ANALYSIS OF CTLA-4 GENE 49A/G POLYMORPHISM IN PATIENTS WITH CHRONIC HEPATITIS C IN UZBEKISTAN

HCV has a high capacity for persistence and causes chronic hepatitis with an increased risk of development of liver cirrhosis. Immunological response of the body has the unique role, since it not only eliminates the virus, but also causes damage of liver cells. Immune status is first conditioned by genetic factors, so immunogenetic aspects determine the features of development and progression of viral hepatitis with transition to liver cirrhosis. The article deals with the analysis of association of CTLA-4 (cytotoxic T-lymphocyte associated antigen-4) gene 49A/G polymorphism with development of liver cirrhosis in patients with chronic hepatitis C.

Keywords: Hepatitis C, liver cirrhosis, immunogenetics, CTLA-4 gene 49A/G polymorphism

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Introduction

The study of susceptibility and development mechanisms of a certain socially significant multifactorial diseases is the main task of molecular medicine. Chronic viral hepatitis C (HCV) is known to belong to multifactorial diseases, in the pathogenesis of which interaction of immunogenetic mechanisms and external factors (virus, intoxication) plays the important role. Due to high degree of chronicity, severity of complications, lack of effective therapies this problem is a field of studies of specialists of various profiles. According to Stephenson (2004) and Roblin et al. (2011), millions of people in the world are infected with hepatitis C virus, but only a relatively small part (about 25%) has the risk of liver cirrhosis (LC) for further 20-30 years after infection, and, at the same time, a significant proportion of patients with the infection do not develop LC throughout life.

Taking into account the crucial significance of immune mechanisms and intensity of oxidative stress in the development of LC, currently the most widely discussed candidate genes are published by Heba (2011) and Danilovic et al. (2012) to be the genes of cytokines, including CTLA-4 gene 49A/G polymorphism.

According to Hodi (2007) and Wang and Chen (2004), cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) is a T-cell receptor and transmembrane glycoprotein, which is expressed on the membranes of T-lymphocytes after their activation by antigen, mediating inhibition of immune response of T-cells. Dariavach et al. (1988) reported that CTLA-4 gene is localized on chromosome 2q33. Ueda et al. (2003) found that receptor isoform (full-length isoform - fCTLA4), which is synthesized in activated T-cells, is encoded in four exons: the leader protein is encoded by exon 1, the ligand binding domain – by exon 2, the transmembrane region - by exon 3, and the cytoplasmic domain – by exon 4.

Studies by Vaidya et al. (2003) and Homann et al. (2006) showed that among the known polymorphisms only polymorphism at position +49 A/G (Thr17Ala) is associated with development of immunopathological processes. In this case, as Kouki et al. (2000) noted, replacement of A/G nucleotides of first exons results in reduced control of proliferation of T-cells (causing decreased functional activity of protein CTLA-4), which in turn leads to change in the nature of the immune response to viral infection. Therefore, the investigation of this polymorphism may be of the greatest scientific and practical value. Noteworthy, no domestic studies have been conducted yet on establishment of genetic markers of liver cirrhosis in patients with HCV-infection, and forcing studies are also few.
The purpose was to evaluate the contribution of CTLA-4 gene 49A/G polymorphism, which encodes the synthesis of immune response protein CTLA-4, to the nature of the course of HCV-infection and formation of liver cirrhosis in these patients.

**Study subjects**

The main study group included 107 patients with chronic HCV-infection. To assess the association of CTLA-4 gene 49A/G polymorphism, the patients with chronic hepatitis C were divided into three subgroups. The first subgroup included 33 patients with moderately active chronic hepatitis C. The second subgroup consisted of 37 patients with high degree of activity of chronic hepatitis C. The third subgroup included 37 patients with liver cirrhosis. The criteria for inclusion of patients in the study were: (1) clinical, biochemical and instrumental verification of diagnosis with determination of stage and severity of the disease, (2) serological markers of hepatitis C, found in blood serum by enzyme immunoassay, (3) HCV RNA (including viral load), revealed using the polymerase chain reaction (PCR) on the device RotorGene 6000 (set by “InterLabService”, Russia).

The control group consisted of 81 apparently healthy Uzbek donors matched by sex and age with the main group. In addition, the control group did not include individuals who suffered any form of hepatitis or any other diseases of the immune system that could affect the results. All the surveyed patients have been living in Uzbekistan and had no family ties with each other. In accordance with current ethical standards, voluntary informed consent was obtained from each patient and the study was approved by local ethical committee.

**Molecular-genetic studies**

Molecular-genetic studies were conducted at the Laboratory of Medical Genetics of the Institute of Hematology and Blood Transfusion of the Ministry of Public Health of the Republic of Uzbekistan. Isolation of total mRNA (to detect viral RNA) and DNA was performed from whole venous blood after stabilization with EDTA using the phenol-chloroform method offered by Sambrook et al. (1989) and Chomczynski et al. (1987). CTLA-4 gene 49A/G polymorphism was identified in accordance with the published procedure by Donner et al. (1997). Amplification of this locus was performed on the programmable thermal cycler of “Applied Biosystems” company (USA).

The main characteristics of the studied genes polymorphisms and sequence of oligoprimers are summarized in Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>The structure of oligoprimers</th>
<th>rs number in database NCBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTLA-4 (2q33)</td>
<td>A49G replacement</td>
<td>F:5′-GCT GTA CTT CCT GAA GAC CT-3′</td>
<td>231775</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:5′-AGT CTC ACT CAC CTT TGC AG-3′</td>
<td></td>
</tr>
</tbody>
</table>

**Statistical analyses**

Statistical processing was carried out in the program OpenEpi (version 9.3). Corresponds to the distribution of genotypes to Hardy-Weinberg equilibrium, the calculation of observed and expected heterozygosity, comparing allelic and genotype frequencies in the subgroups were carried out by standard methods of population biometrics. Odds ratio (OR) was calculated using the formula: OR = ad/bc; where a - analyzed allelic frequency in patients; b - analyzed allelic frequency in the control sample; c and d - total frequency of other alleles in patients and controls, respectively. The value of OR=1 indicates no association, OR>1 - there is a positive association with “risk factor”, and OR<1 – there is
a negative association of alleles and genotypes with the disease. Confidence interval (CI) was established at 5% significance level.

**Results**

Table 2 demonstrates the distribution of alleles and genotypes of the studied DNA polymorphism in patients with chronic hepatitis C, as well as in the control group.

An important task of studying multifactorial diseases is to analyse linkage on disequilibrium, i.e. to estimate conformity of the expected and observed frequencies of distribution of genotypes of the studied genetic polymorphism. The comparative assessment of conformity of the expected and observed frequencies of distribution of genotypes of CTLA-4 gene has not revealed statistically significant (P>0.05) deviation from the canonical distribution of Hardy-Weinberg equilibrium in the combined group of patients with chronic hepatitis C, indicating no effect of systematic or random factors that can change the genetic structure of populations. In the group of patients the expected frequency of distribution of genotypes was as follows: A/A=0.28; A/G=0.50; G/G=0.22, and the observed frequency of genotypes was: A/A=0.25; A/G=0.56; G/G=0.29 (Table 2). The observed frequency of genotypes of this DNA polymorphism (A/A=0.32; A/G=0.46; G/G=0.22) in the control group was also consistent with the expected frequency of distribution of Hardy-Weinberg equilibrium (A/A=0.30; A/G=0.50; G/G=0.20).

**Table 2. The Differences between the Expected and Observed Frequencies of Heterozygosity**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Observed heterozygosity</th>
<th>Expected heterozygosity</th>
<th>D*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main group</td>
<td>0.56</td>
<td>0.50</td>
<td>-0.11</td>
</tr>
<tr>
<td>Control group</td>
<td>0.46</td>
<td>0.50</td>
<td>0.086</td>
</tr>
</tbody>
</table>

Note: * D - relative deviation of the expected heterozygosity from the observed one, calculated by the formula: D=(h_{exp}-h_{obs})/h_{exp}, where h_{obs} and h_{exp} are the observed and expected heterozygosity, respectively

In this case, we established almost maximum level of genetic diversity of this polymorphism in the Uzbek people with the most high rate of heterozygosity (Table 2). Increase of frequency of the heterozygous genotype and simultaneous decrease in the share of individuals with homozygous genotypes among apparently healthy donors may be because of the selective advantage of CTLA-4 gene heterozygous genotype. We found that in the group of patients the observed heterozygosity was prevailed than the expected one. This was also shown by relative deviation of the expected heterozygosity from the observed one: D=-0.11.

The results of analysis of the distribution of frequency of CTLA-4 gene 49A/G polymorphism in subjects of the control group and HCV-infected patients have not confirmed significant differences in allelic frequencies in the comparable groups and subgroups. As in the main group of patients with chronic hepatitis C, as in healthy donors we observed a slight increase in the frequency of wild allele “A” (53.3% and 54.9%, respectively). In this case, the frequency of occurrence of the mutant allele in the main and control groups were 46.7% and 45.1%, respectively, and these differences were not statistically significant ($X^2=0.1; P=0.7; OR=1.1; 95\% CI 0.7099-1.611$).

Since the main group of patients is a combination of patients with hepatitis C with different degrees of inflammatory activity and liver fibrosis, it was appropriate to consider each subgroup studied separately. However, in all subgroups of patients the frequency of the mutant allele “G” of this polymorphism showed a similar level to the control value (45.0%), and ranged from 43.2 to 54.6%.
In the study of the distribution of genotypes of the polymorphic variant, both in the main and control groups we marked prevalence of heterozygous genotype A/G (Table 3). We observed increase in the frequency of occurrence of this genotype in the patients compared with the control group (56.1% and 45.7%, respectively), as well as simultaneous decrease in the frequency of occurrence of genotypes A/A and G/G in both groups. The rate of homozygous genotypes A/A and G/G in the studied main group was 25.2% and 18.7%, while in the control group was 32.1% and 22.2%, respectively.

Despite in the main group the rate of heterozygotes was 1.5-fold higher than in healthy donors, there were not found statistically significant differences ($\chi^2=2.0; P=0.1; OR=1.5; 95\% CI 0.849-2.712$).

However, comparative analysis of each study subgroups separately has shown that rather unexpected was increase of A/G heterozygotes among patients with moderately active chronic hepatitis C (1-subgroup) than in the control group (66.7% vs. 45.7%, respectively). In this case, the risk of moderately active chronic hepatitis C in individuals with this genotype was significantly increased 2.4-fold compared to the control group ($\chi^2=2.4; P=0.04; OR=2.4; 95\% CI 1.021-5.54$). At the same time, in this subgroup of patients homozygous G/G type of this polymorphism was less frequent than in healthy donors (21.1% vs. 22.2%, respectively). The estimated cumulative incidence of unfavorable genotypes in the subgroup of patients with moderate chronic hepatitis C was highly statistically significant ($\chi^2=4.1; P=0.04; OR=3.5; 95\% CI 0.995-12.17$). In the subgroup of patients with high degree of activity of chronic hepatitis C we also revealed increase in the frequency of carriers of heterozygous genotype A/G, in comparison with healthy controls (54.1% vs. 45.7%, respectively). However, this difference was not statistically significant ($\chi^2=0.7; P=0.4; OR=1.4; 95\% CI 0.64-3.053$). This indicates a lack of association of the genetic marker with this form of chronic HCV-infection.

As in most populations studied was found association of CTLA-4 gene heterozygous genotype 49A/G polymorphism with moderately active chronic hepatitis C, the next stage of our research was to analyze the contribution of this genetic marker for susceptibility to LC in Uzbek patients with chronic hepatitis C.

Analysis of the distribution of unfavorable genotypes of this polymorphism has showed no significant differences in these parameters in the subgroups of patients with LC, in comparison with the control group and subgroup of patients with chronic hepatitis C without LC. The frequencies of mutant genotypes were as follows: the frequencies of heterozygous A/G genotype in the group of patients and controls were 48.6% and 45.7%, respectively ($\chi^2=0.1; P=0.8; OR=1.1; 95\% CI 0.51-2.455$); the frequencies of homozygous

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>The frequency of alleles</th>
<th>The frequency of distribution of CTLA-4 genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>abs.</td>
<td>abs. %</td>
<td>A/A</td>
</tr>
<tr>
<td>The main group</td>
<td>107</td>
<td>114</td>
<td>53.3</td>
</tr>
<tr>
<td>Of them:</td>
<td></td>
<td></td>
<td>n %</td>
</tr>
<tr>
<td>1-subgroup Moderately active chronic hepatitis C</td>
<td>33</td>
<td>30</td>
<td>45.4</td>
</tr>
<tr>
<td>2-subgroup Highly active chronic hepatitis C</td>
<td>37</td>
<td>42</td>
<td>56.7</td>
</tr>
<tr>
<td>3-subgroup Liver cirrhosis</td>
<td>37</td>
<td>42</td>
<td>56.7</td>
</tr>
<tr>
<td>The control group</td>
<td>81</td>
<td>89</td>
<td>54.9</td>
</tr>
</tbody>
</table>

Note: * - statistically significant differences

TABLE 3. THE FREQUENCY OF DISTRIBUTION OF ALLELES AND GENOTYPES OF CTLA-4 GENETIC MARKER IN THE INVESTIGATED GROUPS
G/G genotype were 19.0% and 22.2%, respectively (X²=0.1; P=0.6; OR=0.8; 95%CI 0.39-1.644).

Discussion

Chronic viral hepatitis is known to be the most common cause of liver cirrhosis (LC). There is a large number of reports in the world devoted to the search relationship between allelic variants of candidate genes and the rate of LC progression (e.g., Tanaka, Furuta, Suzuki et al., 2003; Gaeta, Stornaiuolo, Precone et al., 2003; Schott, Witt, Pascu, van Boemmel, Weich et al., 2007). CTLA-4 gene alleles, carrying the “A” or “G” at position +49 of the first exon, correspond to threonine or alanine in the leader peptide of CTLA-4 molecule. Allele “G” is characterized by reduced control over proliferation of T-cells and is associated with development of certain immune diseases.

In this research, in the first phase of study, we carried out the analysis of linkage on disequilibrium of the studied polymorphisms, as well as the prevalence of CTLA-4 gene 49A/G polymorphic variant in patients with chronic hepatitis C and healthy volunteers in order to assess the association of carriers of this polymorphism with susceptibility to HCV-infection.

In the second phase of study, we assessed the frequency of CTLA-4 gene polymorphic variants in patients with chronic HCV-infection with different activity of inflammation, including LC. There were no statistically significant differences in the study of the frequency of genotypes of CTLA-4 gene 49A/G polymorphism between the subgroup of patients with chronic hepatitis C complicated with LC and 1- and 2-subgroups of patients with chronic hepatitis C without LC (P>0.05), indicating that there is no direct association of this marker with LC development. Comparative analysis of the frequency of the studied genes with literature data showed that the prevalence of the mutant allele of the studied polymorphic variants in patients with chronic hepatitis C (56.8%) corresponds to the data described by Gaeta et al. (2003) and Heba (2011).

In general, we can argue that CTLA-4 gene 49A/G polymorphism to a lesser extent affects the susceptibility to LC in patients with chronic hepatitis C. This is different from studies by Tanaka et al. (2003), Schott et al. (2007), and Heba (2011), who reported reliable association of this marker with development of LC. Seeff et al. (2001) consider that this inconsistency may determine the degree of influence of this polymorphism in the pathogenesis of liver disease in different populations, as well as is connected with the unequal criteria of division of patients into groups and subgroups, with the survey of relatively small groups of patients, increasing, thus, the possibility of false associations. In addition, development of LC may involve other more serious gene combinations. Schott et al. (2007) reported that, possibly, the combination of several defective genes, the protein products of which are involved in the pathogenesis of chronic hepatitis C, have the significant influence on the course of chronic viral infection with the transition to LC. However, until now, most published researches do not include information on the combined analysis, including attribution to the gene-gene interactions with the clinical course of chronic HCV-infection. In this regard, cytokine genes, which play the major role in the formation and regulation of defense reactions, are of particular interest. Taking this into account, the following could be proposed as recommendations: for final conclusion about the role of CTLA-4 gene +49A/G polymorphism (rs231775) in development of LC in patients with chronic hepatitis C, we recommend to analyze gene-gene interactions of this polymorphism with other cytokines genes.

Thus, taking into account different degrees of inflammatory activity and liver fibrosis, the analysis of the distribution of the studied CTLA-4 gene 49A/G polymorphism in the total group of patients has revealed a number of features. Only heterozygous variant of this DNA marker was characterized by statistically significant deviation in the frequency of occurrence of the corresponding value in the control, or in other words, had a so-called “main effect” on the risk of moderately active HCV-infection. In other cases, the
distribution of alleles and genotypes of the studied polymorphisms corresponded to that in the healthy population, or had a significant trend to deviate from the norm.

**Conclusion**

In conclusion, we would like to note that our results reveal some aspects of genetic factors in the development of diseases associated with dysregulation of defense reactions in the body, and indicate whether further study of candidate genes involved in the pathogenesis of immune disorders is possible. Knowledge about the genetic basis of disorders of the immune system, the study of contributions of combinations of alleles in susceptibility to the disease, will be helpful in the development of new treatments for specific diseases (including HCV), as well as creation of an integrated program for prevention of disease, taking into account the individual genetic characteristics.

In the studied population, the level of observed heterozygosity did not exceed the theoretically expected values, as well as CTLA-4 gene +49 A/G polymorphism was in accordance with Hardy-Weinberg equilibrium, indicating no effect of systematic or random factors that can change the genetic structure.

Heterozygous variant of CTLA-4 gene +49 A/G polymorphism belongs to genetic factors related to dysregulation of defense reactions and is associated with the development of moderately active form of HCV-infection.

The population specificity on this polymorphism was shown. We do not suggest that carriers of unfavorable genotypes of CTLA-4 gene +49A/G polymorphism have an increased risk of liver fibrosis in the case of chronic hepatitis C.

**References**


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