

Role of IL28-B Polymorphism (rs12979860) on Sustained Virological Response to Pegylated Interferon/Ribavirin in Iranian Patients With Chronic Hepatitis C

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Abstract

Background: The current medical treatment for hepatitis C virus (HCV) infection is pegylated interferon plus ribavirin, but just 50% of genotype 1 HCV patients and about 80% of HCV genotype 3 patients are treated completely. Recently, the rs12979860 C/T polymorphism, which is located 3 kb upstream of the IL28b gene that codes IFN λ 3, shows a powerful association in response to the treatment in HCV patients.

Objectives: The aim of this study was to evaluate the relationship between IL28b single nucleotide polymorphism (SNP) and treatment outcomes among chronic HCV patients in Iran.

Patients and Methods: In this cross-sectional study, 108 blood samples were collected from chronic patients in Iran; 50 unrelated healthy subject samples were also collected. Genomic DNA was extracted, and rs12979860 SNP was done by PCR-RFLP. Finally, products were detected on 12% polyacrylamide gel electrophoresis.

Results: The analysis of data for C/T SNP showed that the CC genotype is more common in the control group than in the group of patients. In contrast, the frequency of TT as a mutant genotype is more frequent in patients than in uninfected people. In addition, results showed a statistically significant relationship between CC, CT, and TT genotypes in sensitive and resistant groups (P value: < 0.001, Or: 0.003, CI: 0-0.047). This relationship was also examined in terms of allele frequency, to determine whether the possibility of resistance to treatment in patients with T allele is more than in patients who carry C allele (P value: < 0.001).

Conclusions: These results showed a significant effect between rs12979860 SNP and sustained virological response (SVR) rate in Iranian patients with chronic HCV. To decrease the cost of long treatments and to prevent severe side effects, determining this polymorphism at the beginning of treatment can be very helpful for patients and physicians.

Keywords: Hepatitis C Virus, Sustained Virological Responses, , Interleukin 28B, rs12979860, Iran

1. Background

About 170 million people are affected by the hepatitis C virus (HCV) worldwide, and nearly 70% of those cases broaden to chronic infection, which may progress to cirrhosis and hepatocellular carcinoma (1, 2). This disease can spread by several routes, with blood transfusions and intravenous drugs use being considered the most significant methods of transmission (3-5). Spontaneous clearance occurs in 70 - 75% of chronic HCV patients (6).

Six major HCV genotypes and more than 90 subtypes have been identified (7). In Asia, the most frequent genotypes are 3 and 6. HCV genotype 6 is generally restricted

to Southeast Asia. In the Middle East, however, 4a is the predominant genotype; 1b and 3 are the most frequent genotypes in Turkey and Pakistan, respectively. The predominant genotype in Iran is 1a, followed by 3a and 1b (7). HCV genotype has clinical importance as it determines the treatment duration (7). The recommended treatment for HCV infection is pegylated interferon plus ribavirin, but just 50% of genotype 1 HCV patients and about 80% of HCV genotype 3 patients are treated completely and show sustained virological responses (SVR). Unfortunately, the rest of the patients do not respond to treatment and eventually show cirrhosis; these patients are resistant to treat-

ment (8-10). Some severe side effects, such as influenza-like symptoms, hematological oddity, and psychiatric symptoms, could result in untimely discontinuation of treatment (11, 12). To prevent this side effect in patients who do not gain advantage from treatment and also to reduce the cost of treatment, discovering factors in individuals that can play an independent role in predicting of treatment outcomes in patients would be useful (13). Previous studies show that some factors, such as HCV genotypes and viral load, are linked to treatment outcomes (13). Furthermore, due to a long history of chronic HCV, some events like viral clearance and persistence of treatment are used to assess the success or failure of treatment, and recent studies have discovered that some host genetic factors play a role in the treatment of HCV patients (12). Cytokines are among the predominant mechanisms of a host's defense against infection; they induce an inflammatory response that often leads to tissue injury and also act as antiviral effectors. Cytokine synthesis capacity has a significant genetic component, which explains why there are differences between individuals in their ability to produce cytokines; it can also be due to single nucleotide polymorphisms (SNP) within the coding regions of cytokine genes (14-16). Type III interferon group was classified to three IFN- λ (lambda) molecules called IL29, IL28A, and IL28b. Besides its antiviral properties, INF- λ exhibits antitumor activity. In fact, several experimental studies in cell lines and in animal models demonstrated that the activation of type III IFN induces apoptosis. Recently, the rs12979860 C/T polymorphism located 3 kilo base pairs upstream of the IL28b gene that codes IFN λ 3; this shows a powerful association in response to treatment in HCV patients. This effect is influenced by ethnicity (17-19). IL28A, IL28B, and IL29 signal through the JAK-STAT pathway to inhibit HCV (20).

2. Objectives

The aim of this study was to evaluate the relationship between IL28b SNP and treatment outcomes among Iranian HCV patients.

3. Patients and Methods

3.1. Sampling

This study was performed according to the declaration of Helsinki and relevant local regulations. The sampling protocols were approved by the ethical committee of the Pasteur institute of Iran (Code of ethical approval: 91-0201-7997). In this cross-sectional study, using a convenience sampling strategy from September 2012 to January 2013, patients who were referred to Tehran West health center

and the association of liver disease (Tehran, Iran) were selected for this study if at least 6 months passed from the start of their treatment with peg-interferon plus ribavirin. In total, we collected 108 samples, which included 71 patients sensitive to treatment with a mean age of 42; their viral load was zero after six months of treatment. Additionally, there were 37 resistant patients with a mean age of 49 with a positive viral load six months after the end of treatment. Fifty unrelated healthy subjects were collected to investigate the effect of rs12979860 SNP on susceptibility to HCV infection. It is noteworthy that the sensitivity and resistance was measured based on information available in the clinical records and SVR; the samples were collected in consultation with physicians. 5 cc of whole blood in special EDTA-containing tubes were collected after receiving written consent and according to regulations approved by the medical ethics committee of the Pasteur Institute of Iran.

3.2. Genomic DNA Extraction

Genomic DNA was extracted from 500cc of peripheral blood containing EDTA anticoagulant using a Genomic DNA extraction kit (Thermo Scientific, Lithuania) based on the manufacturer's instructions, and it was stored at 20°C until use.

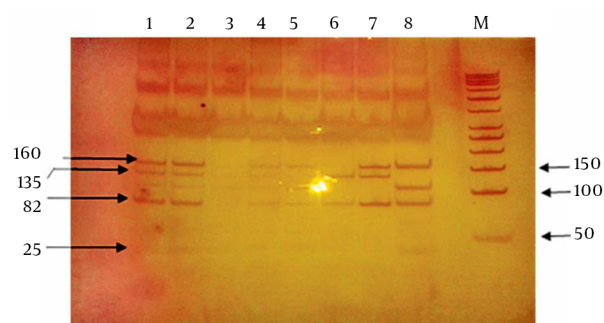
3.3. Proliferation of IL28 Gene by PCR

To amplify the target gene, touchdown PCR was performed by specific forward 5' GCTTATCGCATACGGCTAGG 3' and reverse 5'AGGCTCAGGGTCAATCACAG 3' primers with an annealing temperature of 64°C. Briefly, a gradient of annealing temperature was used in this program; it started from 67°C and decreased 2°C during each cycle. Finally, 35 cycles with denaturation at 94°C for 40 seconds was performed, annealing at 64°C for 40 seconds and extension at 72°C for 45 seconds. A final extension period of 5 minutes was performed. At the end, to ensure the accuracy of the reaction, agarose gel electrophoresis of PCR products were done.

3.4. Genotype Determining by RFLP

In order to determine polymorphisms, a *Bst*UI restriction enzyme was used (Thermo Scientific, Lithuania) to digest the 242 bp fragments of PCR product. Digestion was done in a total volume of 30 μ l containing 1 μ l of *Bst*UI, 2.5 μ l of R buffer, 10 μ l of PCR product, and 16.5 μ l of deionized distilled water for 90 minutes at 37°C. Finally, evaluation of the genotype (C/T) rs12979860 was performed with 12% polyacrylamide gel electrophoresis and a silver staining method (Figure 1).

Figure 1. Polyacrylamide Gel Electrophoresis of Digest Products to C/T Genotype Evaluation in Different Groups



Lines 1, 5, 4, 3, 2: CT, line 6: CC, line 8: TT, and line M: DNA ladder marker 50 bp (Thermo Scientific).

3.5. Statistical Analysis

The information from the sensitive, resistant, and control groups was analyzed with SPSS program version 19. The frequency between groups was analyzed using the chi-square Yates correlation coefficient or Fisher's exact test. The means were compared by ANOVA. Logistic regression analysis was used for correlation between the infection and the polymorphism. P value was significant at < 0.05 .

4. Results

In this cross-sectional study, polymorphic regions of the IL28B promoter were investigated between two sensitive and resistant groups, and their results were compared with the results of a control group. According to the results of agarose gel electrophoresis of samples using two specific primers, the specific band (242 bp) was observed.

To determine genotypes, digest products of *Bst*UI restriction enzyme were electrophoresed on 12% polyacrylamide gel and were stained with a silver staining method. Samples that had three 25, 82, and, 135 bp bands were CC; samples that had two 82 and 160 bp bands were TT; and samples that showed all four 25, 82, 135, and 160 were constructed CT. The results of the statistical analysis of demographic information of the 108 patient samples and 50 controls are shown in [Table 1](#).

There is a correlation between patient and control groups in terms of mean age, sex, and ethnicity (P value: 0.001). However, there was not any significant relationship between BMI and marital status in these two groups (P value: 0.67) ([Table 1](#)). In addition, analyses of two sensitive and resistance groups showed a significant relationship between age (P value: 0.002), sex (P value: 0.02), and liver status (cirrhosis and chronic) (P value < 0.001) in such

a way that 98.6% of chronic HCV patients showed SVR while 76.6% of them were resistant to treatment. However, 1.4% of the patients who had cirrhosis showed SVR, but 25.4% of them were resistant ([Table 1](#)). There is a significant relationship between Iranian Fars populations and other Iranian populations in sensitive and resistant groups (P value < 0.001).

Investigation of HCV transfer risk factors between two sensitive and resistant groups showed that patients that were drug addicted shared HCV-infected syringes for injection, but there is not any relationship between sensitive and resistant groups with this risk factor (IVDU) (P value: 0.12) ([Table 1](#)). There is also a correlation between HCV genotypes and the response to treatment (P value: 0.001). In fact, the 70.4% of patients who were infected by HCV genotype 1 were sensitive to treatment, while 97.2% of them were resistant. The number of patients in these two groups who were infected by HCV genotype 3 was 29.6% and 2.8%, respectively.

[Table 2](#) demonstrates the frequency of IL28b genotypes in the patient and control groups. In fact, CC genotype is more common in the control group than in the patient group. In contrast, the frequency of TT as mutant genotype is more frequent in patients than in uninfected people (P value: 0.024, Or: 11.2, CI: 1.373 - 91.33). These results lead to the fact that people who have TT genotype (in comparison with those who have CC genotype) are more susceptible to infection when they encounter some risk factors. This relationship of Allele frequency was analyzed as well. Based on this analysis, there is a correlation between allele frequency and susceptibility to HCV infection in the patient and control groups (P value: 0.008, Or: 2.008, CI: 1.196 - 1.365) ([Table 2](#)). In addition to the patient and control groups, a comparison between sensitive and resistant groups was performed; results showed a statistically significant relationship between CC, CT, and TT genotypes in sensitive and resistant groups (P value: < 0.001 , Or: 0.003, CI: 0 - 0.047). The frequency of the TT genotype in the resistant group is 35.1%, but in the sensitive group it is 1.4%. The CC genotype's frequency is 39.4% in the sensitive group and 2.7% in resistant patients. This relationship also was examined in terms of allele frequency, so that the possibility of resistance to treatment in patients with T allele is more than patients with C allele (P value: < 0.001 , Or: 4.36, 2.39 - 7.945) ([Table 2](#)).

5. Discussion

In this study, we evaluated the relationship between IL28b rs12979860 polymorphism and treatment outcomes in Iranian HCV patients. In patients with European ancestry, the CC genotype is associated with a twofold higher

Table 1. Demographic and Clinical Information of Patients (Sensitive and Resistant) and Control Group

Variables	Sensitive, No. (%)	Resistant, No. (%)	Healthy, No. (%)	P Value ^a	P Value ^b
Sample	71 (65.7)	37 (34.25)	50	-	
Mean age, y	42	49	29.7	0.002 ^c	< 0.001
Age range	22 - 60	35 - 62	15 - 77		
Sex, male/female	68 (95.8)/3 (4.2)	30 (81)/7 (19)	17 (15.6)/33 (76.7)	0.02 ^c	< 0.001
Mean BMI, mean ± SD	24.1 (3.60)	25.22 (3.40)	24.7±3.7	0.1	0.67
Ethnicity				< 0.001	0.001
Iranian Fars population	69 (97.2)	35 (94.6)	36 (72)		
Other Iranian population	2 (2.8)	2 (5.4)	14 (24)		
Liver status					
Chronic	70 (98.6)	28 (75.6)		< 0.001 ^c	
Cirrhosis	1 (1.4)	9 (25.3)			
Risk factors					
IVDU	45 (72.6)	17 (27.4)		0.12	
Other risk factors	4 (0.44)	5 (0.56)			
ALT, min - max	46 (10 - 316)	56 (18-233)		0.503	0.76
AST min - max	33 (16 - 199)	45 (18-233)		0.143	
Viral load				0.579	
< 10 ⁶	39 (55)	18 (48.6)			
> 10 ⁶	32 (45)	19 (51.4)			
HCV genotype					
1a	50 (70.4)	36 (97.2)		0.001 ^c	
3a	21 (29.6)	1 (2.8)			
Co infection					
HCV	47 (66.2)	27 (73)			
HCV/HIV	24 (33.8)	10 (27)		0.51	

^aP value between sensitive and resistant groups.

^bP value between case and control groups.

^cStatistically significant.

rate (Or: 1.8-2.3) of SVR than the TT genotype; the ratio is the same in African American and Hispanic populations (21). It is reported that East Asians have higher SVR rate than patients of European ancestry (21).

In a study that has been done in China, C allele is connected with higher serum levels of IL28B, IL28A, and IL29, which are all necessities in induction of expression of IFN responsive gene (ISG) expression; this is related to better IFN based therapy (22). A preliminary report from Mahboobi shows that 69% of patients infected by the HCV genotype 3 and 47% of patients infected by HCV genotype 1 were CC (23). In this study, almost 70.4% of patients who were infected by HCV genotype 1 were SVR, and 97.2% of them were resistant to treatment; the percentages of patients

who were infected by HCV genotype 3 were 29.6% and 2.8%, respectively. Frequencies of the favorable CC genotype of rs12979860 polymorphism alter in different nations. In Chinese populations, the frequency of the CC genotype increases from the lowest in resistant patients to the middle in patients who were SVR after treatment, and it increases to the highest in patients with spontaneous clearances (24). Other studies on African, European and East Asian populations demonstrate that Asians show higher SVR than other populations and that the frequency of C allele in Asians is higher than others. This result shows that the cause of different SVRs between different populations is related to C allele as a favorable allele (21, 25).

Furthermore, McCarthy discovered that in Caucasians

Table 2. Genotype Frequencies of Case (Sensitive and Resistant) and Control Groups

Host Genotype	Case, No. (%)	Control, No. (%)	P Value	OR ^a	95% CI ^b
CC	30 (27.7)	24 (48)		1 ^c	
CT	64 (59.2)	25 (50)	0.047	2.048	1.009 - 4.159
TT	14 (12.9)	1 (2)	0.024	11.2	1.373 - 91.33
C allele	124 (57)	73 (73)		1 ^c	
T allele	92 (43)	27 (27)	0.008	2.006	1.196 - 1.365
Host Genotype	Sensitive, No. (%)	Resistant, No. (%)	P Value	OR ^a	95% CI ^b
CC	28 (39.4)	1 (2.7)		1 ^c	
CT	42 (59.2)	23 (62.1)	0.009	0.064	0.008 - 0.511
TT	1 (1.4)	13 (35.1)	< 0.001	0.003	0-0.047
C allele	98 (69.7)	25 (33.7)		1 ^c	
T allele	44 (30.3)	49 (66.3)	< 0.001	4.36	2.39 - 7.945

^aOdds ratio^bConfidence interval.^cReference group.

nearly 61% of patients' SVRs showed the CC genotype, and McCarthy also found that just 21% of sensitive to treatment patients were TT. However, the frequency of the CC genotype in the resistant group was 19%. McCarthy also examined this relationship in an African American population; in the African American population there was not any significant association between rs12979860 SNP and treatment response (24). In this study of Iranian patients, the results showed a significant relationship between IL28b rs12979860 and treatment outcomes. In fact, 39.4% of sensitive to treatment patients showed CC and just 1.4% of them showed TT as a mutant genotype.

McCarthy examined the association between rs12979860 polymorphism and the HCV genotype among 681 Caucasian chronic HCV patients. The results showed that the frequency of the CC genotype as a favorable genotype in HCV genotype 1 is 32%, in HCV genotype 2 it is 46%, and in HCV genotype 3 it is 55%. They also assessed SVR in different kinds of HCV genotypes; they showed that 90.6% of resistant patients were infected by HCV genotype 1 and that only 9.4% of them carried HCV genotype 3 (24). It is noteworthy that in this study of Iranian patients there is a significant association between HCV genotypes and the sensitive and resistant groups. In fact, the results showed that 43.83% (50 out of 89) of patients who were infected by HCV genotype 1 were sensitive to treatment, whereas 95.45% (21 out of 22) of patients who were infected by HCV genotype 3a were sensitive to treatment. These results show that the HCV genotype can also play a predictor role in treatment outcomes; it means that people who carry HCV genotype 3 have a better chance of showing SVRs

when they use medicine. However, patients with HCV genotype 1 show more resistance to treatment. Maybe there is a better reason for long treatment duration for patients who are infected by HCV genotype 1 than in patients who carry HCV genotype 3.

In a study in Italy, Fabris assessed the relationship between IL28 rs12979860 polymorphism and suffering from HCC (26). The carriage of T allele of this SNP was strictly associated with the presence of HCC (26-28). In fact, patients with HCC carried T allele in 43.5% of cases while patients without HCC presented this allele in 31% of cases (26). In this study, we also analyzed the relationship between liver status and SVR and non-SVR groups. Results showed that 98.6% of chronic patients were SVR while only 1.4% of cirrhosis patients showed SVR. The combination of these results demonstrates that rs12979860 polymorphism can play a role in predicting treatment success or failure and also the progression of HCV infection.

Our results develop the observations of Ge et al. (19) and indicate that rs12979860 has a high specificity. In order to decrease the cost of long treatments and to prevent severe side effects, determining this polymorphism at the beginning of treatment can be very helpful for patients and physicians.

Footnotes

Authors' Contribution: Study concept and design: Seyed Mehdi Sadat; analysis and interpretation of data: Solmaz Talebi and Seyed Mehdi Sadat; drafting of the manuscript: Mahtab Daneshvar; sample collection: Mehri Nikbin and

Mahtab Daneshvar; method and experiment designs: Sanaz Mahmazi and Reza Aghasadeghi; laboratory experiments: Reza Aghasadeghi and Fozzieh Javadi; medical consultant: Mehri Nikbin.

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