RESEARCH ARTICLE

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TLR3 Gene Polymorphism in HCV Infection in the Kazakh Population of Western Kazakhstan

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Abstract

Background and Objectives: Hepatitis C virus (HCV) infection is a common cause of cirrhosis worldwide, leading to significant economic and social burdens. Approximately 170 million people (3% of the population) are infected with HCV, with the risk of developing complications such as cirrhosis and hepatocellular carcinoma. In the United States, HCV is the main cause of liver cirrhosis, accounting for 26% of cases. Recent studies have shown an increase in the proportion of HCV-related liver cirrhosis. **Materials and Methods:** A total of 102 patients with chronic hepatitis C in the reactivation phase from the Atyrau and Aktobe regional hepatology centers, who had not previously received antiviral therapy, were examined. A control group, matched by gender and age, included 127 practically healthy individuals of Kazakh nationality. All patients underwent a comprehensive examination, which included a complete blood count, a biochemical blood analysis and PCR for HCV. Venous blood samples were taken from all subjects for molecular genetic analysis. Genotyping of *TLR3* polymorphism (rs5743312, rs5743305, rs3775291, rs5743311, rs1879026) was performed using real-time PCR. Thes study is a case control study. **Results:** In patients with cirrhosis of the liver resulting from chronic hepatitis C (HCV), the results of biochemical analysis were statistically significantly higher than in patients with HCV without liver cirrhosis: the levels of total bilirubin (p 0.017*), alkaline phosphatase (p 0.022*), and gamma-glutamyl transferase (0.041*) were elevated. The results indicated that the CC genotype of *TLR3* rs1879026 was associated with the development and chronicity of HCV infection compared to practically healthy individuals (p=0.001). In the distribution of genotypes and alleles for rs5743312, rs5743305, rs3775291, and rs5743311, no significant differences were found between patients with HCV and the healthy control group. **Conclusion:** The *TLR3* rs1879026 gene polymorphism plays a significant role in the predisposition to HCV infection in the Kazakh population of the Aktobe and Atyrau regions.

Keywords: Hepatitis C- *TLR3*- rs5743305- rs5743312- rs5743311- rs1879026- rs3775291- Kazakhstan

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Introduction

Hepatitis C virus (HCV)-infection is one of the most frequent causes of cirrhosis worldwide. It entails a significant economic and social burden and increased health care costs. According to WHO, 170 million people worldwide (3% of the population) are infected with hepatitis C virus (HCV), with the risk of long-term adverse outcomes such as cirrhosis (LC) and hepatocellular carcinoma (HCC). In the United States, viral hepatitis C is the leading cause of LC, accounting for 26% of cases. In recent years, the structure of viral LC has changed toward increasing its proportion to 30.3% in the outcome of CHC (Xiao et al., 2009). Extrahepatic manifestations of HCV infection include deterioration of quality of life and mood disorders (He et al., 2022).

There are known studies devoted to the study of genetic variants associated with the development of chronic forms of viral infectious hepatitis and its complications. According to MashaelR, Al-Anazietall found a *TLR3* toll-like receptor gene polymorphism associated with the development of liver cirrhosis and HCV-ethiological hepatocellular carcinoma in the Saudi Arabian population (Al-Anazi et al., 2017). *TLR3* recognize hepatitis C virus RNA and trigger primary inflammatory cascades in response to virus penetration. *TLR3* is known to affect the duration and chronicity of the inflammatory process in HCV infection with subsequent pathological changes in liver cells (development of cirrhosis, fibrosis, hepatocellular carcinoma) (Graham and Hill, 2001). Many studies have shown that TLRs play an important role in the pathogenesis of many liver diseases, including viral hepatitis, nonalcoholic steatohepatitis, alcoholic hepatitis, autoimmune hepatitis, fibrogenesis and liver

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carcinogenesis (El-Bendary et al., 2018; Khambu et al., 2019; Nasiri et al., 2020; Tong and Guo, 2019). Al-Anazi et al., (2017) identified allelic variants of *SNP rs8099917* gene polymorphisms among the Central Asian group. TLR gene polymorphisms in viral infectious hepatitis vary among different nations. And virtually no genetic markers of predisposition to hepatitis C have been studied in the Kazakh population of Western Kazakhstan. The aim of the study was to examine the effect of *TLR3* single nucleotide gene polymorphism (SNP) on HCV susceptibility, chronicity and outcomes (liver cirrhosis) in a Kazakh population in Western Kazakhstan.

Materials and Methods

The study protocol corresponded to the Helsinki Declaration of 1,975 and was approved by the local ethical committee of the Marat Ospanov Western Kazakhstan Medical University, protocol №65 of March 13, 2020.

One hundred two patients with CHC in the reactivation phase of Aktobe and Atyrau regional hepatology centers, who had not previously received antiviral therapy, were examined. Distribution by sex: men - 52 (51%), women - 50 (49%). The mean age of the patients was 46.8 (18-60) years with 2.65±1.8 years of infection from the moment of detection. The control group, comparable in sex and age, included 127 essentially healthy people. All patients signed an informed consent to participate in the study.

The diagnosis was made according to the "Clinical Protocol for the diagnosis and treatment of chronic HCV in adults" №23 of October 23, 2020 Хронический гепатит С у взрослых > Клинические протоколы МЗ РК - 2020 (Казахстан) > MedElement on the basis of clinical, epidemiological and laboratory data, detection of antibodies (antibodies) to HCV antigens by immunofluorescence (ELISA) and verification of HCV ribonucleic acid (RNA) by polymerase chain reaction (PCR) on a PR 4100 photometer manufactured by BIORAD. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using an automatic biochemical analyzer "Architect-4000" (USA). HCV genotype 1 was detected in 38 (37.2%) of the 102 patients studied, genotype 2 in 15 (14.7%), and most frequently genotype 3 was detected in 49 (48%) patients. At the time of inclusion in the study, low viremia ($<8x10^5$ IU/ml) was observed in 38.2% (n=39) of patients, moderate ($>8x10^5$ IU/ml), - in 46% (n=47), high ($>2.4x10^7$ IU/ml) - in 14.7% (n=15). During antiviral therapy sofosbuvir + daclatasvir at 24 weeks of follow-up, a sustained virological response was achieved in the majority of patients (89.2%).

All subjects had venous blood sampling for molecular genetic analysis. Genomic DNA isolation from peripheral blood of the subjects was performed using TestGen DNA-Blood-M-100 reagent kits. The principle of the method used in the kit is based on reversible binding of nucleic acids on the surface of magnetic particles. Genotyping of the *TLR3* polymorphism (rs5743312, rs5743305, rs3775291, rs5743311, *rs1879026*) was performed by real-time PCR on a DT-Prim amplifier using commercial kits from TestGen using fluorescent detection based on destructible oligonucleotide probes using synthetic analogues of oligonucleotides.

Collection, accumulation, and systematization of the results of the study were carried out in a MS Excel 365 database. Statistical processing of the results was performed using Statistica 10 software. Quantitative measures were assessed for normality using the Shapiro-Wilk test (for less than 50 subjects) or the Kolmogorov-Smirnov test (for more than 50 subjects). Quantitative data with a normal distribution were described using arithmetic mean (M) and standard deviations (SD), 95% confidence interval (95% CI) limits. Categorical data were described with absolute values and percentages.

Comparison of two groups according to the quantitative indicator having normal distribution, under the condition of equality of variance, was performed using Student's t-criterion. Comparison of percentages in the analysis of four-field contingency tables was performed using Pearson's chi-square test (with values of the expected phenomenon greater than 10). Comparison of percentages in the analysis of multifield contingency tables was performed using Pearson's chi-square test. A predictive model of the probability of a particular outcome was constructed using the logistic regression method. The measure of certainty, indicating the part of variance that can be explained by logistic regression, was Nigelkerk's R^2 coefficient. To assess the diagnostic significance of quantitative features in predicting a particular outcome, we used the ROC-curve analysis method. The dividing value of a quantitative trait at the cut-off point was determined by the highest value of the Yuden index. Results are given as: arithmetic mean values for quantitative indicators presented in the text as M±SD, where M is arithmetic mean, Me[Q_1 - Q_3], where Me is median, [Q_1 - Q_3] is upper and lower quartile, SD is standard deviation, for qualitative indicators as %, CI for fraction.

Results

We analyzed the distribution of allele frequencies of eight *TLR3* SNPs in patients with HCV (n=102) compared with those in essentially healthy subjects in the Kazakh population of Western Kazakhstan (n=127). Older age was significantly associated with a higher risk of liver cirrhosis in HCV outcome. Indicators of cytolytic and cholestatic syndromes: ALAT and ASAT levels, total and direct bilirubin, alkaline phosphatase, gammaglutamyl transpeptidase were evaluated in patients with HCV infection (Table 1).

The presence of liver cirrhosis in patients was characterized by statistically significant intensification of cholestatic component. Thus, the results of their biochemical study were statistically higher than in patients with HCV infection without cirrhosis: the content of total bilirubin (p 0.017*), alkaline phosphatase (p 0.022*), gammaglutamyltranspeptidase (0.041*).

We confirmed the significance of *TLR3* SNPs as a marker of chronic HCV infection in patients compared to uninfected healthy control subjects. The distribution of genotypes and allele frequencies of *TLR3* polymorphisms

between HCV patients and healthy control subjects is presented in Table 2.

The results showed that the CC *TLR3* genotype *rs1879026* was associated with the development of HCV infection and its chronicity in comparison to essentially healthy individuals (p=0.001). There were no significant differences in the distribution of rs5743312; rs5743305; rs3775291; rs5743311 genotypes and alleles in HCV patients compared with healthy controls.

A predictive model was developed to determine the probability of a distribution group index depending on the index (alleles) of the *TLR3* gene polymorphism by binary logistic regression. The observed dependence is described by the equation:

$$P = 1 / (1 + e^{-z}) \times 100\%$$
$$z = 4.712 - 1.230X_{TA} - 1.696X_{TT} - 3.300X_{CA} - 5.327X_{AA}$$

where P is Probability Control, X_{TA} is rs5743305 (0 - AA, 1 - TA), X_{TT} is rs5743305 (0 - AA, 1 - TT), X_{CA} is *rs1879026* (0 - CC, 1 - CA), X_{AA} is *rs1879026* (0 - CC, 1 - AA).

Analysis of the predictor linkage model (alleles) with the probability of HCV infection revealed statistically significant differences ($p < 0.001$) between the Unadjusted and Adjusted groups due to *rs1879026*: CA *rs1879026*: AA *TLR3* gene variants (Table 3).

Based on the Nigelkerk determination coefficient value, the model explains 58.3% of the observed variance of the group indicator. When assessing the "rs5743305" index, the risk of HCV infection increased 3.422-fold in the presence of the TA allele and 5.450-fold in the presence of the TT allele. The "*rs1879026*" gene polymorphism showed a similar pattern with higher multiplicity: the CA allele genotype increased 27.102-fold, and the AA allele increased 205.737-fold. When assessing the dependence of the probability of HCV infection in patients with hepatitis C in the Kazakh population compared with healthy people, the following curve was obtained using ROC analysis (Figure 1).

ROC-curve of HCV-infection chronicity probability estimation characterizes the dependence of "group" indicator probability on the value of logistic function P. The resulting model was statistically significant ($p<0.001$) and the area under the ROC curve was 0.892 ± 0.021 with 95% CI: 0.850 to 0.933.

Discussion

Study of *TLR3* gene polymorphism: rs5743312, rs5743305, rs3775291, rs5743311, *rs1879026* in residents with HCV infection in Aktobe and Atyrau regions was

Table 1. Baseline Data and Indices of Biochemical Blood Analysis of Patients with HCV Infection in the Kazakh Population of Western Kazakhstan

Variable	Patients with chronic viral hepatitis C (n=85)	Patients with cirrhosis (n=17)	Healthy people (control) (127)	p value
Age (years), M ± SD	44 ± 11	54 ± 6	45 ± 10	<0.001*
				0.001**
Number of men, n (%)	46 (54.1)	6 (35.3)	80 (63.0)	0.067
Number of women, n (%)	39 (45.9)	11 (64.7)	47 (37.0)	
ALT, Me [Q_1 - Q_3]	33 [19-54]	41 [28-114]	-	0.101
AST, Me [Q_1 - Q_3]	30 [16-44]	50 [28-110]	-	0.017
Total bilirubin, Me [Q_1 - Q_3]	11 [8-16]	14 [12-21]	-	0.017*
Direct bilirubin, Me[Q_1 - Q_3]	5 [3-7]	7 [4-9]	-	0.102
Alkaline phosphatase, M ± SD	86 ± 31	107 ± 47	-	0.022*
GGT, Me [Q_1 - Q_3]	42 [26-68]	58 [49-79]	-	0.041*

*, the difference of indicators in Group 1 and Group 2; **, the difference of indicators in group 2 and 3

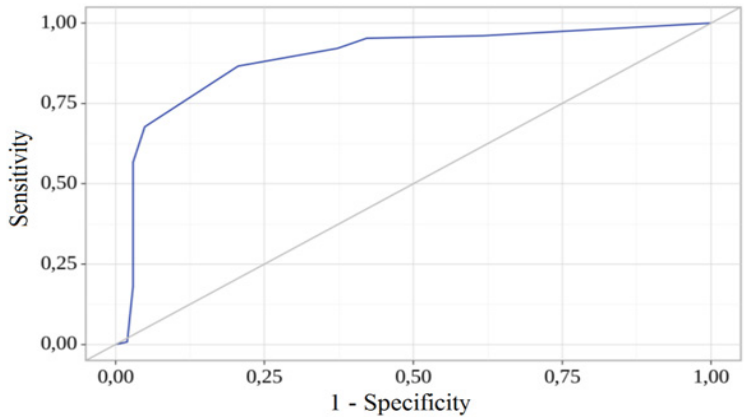

Figure 1. ROC-Curve for Estimating the Probability of Chronic HCV Infection in the Kazakh Population of Western Kazakhstan

Table 2. Genotypic distribution of *TLR3* Gene Polymorphisms in Patients with HCV Infection Compared to Healthy Individuals in the Kazakh Population of Western Kazakhstan, n (%)

Polymorphism variants	Genotypes	Group		p
TLR3		Patients with hepatitis C	Comparison group	
rs5743312	CC	72 (70.6)	81 (63.8)	0.063
	CT	23 (22.5)	43 (33.9)	
	TT	7 (6.9)	3 (2.4)	
rs5743305	AA	9 (8.8)	19 (15.0)	0.321
	TA	37 (36.3)	47 (37.0)	
	TT	56 (54.9)	61 (48.0)	
rs3775291	CC	44 (43.1)	67 (52.8)	0.347
	CT	48 (47.1)	49 (38.6)	
	TT	10 (9.8)	11 (8.7)	
rs5743311	CC	102 (100.0)	127 (100.0)	-
rs1879026	CC	3 (2.9)	72 (56.7)	< 0.001*
	CA	35 (34.3)	45 (35.4)	
	AA	64 (62.7)	10 (7.9)	

*, Differences in the indicators are statistically significant (p<0.05)

Table 3. Predictive Model of Association of Predictors (Alleles) with the Probability of HCV Infection in the Kazakh Population of Western Kazakhstan

Predictors (alleles)	Uncorrected attitude (Unadjusted)		Adjusted ratio (Adjusted)	
	COR; 95% CI	p	AOR; 95% CI	p
rs5743305: TA	0.602; 0.244 - 1.484	0.270	0.292; 0.099 - 0.864	0.026*
rs5743305: TT	0.516; 0.216 - 1.234	0.137	0.183; 0.061 - 0.554	0.003*
rs1879026: CA	0.054; 0.016 - 0.185	< 0.001*	0.037; 0.010 - 0.134	< 0.001*
rs1879026: AA	0.007; 0.002 - 0.025	< 0.001*	0.005; 0.001 - 0.019	< 0.001*

*, The effect of the predictor is statistically significant (p <0.05)

conducted to identify genetic markers of predisposition to hepatitis C and predict its outcomes in the Kazakh population of Western Kazakhstan. Of all *TLR3* polymorphism variants, *TLR3* rs3775291 was the most studied. This particular variant of the *TLR3*rs3775291 polymorphism corresponded to the highest value of the Juden index when assessing the dependence of the probability of HCV infection in patients with hepatitis C in the Kazakh population compared with healthy controls on the threshold value of the logistic P cut-off point function (0.545); the sensitivity and specificity were equal to 86.6% and 79.4%, respectively. This is consistent with the literature that the primary developmental point for *TLR3* polymorphisms is rs3775291 (C1234T). T mutates from C in exon 4, which provokes the replacement of leucine 421 with phenylalanine. Such mutations are associated not only with HCV development but also with active chronic HBV, encephalitis, human immunodeficiency virus, and other diseases. Data from a contemporary meta-analysis on the role of the *TLR3* polymorphism in HCV development show that the *TLR3* rs3775291 single-nucleotide polymorphism plays a vital role in the pathological process of HBV development (Fischer et al., 2018). It is also known about the risks of developing macular degeneration when carrying the *TLR3* type rs3775291 mutation (Klettner and Roider, 2021). TLR recognizes viral RNA and signals using two different pathways: through the TLR7 and TLR8 receptor via the molecular adaptor MyD88 and through the *TLR3* receptor via the molecular adaptor TRIF into the cell nucleus. In the case of *TLR3* mutation types rs3775291, rs13126816, rs5743305 the processes of interferon synthesis, which are responsible for viral RNA deactivation, are damaged as well as pathological changes in the expression level of other innate immunity genes.

The C allele of the three *TLR3* SNPs (rs3775290, rs3775291, and rs5743312) was known to be significantly higher in HCV. However, no significant correlation was found between healthy and HCV patients, indicating a strong association of this SNP with protection against the development of chronic HCV infection. A study of the *TLR3* rs3775291 gene showed no association of the polymorphism with susceptibility to HCV infection, as previously no significant difference in the C *TLR3* rs3775290 allele between HCV-positive patients and healthy subjects, but the T allele was associated with late-stage fibrosis and liver cancer (El-Bendary et al., 2018). However, subgroup analysis by polymorphism sites showed a significant increase in the risk of HCV infection associated with genotypes at rs3775291. Interestingly, a significantly reduced risk was associated with the rs3775291-T allele. A strong correlation was observed in the rs3775291 and HCV infection assays. But the TT/CT genotypes accounted for the increased

risk of HCV infection, and the T allele protected against viral infection. The association between the rs3775291 polymorphism in the coding region of the *TLR3* gene and the risk of HCV infection and HBV-related diseases is also biologically plausible. The mutation (replacement of leucine 421 with phenylalanine) can destroy cell structure and impair protein function because of the highest purifying selection pressure associated with allele C. The amino acid leucine in rs3775291 is adjacent to asparagine, whose glycan fragment binds double stranded RNA (dsRNA), a replication intermediate of many viruses. Because mutations in an adjacent region (Asp413) result in a significant decrease in TLR-3 signaling activity, rs3775291 may prevent the glycan fragment of Asp413 from binding to dsRNA or affect its glycosylation, which seems to be able to explain the reduced signaling activity induced by rs3775291. Reduced TLR-3 signaling activity causes a higher incidence of infections and related diseases. Thus, it is likely that the rs3775291 polymorphism is associated with the risk of HCV infection.

ROC-analysis of probability dependence assessment of HCV infection in patients with hepatitis C in the Kazakh population compared with healthy controls revealed the lowest levels of sensitivity (Se) for *TLR3* gene variants rs5743312 (56.7%), rs5743305 (67.7%), but the highest levels of specificity (97.1%, 95.1% respectively, Table 4). *TLR3* gene polymorphisms rs5743312, rs5743305 are positive (95.1% and 93.2, respectively) in predicting the clinical course of HCV infection. Similar assumptions were made by the authors, who observed that the C allele of the three *TLR3* SNPs (rs3775290, rs3775291 and rs5743312) was strongly associated with protection against developing chronic HCV infection, even though they were significantly higher in HCV, but without a significant association between healthy and HCV infected patients. Among healthy individuals, it appeared that the GCGA haplotype could protect people from hepatitis [11]. The *TLR3 rs1879026* CC genotype polymorphism draws particular attention, as shown by our findings on the genotypic distribution of *TLR3* gene polymorphisms in patients with HCV compared to healthy individuals in the Kazakh population of Western Kazakhstan (Table 2). It was accurately associated with the development of HCV chronic infection (p=0.001) than other gene variants rs5743312, rs5743305, rs3775291, rs5743311. The high significance of *TLR3* gene *rs1879026* in predisposition to HCV infection, acceleration of the replicative phase and chronicity was confirmed by binary logistic regression data. It was the *TLR3 rs1879026* CA, *rs1879026* AA gene variants that were statistically more different (p<0.001) between the Unadjusted and Adjusted groups in calculating the prognostic association of alleles with the probability of HCV detection (Table 3). The Nigelkerk observed variance determination coefficient showed that the risk of infection increased with the *rs1879026* gene polymorphism: with the CA allele genotype 27.102-fold and the AA allele 205.737-fold. Whereas polymorphism of the "rs5743305" gene leads to the lowest risk of HCV infection: with the TA allele by 3.422 times and the TT allele by 5.450 times. We have calculated similar results by ROC-analysis of the probability of chronic HCV-infection in the Kazakh population of Western Kazakhstan, in which *TLR3* gene *rs1879026* was recorded by the probability dependence as the most negative (92.4%) in the trajectory of further clinical course, that is chronicity with development of liver cirrhosis. This gene variant was the least specific (57.8%) and with the highest level of sensitivity (Se) - (95.3%). Chinese scientists, when studying only the surface correlation between SNPs and susceptibility to severe hepatitis, concluded (Qiu et al., 2018), that *TLR3* (e.g., *rs1879026*) along with other TLR2 gene polymorphism variants (e.g., rs1898830 and rs3804100), TLR4 (e.g., rs2149356) and TLR9 (e.g., rs187084, rs352139 and rs352140) may serve as risk and predictive biomarkers of neonatal severe hepatitis in the Chinese population. However, the frequency of the *TLR3* gene *rs1879026* allele T was significantly lower among residents of Saudi Arabia with hepatitis than residents of other countries (Al-Qahtani et al., 2012).

In conclusion, genetic typing of *TLR3* genes: rs5743312, rs5743305, rs3775291, rs5743311, *rs1879026* in patients with HCV of Western Kazakhstan showed that *TLR3rs1879026* gene polymorphism is significant in predisposition to HBV infection in Kazakh population of Aktobe and Atyrau regions. Despite the small sample size and the need for further research with larger cohorts, the results obtained are relevant for identifying the prognostic relationship of genotypes with the likelihood of HCV outcomes, to personalized approach to antiviral therapy, its optimization based on molecular

Author Contribution Statement

All authors participated in designing the study. All authors except AAR participated in data collection. AAR and GNN analyzed the data. GNN and AAR prepared the first draft of the manuscript. All authors provided critical comments to the first draft. All authors approved the final version of the manuscript.

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Institutional Review Board Statement

The study protocol corresponded to the Helsinki Declaration of 1975 and was approved by the local ethical committee of the Marat Ospanov Western Kazakhstan Medical University, protocol №65 of March 13, 2020.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

The data presented in this study are available on request from the corresponding author.

Conflict of interest

The authors declare no conflict of interests.

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