH INFECTIOUS MONONUCLEOSIS CAUSED BY EPSTEIN-BARR VIRUS

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Annotation. The prevalence of polymorphism -1486 T/C of TLR-9 gene in 52 patients with infectious mononucleosis (IM) caused by the Epstein-Barr virus was studied. Based on the results obtained, three main genotypes -1486 T/C of the gene TLR-9-TT, TC, CC, were identified. The study of the frequency of occurrence of individual genotypes in patients with IV revealed dominance of CC and TT genotypes in comparison with the control group. The study of the frequency distribution of the -1486 T/C polymorphism of the TLR-9 gene for different genotypes showed the specificity of the changes for the CC genotype in patients with IM and the absence of such changes for the TT and TC genotypes.

Aim of research. To establish the frequency of the polymorphism -1486 T/C of the TLR-9 gene in patients with IM caused by the Epstein-Barr virus.

Materials and methods. A study to determine the polymorphism -1486 T/C of the TLR-9 gene was conducted in 52 patients with IM. Among them, women - 31 (59,6%), men - 21 (40,4%) at the age of 18 to 34 years. The control group for studying the prevalence of the polymorphism -1486 T/C of the TLR-9 gene was 40 healthy donors. The mean age was 24,2±2,4 years, with a range from 18 to 44 years. To detect DNA VEB using the reverse transcription PCR method with hybridization-fluorescent detection of amplification products, Amplisens (Russia) reagent kits were used. The

polymorphic region -1486 T/ C, rs187084 of the TLR9 gene was studied by real-time PCR amplification by determining the length of the restriction fragments-PCR using NcoI restriction enzyme and oligonucleotide primers.

Results. An analysis of the results of polymorphism -1486 T/C of the TLR-9 gene made it possible to identify three main genotypes - TT, TC, CC. The allotment frequency of the discovered -1486T/C SNP genotypes of the gene TLR-9 in patients with IM was the following: TT genotype – 17 % (9 patients), TC – 46 % (24 patients) and CC – 37 % (19 patients). In patients of the control group wild type of TT genotype was found in 40,0% (16 patients), heterozygous TC genotype - in 45,7% (18 patients), while homozygous CC genotype was found in 14,3% (6 patients). An investigation of the frequency of occurrence of individual genotypes revealed the dominance of CC and TT genotypes in comparison with the heterozygous genotype of the TC. The study of the frequency distribution of the -1486 T/C polymorphism of the TLR-9 gene for different genotypes showed the specificity of the changes for the CC genotype in patients with IM and the absence of such changes for the TT and TC genotypes. Analyzing the occurrence frequencies allotment of -1486 T/C genotypes of the gene TLR-9 in patients with IM statistically significant differences of the level $p < 0,05$ were stated for TT and TC genotypes in the group of the patients with IM and the control group. Thus for homozygous TT genotype this index comprised 17% versus 40% ($p < 0,05$), for CC genotype - 37% versus 15% ($p < 0,05$), while for heterozygous TC genotype the allotment of frequencies had no statistically significant difference in comparison with the control group indices and was found with the same frequency in the groups of the patients under study 46% versus 45% ($p > 0,1$). According to the calculated index of the odds ratio presence of homozygous CC genotype in the genome of the patients with IM is specific for the patients with IM (CI:1,16-9,2 i OR=3,26, consequently) allowing to estimate it as a positive association in comparison with the received indices for homozygous TT genotype (CI:0,12-0,82 i OR=0,31, consequently) and heterozygous TC genotype (CI:0,46-2,4 i OR=1,05), which are estimated as a negative TT genotype association with IM and absence of associations with IM for TC genotype. Our study on polymorphism -1486 TLR-9 C/C revealed a correlation with the disease of IM, which confirms the important role of TLR-mediated signaling in the pathogenesis of EBV infection. Investigation of polymorphism among the receptors involved in virus recognition is necessary to determine the genetic background associated with the risk of infection, the course of the disease and the possible consequences of MI. This will allow to identify risk groups among patients and to conduct timely therapy.

Conclusions. 1. It was proved that in patients with IM, the polymorphism -1486 T/C of the gene TLR-9 was detected more reliably than in the control group. 2. Distribution of frequency of occurrence of polymorphism-1486 T/C of gene TLR-9 allowed to reveal association of genotype CC with manifest forms of IM.

Keywords: infectious mononucleosis, Epstein-Barr virus,
Toll-like receptors, polymorphism