Polymorphism of IL-28B Gene (rs12979860) in HCV Genotype 1 Patients Treated by Pegylated Interferon and Ribavirin

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**ABSTRACT**

**Background:** Nowadays, the immune response to hepatitis C (HCV) treatment has become a crucial issue mostly due to the interleukin 28B (IL-28B) polymorphism effects in chronic HCV patients. The aim of this study was to detect the polymorphism of IL-28B gene (rs12979860) in HCV genotype 1 patients treated with pegylated interferon and Ribavirin.

**Methods:** From the yr 2010 to 2012, a total of 115 peripheral blood mononuclear cells (PBMCs) of HCV patients who presented to Gastrointestinal & Liver Disease Research Center (GILDRC), Firoozgar Hospital, Tehran, Iran were enrolled in this retrospective cross sectional study. Samples were then categorized based on the presence of sustained virologic response (SVR and no-SVR). Variables including age, gender, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels of the two groups were investigated based on different IL-28B genotypes.

**Results:** Analysis by the variables of age and gender showed a mean age ± SD of 42.1±14.0 and gender variability of 44 females (38.2%) and 71 males (61.8%). Adding up these results, the analysis of ALT levels revealed that there was between 293 and 14 mg/ml; AST levels ranged between 217 and 17 mg/ml; the viral load (HCV RNA) ranged between 7,822,000 and 50 IU/ml; the prevalence of CC, CT and TT genotypes were 90.9%, 54% and 25.0%.

**Conclusion:** IL-28B polymorphism has an effective impact on the therapeutic response to ribavirin and peginterferon combination therapy in chronic HCV patients infected by different genotypes. This polymorphism is crucial in natural clearance.

**KEY WORDS**

Chronic HCV infection
Sustained virologic response
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**ABSTRACT**

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Infected patients (20-30%) recover spontaneously and the rest (around 70-80%) became chronic carriers, 25% of which may develop cirrhosis or hepatocellular carcinoma (HCC) later on (1, 2). HCV has several genotypes and subgenotypes with different distribution patterns around the globe.

Antiviral therapy of HCV infected individuals could dramatically decrease the rate of complications. The most common drug therapy for HCV infection currently used is the combination of Pegylated interferon plus ribavirin (PegIFNα/RBV) that has showed variable responses in the reduction of viral replication rate and the clinical presentation considering different genotypes and subgenotypes, an observation termed sustained virologic response (SVR) (3-5). SVR is defined as six months of true negative test results of HCV PCR, performed following the completion of drug therapy (3, 4). Treatment of HCV genotype 1 results in 30% SVR rate after 6 months, an issue that imposes major difficulties not only for virus clearance but also for patients enduring therapy (4). Genotypes 2 and 3 yield about 65% SVR rate, collectively, which implies better response than genotype 1. Meanwhile, there are some extra factors to affect the response to treatment (5, 6). Currently, Iranian HCV infection prevalence in general population is about 1%, with subtype 1a being the most common subtype (44.9%) followed by subtype 3a (39.6%), and subtype 1b (11.3%), respectively (2).

Clearance of HCV infection is also related to host genetic factors in infected individuals (6, 7). One of the best described factors is interferon lambda 3 (IFN-λ3), an element assumed to cause a great response against HCV infection and is located on chromosome 19 of the IL-28B gene (8, 9). IFN-λ3 regulates Treg cells and increases adaptive cellular immunity. The IL-28B cytokine acts as an antiviral by induction of interferon-stimulated genes through JAK-STAT pathway.

Recent studies (9-13) illustrate that the single nucleotide polymorphism (SNP) on substantial parts of the IL-28B gene (on its genetic variant) is vital to the IFN-λ3 function and its behavior in response to PEG-IFN treatment. Suppiah et al. (10) and Tanaka et al. (11) showed the importance of SNP rs8099917 and SVR which T allele were associated with higher SVR (78% for genotype TT). rs12979860 SNP has the same relationship with SVR (12, 13). Generally, African individuals show lower response to therapy compared to Europeans and SVR in CC genotypes is 2-3 times higher than TT genotypes (13).

The aim of this study was to determine the polymorphism of IL-28B gene (rs12979860) in HCV genotype 1 patients treated by Pegylated interferon and ribavirin.

Materials and Methods

Patient selection

This retrospective cross sectional study was conducted by Gastrointestinal & Liver Disease Research Center (GILDRC), Firoozgar Hospital, Iran University of Medical Sciences, Tehran, Iran. Sampling was performed from May 2010 to March 2012 by all referred patients that filled the inclusion criteria. Our study population was categorized in 2 groups of SVR and non SVR. Our Sample patients were all suffering from chronic HCV genotype 1 infection treated by PegIFNα/RBV and had completed treatment for over 6 months. SVR group criteria were: 1) patients suffering from HCV genotype 1 that completed treatment 6 months ago, 2) patients manifested no evidence of cirrhosis, 3) there was no co-infection by other hepatitis viruses and 4) there was no co-infection with other diseases.

Informed consent was obtained from each patient. This study has been approved by the Ethics Committee of Iran University of Medical Sciences, Tehran, Iran.
Venous blood samples were collected for routine biochemical tests with an automated device using enzyme immunoassay method (anti-HCV and anti-HIV: Architect System, Abbott Diagnostics, Germany; HBsAg: Roche Diagnostics, USA) used for HBsAg, anti-HCV, and HIV antibodies examinations. To assess HCV RNA levels the COBAS AmpliPrep/COBAS TaqMan 48 System was used (limit of detection 15 IU/ml) at baseline, at 6 months (24 weeks) and after the end of treatment (SVR).

DNA collection and extraction

Ethylene di-amine tetra-acetic acid (EDTA) tubes were used for whole blood collection. Peripheral blood mononuclear cell (PBMCs) (200 µl) of all specimens were used for DNA extraction performed by Qiagen Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol and then the extracted DNA stored at -20 ºC till use. Ficoll Hypaque (FH) gradient centrifugation (Lympholyte-H, Cedarlane, Canada) was used for PBMCs iso-lation. Phosphate-buffered saline (pH = 7.3 ± 0.1) was used for the three-step washing of the pellet of PBMCs. Cells were then counted and after adding RNALater (Ambion Inc., Austin, TX) solution, they were stored at -80 ºC until use. NanoDrop model ND-1000 (PeqLab, Erlangen) assessed the quality of DNA using absorbance ratio calculation (OD 260/280 nm).

IL28B Genotyping

We chose the most related SNP rs12979860 which included bi-allelic CC, CT, and TT polymorphisms to evaluate treatment response to peg-interferon and ribavirin. This region located on chromosome 19, contains multiple single-nucleotide polymorphisms (SNPs) in linkage disequilibrium around the IL28B gene.

Genotyping of the rs12979860 was performed using a pyrosequencing method for detection of IL28B variants TT, CT and CC from PBMCs specimens. The primers for rs12979860 PCR were as follows: forward primer 5'-ATT CCT GGA CGT GGA TTT GTA CT-3', reverse primer 5'-biotin-GGA GCG CGG AGT GCA ATT-3'. Sequence primers for rs12979860 pyrosequencing analysis were: 5'-AGC TCC CCG AAG GCG-3'.

Extracted DNA samples were amplified using Veriti 96 well Applied BioSystem thermal cycler. PCR reactions for bi-allelic polymorphism were performed under the following condition: A total volume of 50 µl reaction mixtures consisted of template DNA or control (beta-globin) corresponding to 0.2–0.5 µM concentration, a dNTP mix (Fermentas GmbH, Germany) with equal concentrations of each of the four dNTPs at a 0.5 mM concentration, each forward and reverse primers at 0.5 µM concentration, MgCl2 solution (Fermentas GmbH, Germany) at 1.5 µM concentration, 1× reaction buffer (Fermentas GmbH, Germany), Taq DNA polymerase (Fermentas GmbH, Germany) at 5 units/µl concentration and sterilized D.W. added to round out the total volume. the mixture was heated at 95 ºC for 5 min, 45 cycles of 95 ºC for 15 sec, 57 ºC for 30 sec, 72 ºC for 15 sec, then kept at 72 ºC for 5 min. PCR components’ final concentrations were: 1x PCR buffer, 2 mM MgCl2, 0.125 mM dNTPs, 0.2 µM Forward primer and 0.2 µM Reverse biotinylated primer, 1U of AmpliTaq polymerase (Perkin Elmer, Waltham, USA) and 2 ng/µl DNA template.

Running of 12 µl PCR material on 1.5% agarose gel (Sigma-Aldrich, St. Louis, USA) was used to confirm successful amplification. PyroMark Q96 analysis Software was used for evaluation of each SNP of sequenced PCR products by PyroMark ID Pyrosequencing machine (QIAGEN, Germany). Purification of PCR products was performed by illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK), according to manufacturer’s instructions. Genotyping was conducted in a blinded fashion relative to HCV treatment status and other patient or treatment response characteristics.
Statistical analysis

SPSS version 20 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses including the basic descriptive and frequency features. After the calculation of arithmetic and standard mean, double-sided chi square/fisher-exact tests and t tests were used to compare genotype frequency and incidence between the various groups. P values less than 0.05 were considered to be statistically significant.

Results

A total of 115 samples were analyzed. Variables including age, gender and serum transaminases of the two groups (SVR & non SVR) were compared by their related IL-28B genotype.

Analysis by variable characteristics yielded the following results: mean age ± SD of 42.1±14.0 yr (age range: 10-65 years), gender distribution of 44 females (38.2%) and 71 males (61.8%). Alanine aminotransferase (ALT) levels ranged between 293 and 14 mg/ml; Aspartate aminotransferase (AST) levels ranged between 217 and 17 mg/ml; the viral load (HCV RNA) ranged between 7,822,000 and 50 IU/ml; and genotyping of rs12979860 with Pyrosequencing method showed the CC variants were dominant which followed by CT and then TT, respectively. Table 1 summarized more details about our 115 patients.

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. (%)</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (µl/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>34</td>
<td>29.5</td>
</tr>
<tr>
<td>41-80</td>
<td>55</td>
<td>47.9</td>
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<tr>
<td>&gt;80</td>
<td>26</td>
<td>22.6</td>
</tr>
<tr>
<td>AST (µl/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>55</td>
<td>47.9</td>
</tr>
<tr>
<td>41-80</td>
<td>45</td>
<td>39.1</td>
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<tr>
<td>&gt;80</td>
<td>15</td>
<td>13.0</td>
</tr>
<tr>
<td>Viral load (IU/ml)</td>
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<td></td>
</tr>
<tr>
<td>≤800,000</td>
<td>43</td>
<td>37.3</td>
</tr>
<tr>
<td>&gt;800,000</td>
<td>72</td>
<td>62.7</td>
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<tr>
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<td></td>
</tr>
<tr>
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<td>66</td>
<td>57.4</td>
</tr>
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<td>37</td>
<td>32.2</td>
</tr>
<tr>
<td>TT</td>
<td>12</td>
<td>10.4</td>
</tr>
</tbody>
</table>

Regression analyses showed that CC genotype induced higher SVR than TT genotype (OR=29.333, CI=2.558, -336.387, P=0.007) but the effect of CT genotype was not significant in comparison with TT genotype (OR=2.887, CI=0.618-13.496, P=0.177). 90.9% of CC genotypes, 54% of CT genotypes and 25.0% of TT genotypes were in SVR group.

Discussion

There are several viral and host factors including viral load, genotype, age, sex, body mass index, ethnicity, co-infections, and host genome involved in treatment response rate of chronic HCV infection (5, 13, 14). Correlation between SVR and HCV, especially for genotype 1, has been shown in many studies (7, 10, 13-16). Virologic response to drug is one of the outcomes to predict the response of the patient, before beginning treatment (7, 14, 16).

Actually, at the current study we targeted the SNPs of IL-28B (rs12979860) and their related SVR. SVR and non SVR groups were compared by their genotypes and variables.

Some studies have reported the probable variety of dominancy of the three genotypes on SVR...
related to different individuals and viral factors. There was 52.9% CC genotype, 39.7% CT genotype, and 7.4% TT genotype in HCV patients registered at Hepatitis Center in Iran (9); a finding that is similar to our findings. The slight differences might have been caused by the combinatorial HCV genotypes 1, 2, 3 and 4 patients which enrolled in that study, so in comparison with our study, we performed HCV genotype 1 patients lonely in broader population which might be better applicable for SVR prediction.

Aziz et al. (12) reported higher frequency of CC (54.3%) than CT (37%) and TT (8.6%). In comparison with our results, there is a brief difference that could be caused by the almost near rationales and close distance between Iranian and Pakistani nations. They calculated higher SVR in patients with genotype CC (84.2%) as followed by CT and TT (by 56.4% and 22.2%) in comparison with our results we found almost similar SVR in CC genotype (90.9%) followed by CT and TT genotype (by 54% and 25%).

Study of African or European HCV infected patients showed that the spontaneous clearance of HCV in patients with IL-28B CC genotype (rs12979860) was three-fold higher than those with the CT and TT genotypes (13). The baseline ALT levels were not significantly different among the three IL-28B subgroups ($P>0.05$) but our results showed the significant increased level of ALT from baseline in undesirable TT genotype, a finding that resulted in higher SVR.

In Egypt, the occurrence of CC genotype (48%) was higher than the CT (38%) and TT (14%) genotypes (16). CC genotypes resulted in significantly higher spontaneous resolve than others. In comparison with our study, we found the same results and suggested that CC genotype results in higher SVR than others (TT & CT).

Furthermore, the ratio of C allele is higher than T, indicating that the protection by spontaneous clearance of IL-28B CC genotype against chronic infection progression was better during the acute phase (17) which in comparison with our results we fund the same ratio of C:T allele.

Conclusion

There is a considerable prevalence of CC, TT and CT genotypes in our country like other distances. This factor could have a significant impact on our strategy in drug therapy especially in HCV genotype 1 infected individuals. The high level of HCV genotype 1 in our country also suggests the higher need for health systems to use these markers to predict the outcome of antiviral therapy and genotyping of regions like rs12979860 and rs8099917 of IL-28B on chromosome 19 that has a significant role in inefficiency of Peg-IFN plus Ribavirin drug therapy for HCV especially genotype 1 infected individuals which will ultimately reduce the costs and side effects of therapy.

Conflict of interest

No conflict of interest.

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