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Investigation of rs11536889 + 3725G/C Polymorphism of the *TLR4* Gene in Patients with Autoimmune and Chronic Viral Hepatitis C

A. M. Kucherenko[a], L. V. Moroz[b], T. I. Bevz[b], V. I. Bulavenko[b], Y. G. Antypkin[c], V. S. Berezenko[c], M. B. Dyba [c], V. M. Pampukha[a], O. V. Gorodna[a], and L. A. Livshits[a, *]

[a] *Institute of Molecular Biology and Genetics of NASU, Kyiv, Ukraine*

[b] *National Pirogov Memorial Medical University, Vinnytsia, Ukraine*

[c] *SI "Institute of Pediatrics, Obstetrics and Gynecology named after academic E.M. Lukyanova of NAMS of Ukraine", Kyiv, Ukraine*

**e-mail: livshits@imbg.org.ua*

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Abstract—Toll-like receptor 4 (encoded by *TLR4* gene) has a variety of functions, including tissue homeostasis, regulation of cell death and survival via activation of signaling pathways which lead to interferon regulatory factor 3 (IRF-3) activation and type I interferon production. TLR4 may have a crucial role in pathogenesis of complex and infectious diseases. Functional polymorphism *TLR4* +3725G/C substitution (rs11536889) leads to faster transcript degradation and receptors number decrease. The study investigated *TLR4* rs11536889 genotype and allele distribution in healthy volunteers from Ukraine (n = 155), autoimmune hepatitis (AH) children (n = 56) and chronic hepatitis C (CHC) adult patients with various fibrosis severity stages (n = 78). Genotyping was performed by allele-specific PCR. The obtained genotype frequencies in Ukrainian population were: GG genotype—0.813, GC—0.168, CC—0.019 and showed no significant deviation from the ones expected according to Hardy-Weinberg equilibrium. AH and CHC patients were divided according to METAVIR fibrosis score into two groups—with stages F1–F2, and with F3–F4. The frequency of rs11536889 C allele carriers were higher in the group of AH patients with F3–F4 (0.179) comparing to patients with F1–F2 (0.071). This data did not reach the threshold for significance but showed a trend toward association between C allele carriers and higher fibrosis degree. Moreover, the significantly ($p < 0.05$) higher frequency of C allele carriers was observed in CHC patients with higher fibrosis degree (0.400) compared to patients with lower degree (0.057). Severe liver damage risk in such individuals is 11 increased (OR = 11.11, 95% CI: 2.70–45.66). Thus, *TLR4* rs11536889 C allele is associated with higher level of fibrotic liver damage in patients with chronic hepatitis.

Keywords: autoimmune hepatitis, viral hepatitis C, liver fibrosis, single nucleotide polymorphism, Toll-like receptor TLR4

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INTRODUCTION

Today the problem of chronic diffuse liver disorders is extremely relevant taking in consideration the increase in the incidence of chronic hepatitis (CH), which as a result of the progressive pathological process in the liver can lead to the development of severe complications with high mortality – liver cirrhosis (LC) and hepatocellular carcinoma (HCC). Viral hepatitis C is the most common diffuse liver disease in adults. About 2% of the world population is infected by it. The incidence of viral hepatitis C is 3 to 5% (World Health Organization. Global hepatitis report, 2017). The prevalence of autoimmune hepatitis is low in the world and ranges from 0.1 to 1.9 per 100000 per year, however, the rapid progression with the formation of liver cirrhosis causes the relevance of this problem.

Progressive course and inadequate efficacy of etiopathogenic therapy of CH leads to a more in-depth study of the pathogenetic mechanisms of their development and progression, the search for factors that influence the course and therapeutic efficacy.

In order to find pathogenic factors in the development of fibrosis and to identify genetic markers associated with the individual characteristics of this process, a large-scale studies using the GWAS (Genome-Wide Associations Study) strategy were conducted, which reviled the statistically significant association of the polymorphic variant +3725G/C (rs11536889) of the *TLR4* gene with stage of fibrosis [1, 2]. Several studies have also shown that this polymorphous variant of the *TLR4* gene is associated with the risk of development and the degree of progression of a number of pathological conditions [3–6].

The *TLR4* gene encodes a protein that belongs to the family of Toll-like receptors (TLRs – Toll-like receptors). Toll-like receptors are transmembrane proteins that play a key role in the manifestation of early innate immunity. They recognize a wide range of pathogen-related molecular structures, such as bacterial lipopolysaccharide, viral proteins, nucleic acids, and endogenous structures associated with damage (https://www.genecards.org/cgi-bin/carddisp.pl?gene=TLR4).

TLR4 is functionally involved in such processes as maintaining tissue homeostasis, regulating cell death and activating signaling pathways which, in turn, induce interferon products. The functional polymorphism of the *TLR4* gene, the substitution of +3725G/C (rs11536889), affects the stability of mRNA, the level of expression and signaling induced by lipopolysaccharides and other ligands, and also leads to a decrease in the number of receptors [7]. Thus, the polymorphisms of the *TLR4* gene significantly affect the immune response and the characteristics of the development of infectious, allergic and autoimmune diseases [8].

In previous studies, several polymorphic markers have been identified that have shown a significant association with the development of fibrosis in patients with chronic viral hepatitis C. The authors emphasized the further studies to clarify the role of individual genetic markers and, in particular, the rs11536889 of the *TLR4* gene as a genetic risk factor of the development of liver fibrosis in patients with hepatitis are necessary [1, 2]. The aim of our work was to study the association of polymorphism rs11536889 +3725G/C of the *TLR4* gene with clinical manifestations of fibrous changes in adult patients with chronic viral hepatitis C and children with autoimmune chronic hepatitis.

MATERIALS AND METHODS

Patients

The allelic polymorphism of the *TLR4* gene rs11536889 (+3725G/C) and allele distribution was investigated in a population sample of 155 healthy unrelated blood donors from different regions of Ukraine. This analysis was also performed in a group of children with autoimmune hepatitis (AH) ($n = 56$) and patients with chronic hepatitis C (HCV) ($n = 78$) with different stages of liver fibrosis, which were provided by Ukrainian medical institutions: Vinnytsia National Medical University named after. M. Pyrogov, LTD Ukrainian Therapeutic Diagnostic Center (Kyiv), SI Institute of Pediatrics Obstetrics and Gynecology named after Academician O.M. Lukyanova of NAMS of Ukraine (Kyiv).

In the Center of Pediatric Hepatology, the Institute of Pediatrics, Obstetrics and Gynecology named after Academician O.M. Lukyanova of NAMS of Ukraine, in the period from 2016 to 2018, 56 children aged 1 to 18 years old were diagnosed with autoimmune hepatitis (AH).

Diagnosis of hypertension is based on international guidelines for the study of liver diseases (EASL) Clinical Practice Guidelines: Autoimmune Hepatitis, 2015. All children, in addition to general clinical trials (general blood and urine tests, biochemical blood tests, immunological examination), have been tested for serum autoantibodies (ANA, Anti-LKM-1, Anti-SMA, anti-LC1). A virological study to exclude the viral nature of the disease was carried out using the following tests: anti-HAVIgM, HBsAg, anti-HBsIgM, anti-HBsIgG, PCR DNA HBV, anti-HCV IgG and PCR RNA HCV. Children with metabolic diseases, insufficiency of alpha-1 antitrypsin and Wilson's disease were excluded from study.

In order to verify the diagnosis, all patients underwent a puncture liver biopsy with a morphological and immunohystochemical study of the liver biopsy (with determination in the tissue of the liver of expression of CD138 – the cluster of differentiation 138: membrane protein, which is used as an immunological marker of the plasma cells). These studies were performed using a microscope "OLYMPUS BN-2."

Determination of rigidity of the liver (elastometry) was performed on the scanner Radmir ULTIMA. To interpret the obtained parameters and determine the stage of fibrosis, evaluated on the METAVIR scale [9] (for children), according to which the F0 fibrosis stage corresponded to elastography values below 5.8 kPa, F1 fibrosis stage, minimal changes of 5.8–7.2 kPa, F2 fibrosis stage – moderate manifestations of 7.2–9.5 kPa, F3 fibrosis stage – was defined as significant changes of 9.5–12.5 kPa and fibrillary stage F4—more than 12.5 kPa (cirrhosis of the liver).

Also, the study included 82 patients with chronic viral hepatitis C (HCV) aged from 20 to 65 years old who were on a dispensary record at the Vinnytsia hepatologic center.

The diagnosis of HCV was in accordance with the classification given in ICD-10 and confirmed by the detection of total anti-HCV in the blood of all examined patients, antibodies to the structural and non-structural HCV proteins: anti-HCVcor, anti-HCVNS3, anti-HCVNS4, anti-HCVNS5, as well positive qualitative and quantitative HCV-RNA PCR-test and genotyping of HCV.

After clinical and anamnestical analysis patients with any infection diseases of another etiology, exacerbations of chronic inflammatory processes, hereditary, mental illnesses as well as alcohol abuse and the administration of hepatotoxic drugs were excluded from investigation.

Determination of total anti-HCV was performed using Roche Diagnostics ELISA test systems (Switzerland), the determination of anti-HCVcor, anti-HCVNS3, anti-HCVs4 and anti-HCVNS5 was per-

formed on the Orgenxx (Israel) test systems by immunoblotting.

PCR method was used to determine HCV-RNA (sensitivity of the method – >100 IU/mL), a qPCR was used to evaluate the concentration of HCV-RNA (low viral load ≤600000 IU/mL, high viral load – >600000 IU/mL), using analyzer and test system, Cobas 6000, Roche Diagnostics (Switzerland).

Methods for the genotyping of HCV-RNA were based on the use of PCR with type-specific primers for obtaining amplification products of varying lengths. The research was carried out on Roche Diagnostics test systems (Switzerland).

The research was conducted at the Ltd Ukrainian Medical and Diagnostic Center (Kyiv) and at the Ltd. "Sinevo Laboratory."

Elastometry of the liver was performed on the apparatus "FibroScan" (France). Indicators were evaluated on the METAVIR scale [9] according to the fibrosis stage: F0 – <6.2 kPa – no fibrosis, F1 – 6.2–8.3 kPa – minimal changes, F2 – 8.3–10.8 kPa – moderate manifestations, F3 – 10.8–14 kPa – significant changes, F4 – >14 kPa – cirrhosis of the liver.

Participation in the study and blood collection was conducted with the consent of the patients. DNA samples, that had a digital code, were entered. The comparative analysis of genotypes was performed in groups of patients with moderate liver damage (F1–F2 fibrosis) and significant liver damage (F3–F4).

DNA Samples

DNA was isolated by phenol-chloroform extraction using proteinase K [10]. The quality of DNA preparations was measured by the spectral characteristics of the ND-1000 Spectrophotometer (NanoDrop, USA). To analyze the single nucleotide polymorphism of rs11536889 +3725G/C of the *TLR4* gene, the allele-specific PCR technique and primers described in Hishida et al., 2009 were used [11]. Polymerase chain reaction was performed automatically on the iCycler thermocycler manufactured by "BIO-RAD" (USA). The PCR products were fractionated in a 1.5–2% agarose gel and stained with 1% bromide ethidium (an intercalating dye of DNA). The presence of the products was visualized with UV-transilluminator. On the region which flanked by sequences homologous to oligonucleotide primers F1 and R2, the constant product of 397 bp was amplified, which serves as a positive control of the reaction (Fig. 1).

In the case where the DNA-matrix belonged to the individual – homozygote for the wild-type allele (GG), the fragment 184 and 397 bp (constant) products were generated in vitro as a result of DNA amplification. In the case of homozygotes for the mutant allele – CC, fragments 256 and 397 bp (constant) products were amplified. If the tested sample contained DNA-matrix which belongs to heterozygous

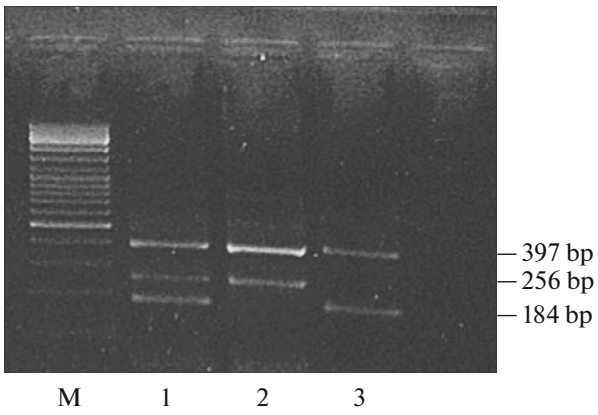

Fig. 1. Electrophoregram of allelic variants +3725 G/C of the *TLR4* gene. Fractionation of PCR products by electrophoresis in 1.6% agarose gel. M is a molecular weight marker (100 bp); 1—heterozygote GC; 2—homozygotes CC; 3—homozygote GG.

individual (GC), all three products: 397, 256, 184 bp were observed on the electrophoregram.

To evaluate the allele frequencies and the correspondents theoretically expected and actual distribution of genotypes, theoretical and actual heterozygosity, the χ^2 parameter was used, calculation were performed using "Genepop" software [12].

Fisher's criterion was used to assess the statistical significance of the frequency differences between the genotypes frequencies and allele frequencies. In the case-control studies, for the purpose of assessing the association strength of the genetic factor with pathology (evaluation of the association with the stage of fibrosis), the odds ratio (OR) was calculated. The odds ratio is a certain state of the dichotomous variable in two groups of subjects [13, 14].

To calculate the odds ratio, the probability of influencing the risk factors (in this case, the polymorphic allele or a certain genotype) in the control and investigated groups was calculated:

The chance to find a genetic marker in research group

$$= (A(A + B))/(B(A + B)) = A/B.$$

The chance to find a genetic marker in the control group

$$= (C(C + D))/(D(C + D)) = C/D, \text{ where}$$

A—is a number of individuals with a genetic marker in a research group,

B—is the number of individuals without a marker in a research group,

C—is a number of individuals with a genetic marker in the control group,

D—is the number of individuals without a marker in the control group.

Table 1. Distribution of genotypes and polymorphic alleles for rs11536889 (+3725 G/C of *TLR4* gene) for healthy individuals from Ukraine

Genotypes and alleles		Quantity	Frequency
Genotype	GG	126	0.813
Genotype	GC	26	0.168
Genotype	CC	3	0.019
Allele	G	278	0.897
Allele	C	32	0.103
	Total	155	

Table 2. Distribution of allelic variants for rs11536889 (+3725 G/C *TLR4* gene) polymorphism in world populations

Populations	Frequency of C allele	P*
Ukraine	0.103	
European superpopulation	0.158	0.018
Mixed American superpopulation	0.117	0.611
East Asian superpopulation	0.177	0.002
Asian superpopulation	0.262	<0.0000001
African superpopulation	0.010	<0.0000001

* Probability based on the results of the Fisher's exact two-tail criterion calculation.

Thus, the OR indicator is calculated by the formula:

$$OR = (A/B)/(C/D) = (A \times D)/(B \times C).$$

The interpretation of the calculated index was carried out as follows. If OR = 1, then the marker being studied does not affect the manifestation of the sign. OR > 1 means that the marker is associated with the increased chance of manifestation of the sign, and OR < 1, on the contrary – with the reduced.

In order to assess the accuracy of OR, a 95% confidence interval (CI) was calculated. The larger the interval, the lower the accuracy of OR. If the confidence interval included a unit, the OR score was considered to be false. It should be borne in mind that the OR value itself is not sensitive to the size of the sample, but the size of the sample depends on the size of the standard deviation and the confidence interval [14]. The OR calculation was performed using the "OpenEpi" software package [15].

RESULTS AND DISCUSSION

Using the described methods of analysis of the rs11536889 +3725G/C of the *TLR4* gene allelic polymorphism, the molecular genetic analysis was performed in the population sample of individuals from different regions of Ukraine (of 155 individuals). The results of the distribution of genotypes and allele frequencies in this group of individuals are presented in Table 1.

It should be noted that the most common genotype for the polymorphism of the *TLR4* gene was homozygotes – GG (frequency – 0.813), while the frequency of homozygotes CC (0.019) was the lowest.

The analysis of the correspondence of the actual frequencies of genotypes to theoretically expected in the population group indicated a random distribution of genotypes in accordance with the Hardy-Weinberg ratio.

According to the results of the comparative analysis for the frequency distribution of the polymorphic +3725 G/C *TLR4* gene alleles in population from Ukraine with the corresponding indices in the world populations obtained in the framework of the "1000 Genomes" project (Table 2), we can conclude that by distribution of allelic variants, the population of Ukraine did not differ from the European and mixed American populations. At the same time, statistically significant differences were found between the population of Ukraine and Asia, East Asian and African superpopulations [http://www.internationalgenome.org/].

In order to determine the possible role of the polymorphic variant rs11536889 of the +3725 G/C of the *TLR4* gene as a genetic marker for predicting various degrees of liver damage development in children with autoimmune hepatitis, we analyzed the distribution of genotypes in the group of patients ($n = 56$). The results of genotypes distribution in this group of patients are presented in Table 3.

According to the results of the comparative analysis, there were practically no differences in the distribution of genotype frequencies, but it was found that the frequency of allele C carriers with a significant liver damage was 0.179, which is 2.5 fold the frequency of individuals with such a genotype in the group of patients with moderate liver damage (0.071). It should be noted that the given differences did not reach the threshold of significance. That may be due to the small number of the study group.

It is important to note that homozygous individuals for the allele C (CC) were found only in patients with significant liver damage (0.036) and accounted for 3.6% of their group, while in patients with moderate liver disease patients with this genotype were absent. The revealed patterns may indicate that allele C may be a factor of the increased risk for liver damage in patients with autoimmune hepatitis.

Table 3. Genotypes distribution for polymorphic variant rs11536889 (+3725 G/C *TLR4* gene) in patients with autoimmune hepatitis

Genotypes/alleles	Control group; $n = 155$	AG group; $n = 56$	Patients with AG, various degrees of liver damage moderate $n = 28$; significant $n = 28$	
		F1 + F2 + F3 + F4	F1 + F2	F3 + F4
GG	0.813	0.875	0.929	0.821
GC	0.168	0.107	0.071	0.143
CC	0.019	0.018	0.000	0.036
G	0.897	0.929	0.964	0.893
C	0.103	0.071	0.036	0.107
GC + CC	0.187	0.125	0.071	0.179

Subsequent studies were aimed at assessing the possibility of allele C carriage association with the degree of liver damage in patients with chronic hepatitis C.

In order to determine the possible role of the polymorphic variant rs11536889 (+3725G/C of the *TLR4* gene) as a genetic marker for the prognosis of individual characteristics of the course of chronic viral hepatitis C, the analysis of the distribution of genotypes and allelic variants for this locus was performed in the groups of patients with chronic viral hepatitis C, with observed different degree of fibrous lesion of the liver. The obtained data on the distribution of genotypes and allelic variants of the +3725 G/C of the *TLR4* gene are presented in Table 4.

According to the results of the analysis, a significantly higher frequency of carriers the allelic variant C rs11536889 +3725G/C of the *TLR4* gene was found in the group of patients with severe liver disease (0.400) compared to patients with moderate lesions (0.057).

The results of the study indicate the association of allele C with the risk of hepatic fibrosis in hepatitis C. Individuals who are carriers of the allelic variant (C) are more likely to suffer from complicated hepatitis, while in the homozygote for the allele G, the disease progresses with less complications.

Frequency of allele C (rs11536889) carriers was statistically significantly higher ($p < 0.05$) in patients with a most significant degree of liver fibrosis (0.400) compared to patients with fewer severe liver damage (0.057). It is important to note that the risk of significant liver damage in patients (allelic C carriers) with hepatitis C is 11 folds higher than in patients with the genotype GG (OR 11.11, 95% CI: 2.70–45.66).

The initial link of fibrosis is the death of hepatocytes by apoptosis, necrosis or necroptosis. The death of hepatocytes induces the activation of Kupffer cells (KCs) and hepatic stellate cells (HSCs) [16–19]. There is a few studies have shown that TLR receptors, in particular TLR4, play an important role in the development and progression of liver fibrosis [20–23].

The role of TLR4 in proinflammatory activation of Kupffer cells (liver macrophage) is sufficiently studied [24]. Recently, new data on the important role of TLR4 in fibrogenesis (activation of star liver cells) have been obtained. Linking lipopolysaccharides (LPS) with TLR4 leads to activation of the inflammatory phenotype of star liver cells [24, 25]. Liver stellate cells are the main cells that take part in liver fibrogenesis [26–28]. In addition, activated star cells acquire resistance to pro-apoptotic stimuli [17].

Several studies have described genetic variants associated with progression of liver fibrosis. The presence of TLR4 D299G and T399I polymorphisms has been shown to reduce the risk of developing liver fibrosis by reducing inflammatory and fibrogenic signaling and reducing the threshold for apoptosis in stellate liver cells [1, 29]. These polymorphisms are also associated with susceptibility to infectious diseases and sepsis [30]. It also has been established that the presence of polymorphism TLR4 D299G and T399I contributes to the more severe course of Crohn's disease, helicobacter infection, cancer, pregnancy loss etc. [6, 31].

According to our research, it can be concluded that +3725G/C polymorphism of the *TLR4* gene

Table 4. Distribution of genotypes for polymorphic variant rs11536889 (+3725 G/C *TLR4* gene) in patients with chronic viral hepatitis C and varying degrees of liver tissue damage

Genotypes/ alleles	Moderate damage, frequency ($n = 53$)	Significant damage, frequency ($n = 25$)
	F1 + F2	F3 + F4
GG	0.943	0.600
GC	0.057	0.320
CC	0.000	0.080
G	0.972	0.760
C	0.028	0.240
GC + CC	0.057	0.400

(rs11536889) is a risk factor for severe fibrotic liver damage in patients with hepatitis of different etiology.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflict of interest.

Statement of compliance with standards of research involving humans as subjects. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants involved in the study.

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