A Tetra-Primer Amplification Refractory Mutation System–Polymerase Chain Reaction for genotyping of of rs8099917& rs12979860 IL28B Polymorphisms in Iranian HCV patients

Running title: Genotyping of rs8099917 & rs12979860 IL28B Polymorphisms with tetra-ARMS-PCR method in Iranian HCV patients

Abstract:

Background: Selecting patients for new direct acting antiviral treatment of HCV have prompted a conflicting matter worldwide because of high cost and limited availability of them. Genotyping of IL28B polymorphisms will aid clinical decision making for identifying priorities of urgent treatment in resource-limited countries.

Objectives: The aim of the present study was to design a simple tetra-primer amplification refractory mutation system–polymerase chain reaction (T-ARMS-PCR) for genotyping of the rs8099917 and rs12979860 IL28B gene polymorphisms . Furthermore, We identify the correlation of variables such as gender, serum Alt level, histology of liver and baseline viral load with these polymorphisms.

Patients and Methods: We efficiently designed a T-ARMS-PCR for detection of rs12979860 and rs8099917 IL28B gene polymorphisms. Using this method, we genotyped 148 hepatitis C patients. To ensure T-ARMS genotyping quality, we, regenotyped samples with the PCR- sequencing method.

Results: Results of genotyping of rs12979860 and rs8099917 by T-ARMS PCR method were 100% concordant with sequencing. Among these 148 patients with chronic hepatitis C, the frequency of the rs12979860 CT, TT and CC genotypes were 72.3%, 14.2% and 13.5% and the frequency of the rs8099917 TT, GT and GG genotypes were 58.1%, 38.5% and 3.4%, respectively. Low frequency (2.7%) of association of two unfavourable homozygot genotypes (TT rs12979860 / GGrs809917) as well as 56.7% of association of 3 or 4 favourable allels could explain good response of Iranians to HCV treatment with interferon- based regimens. About correlation of polymorphisms with different variables, only high viral load showed a statistically significant correlation to unfavourable genotype of TT rs12979860 (p value = 0/03) and there was no correlation of serum Alt level, gender and histology of liver to IL28B genotypes.

Conclusions: we designed a simple, inexpensive, and reproducible T-ARMS-PCR for detection of rs8099917 and rs12979860 IL28B polymorphisms which can be used for identifying patients who has a high risk of fibrosis progression and require urgent antiviral treatment. Considering similarity of rs8099917 frequencies results in different studies in Caucasian ethnicity **,** We suppose that rs8099917 polymorphisms could predict outcomes better than rs12979860 in Iranian HCV patients.

Key Words: Hepatitis C, IL28B, Single nucleotide polymorphism, Tetra-ARMS-PCR, Iranian,

1.Background

As a global health problem, Hepatitis C affects over 170-200 million people worldwide. [1] Infection with the hepatitis C virus (HCV) often causes a chronic disease which enhances the risk of the development of liver cirrhosis and HCC. [2]

According to Genome Wide Association Studies (GWAS), single nucleotide polymorphisms of genes in the area of the IFN-λ can predict the response to treatment with peg-IFN based regimens in patients infected by hepatitis C genotype 1 and of spontaneous viral clearance during acute HCV infection.[3]

The finding of studies carried on patients infected with HCV genotypes 2 and 3 exhibited some controversies. [4]

According to large studies consisting of subjects with different ethnic backgrounds, rs12979860, rs8099917, and rs12980275 were the most significant single nucleotide polymorphisms (SNPs) near the *IL28B* gene. [5]

It is now widely accepted that most of the racial differences in HCV outcomes may originate from different frequencies of IL28B genotypes between ethnic populations. [6]

The tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR) is one of the simplest, most rapid and economical methods of genotyping SNPs. Two outer primers and two allele-specific inner primers are combined in, genotyping, along with only a single PCR followed by electrophoresis separation. [7]

More potent direct acting antiviral therapy including interferon-free regimens for chronic hepatitis C has been developed and approved recently, which drew much attention to the need and value of genotyping IL28B polymorphisms. [8]

The relevance of IL28B has been suggested even in cases with no IFN treatment. It is not quite clear how IL28B polymorphism is relevant to IFN-free treatment regimens. [9] This might be consistent with viral suppression which causes differential immune 'restoration' according to IL28B genotype. [10]

Week 12 response was also higher in good-response IL28B patients among HCV patients of genotype 1a who received RBV-free therapy. This indicates the particularly important role of RBV for poor-response IL28B patients. [11]

Other studies showed that high rates of SVR were achieved even in patients with unfavorable IL28B genotypes. [12,13]This interesting issue will require further evaluation.

Various studies analyzed potential relationships between IL28B variants and other factors suggested to influence outcomes in hepatitis C. IL28B polymorphisms might influence the natural history of chronic hepatitis C. In the era of DAA it is crucial to identify patients at early disease stages with high risk of fibrosis progression who would consequently require urgent HCV treatment with new drugs.

IL28B polymorphisms can act as a predictor of rapid fibrosis progression in chronic hepatitis C. The effect of the IL28B genotype on the natural course of chronic hepatitis C has been examined by several studies, especially with regard to the fibrosis progression and liver inflammation. [12] In an interesting paper by Noureddin et al. The association of the IL28B rs12979860 CC genotype with hepatic inflammation and worse clinical outcomes was reported. Also, IL28B TG/GG was significantly associated with liver fibrosis progression in Caucasian patients. [14-16]

The high cost of new DAA regimens is a significant hindrance, limiting access. [17]This has raised a global discussion among competing health priorities, regarding the unrestricted access of all patients to new therapies. It has been suggested to consider IFN- based regimens as valuable treatment of HCV patients with favorable genotypes of IL28B, who are still unable to afford the newer DAAs, especially in resource-limited Asian countries where favorable genotypes of IL 28B is highly prevalent. [18]

Thus, it is premature to determine if the need for *IL28B* testing will diminish in the era of potent DAA treatment of HCV.

Considering the role of IL28B polymorphisms, even in treatment responses to new DAA reported in the results of some researches, as well as their role in the natural course of HCV, it is important to determine IL28B polymorphisms with a rapid and simple method in order to make decision about HCV patients.

2. Objectives

The aim of the present study was to develop a rapid single-step tetra primer amplification refractory mutation system–polymerase chain reaction (T-ARMS-PCR) for the detection of rs8099917 and rs12979860 IL28B genotypes in Iranian HCV patients. Also the correlation of these polymorphisms with other variables such as gender, serum Alt level, histology of liver and viral load, were identified.

3. Patients and Methods

3.1. Study population

This study was conducted in the Research Center for Gastroenterology and Liver Disease at Mashhad's Emam Reza - university hospital. The study groups consisted of 148 Iranian patients with chronic hepatitis C infection.

All of the patients were anti-HCV antibody positive and had positive Plasma HCV RNA by RT-PCR for more than 6 months. All individuals were Caucasian. The HCV genotype of patients with chronic hepatitis C were assessed in different medical diagnostic laboratories.

The IL28B rs12979860 SNPs were genotyped by the T-ARMS-PCR method. Patients with viral hepatitis B or alcohol intake were excluded from the study. Blood samples were collected in Na-EDTA tubes from

patients during a period from 2010 to 2013 and stored at -80°C. DNA were extracted from peripheral blood by salting-out method. [19]

HCV RNA viral load was detected using Cobas Amplicor Test (Roche Molecular Systems) in patients. Serum Alt levels were determined in all samples. percutaneus liver biopsy was done in 70 patients. Each patient's data were collected in a specially designed study file that included patient demographics, genotype of HCV, baseline viral load, severity of fibrosis and necroinflammatory activity evaluated on liver biopsy by modified HAI scoring system.

The study was approved by the ethics committee of Mashhad University of Medical Sciences and informed consent was obtained from all subjects.

3.2. IL28B Genotyping by Tetra Primer Amplification Refractory Mutation System PCR (tetra ARMS-PCR)

In this study we designed a Tetra amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR) for the detection of rs8099917 T/G and rs12979860 C/T polymorphisms of IL28B. This method is simple, rapid and sensitive for the detection of single nucleotide polymorphisms. To certify genotyping quality, we regenotyped samples with the PCR- sequencing method, and found no genotyping errors.

Primers for T-ARMS-PCR were designed. For IL28B rs8099917 polymorphism, we used two external primers (forward outer: 5"- CATCACCTATAACTTCACCATCCTCCTC-
3", Reverse outer: 5"-GGTATCAACCCCACCTCAAATTATCCTA-3") and the two allele-specific internal primers were (forward inner [G allele]: 5"-CTTTTGTTT
TCCTTTCTGTGAGCAGTG-3", Reverse inner [T allele]: 5"-TATACAGCATG
GTTCCAATTTGGGTAAA-3").

Polymerase chain reaction (PCR) was performed using commercially available PCR premix (AccuPower PCR PreMix, BIONEER, Daejeon, South Korea) according to the manufacturer-recommended protocol.

Into a 0.2-mL PCR tube containing the AccuPower PCR PreMix, 1 μL template DNA (#100 ng/μL), 1 μL of each primer (10 μM), and 15 μL DNase-free water were added. The PCR cycling conditions were 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C, with a final step at 72°C for 10 min. Each reaction was verified on a 2% agarose gel containing ethidium bromide.

The Product sizes for rs8099917were 197- bp for G allele, 295 bp for T allele, and 437 bp for the two outer primers (control band).

Primers for the IL28B rs12979860 polymorphism were designed as follows:

Forward outer: 5"- GTCACTCACGCAGGCCGCCACATC -
3", reverse outer: 5"- ACCGCTACGTAAGTCACCGCCCAGCC -3") and two allele-specific internal primers (forward inner [T allele]: 5"- CTGAACCAGGGAGCTCCCCGAAGGAGT-3", reverse inner [C allele]: 5"- CGGAGTGCAATTCAACCCTGGTGCG -3"). Polymerase chain reaction (PCR) for rs12979860 was performed using commercially available PCR premix (2X primer Taq premix, Genet Bio, South Korea) according to the manufacturer-recommended protocol. Into a 0.2- mL PCR tube containing the AccuPower PCR PreMix, 1 μL template DNA
, 0.8 μL of each primer (10 μM), and 5.3 μL DNase-free water were added. The PCR cycling conditions were 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 58°C, and 40 s at 72°C, at 72°C for 10 min with a final step at 15°C for 30 s. Each reaction was verified on a 2% agarose gel containing ethidium bromide. To certify genotyping quality, we regenotyped samples with the PCR- sequencing method and found no genotyping errors.

The Product sizes for rs12979860 polymorphism were 317 bp for T allele, 473 bp for C allele, and 738 bp for the two outer primers (control band).

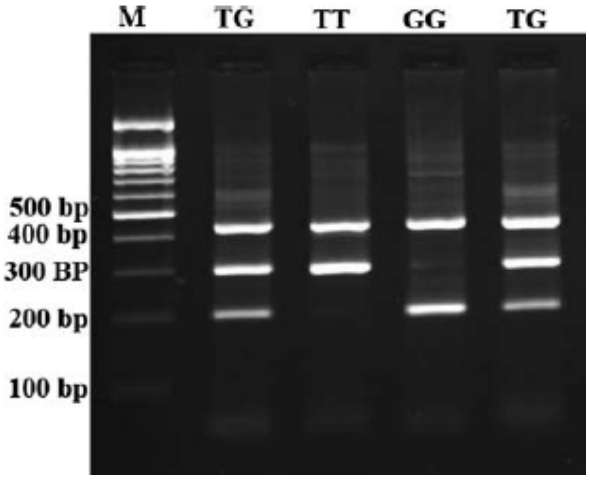

FIGURE 1 Electrophoresis pattern of tetra amplification refractory mutation system–polymerase chain reaction for detection of IL28B rs8099917 T/G polymorphism..The product sizes were 299 bp for control band, 155 bp for C allele, and 201 bp for G allele. M, DNA marker;

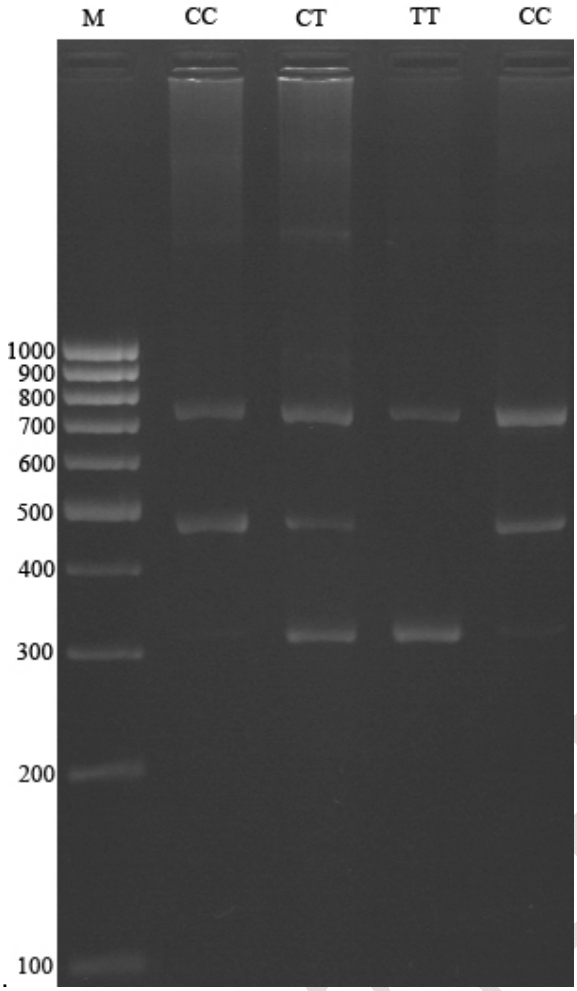

FIGURE 2 Electrophoresis pattern of tetra amplification refractory mutation system–polymerase chain reaction for detection of IL28B 12979860 C/T polymorphism. M = DNA marker. The product sizes were 299 bp for control band, 155 bp for C allele, and 201 bp for G allele. M, DNA marker.

3.3. Statistical analysis

Statistical analysis was performed using commercial software (SPSS for Windows, V 18, SPSS Inc, Chicago, IL, USA). Descriptive statistics were used to calculate the mean and standard deviation.

To determine the association of the IL28B single nucleotide polymorphism with other predictors of treatment response, we compared genotypic frequencies in three genotypic variation between the groups by chi-squared test .The significance level was set at a *P*-value of ≤ 0.05. Variables included in our study

were baseline viral load (log10 IU/mL), stage of liver fibrosis, grade of necroinflammatory activity, sex, serum alanine aminotransferase level.
In another analysis, IL28B polymorphisms were evaluated according to CC versus non-CC for polymorphism of rs12979860 and TT versus non-TT for polymorphism of rs8099917 (favorable versus unfavorable). Comparisons between groups for different variables were made using Mann-Whitney test.

4. Results

The study group included 119 (80/4 %) men and 29 (19/6 %) women with a mean age of 42.4 ± 12.4 years. Among 148 patients with chronic hepatitis C, 66.3% were infected with HCV genotype 1, 26% with HCV genotype 3, 6.7% with HCV genotype 2 and 1% with HCV genotype 4.
The baseline viral load was determined in 101 patients, which was > 400,000 IU/mL in 43/6% and < =400000 IU/mL in 56/4% of patients. The T-ARMS-PCR methods were effectively applied to genotyping rs8099917 T/G and rs12979860 C/T polymorphism of IL28B. The genotypes determined by this method were concordant with those determined by sequencing.
The frequencies of IL28B genotypes for rs12979860 were as follows: 107 (72.3.6%) were of genotype C/T, 21 (14.2%) patients were of genotype T/T and 20 (13.5%) were of genotype C/C. In addition, the distribution of IL28B rs8099917 genotypes among the patients was as follows: 86(58.1%) were TT, 57(38.5%) were GT and 5(3.4%) were GG. The differences in the distribution of IL28B genotypes and alleles between two sexes were not statistically significant (P = 0.49 for rs12979860 and P = 0.99 for rs8099917 by Chi-square test).
The serum Alt level in CC genotype of rs12979860 with median level of 50.5+_160 IU was not significantly different from unfavorable non CC genotypes with median level of 53.5+_59.33 IU (P= 0.76 by Mann-Whitney test) . Moreover, in favorable genotype of rs8099917 TT with median Alt level of 48+_91.26 IU was not significantly different from unfavorable non-TT genotypes with median level of 59+_64.6 IU (P= 0.59 by Mann-Whitney test).
When we compared the distribution of IL28B rs12979860 genotypes of HCV patients with high viral load to low viral load, we found a 20% higher frequency of rs12979860 TT genotype in the patients with high viral load, compared to patients with low baseline viral load, which is statistically significant (P = 0.03) . On the other hand, the proportion of patients with C/C polymorphism was highest in the low baseline viral load (< 400.000 IU/mL) group (17.5%) compared to high viral load (> 400000) group (6.8%).
There was not such an association of rs8099917 genotypes with viral load.
Liver biopsy was performed in 70 patients and histologic evaluation was done according to Modified HAI - scoring system by two expert pathologists. Results were as follows: 35(50%) mild fibrosis (stage 1&2), 8 (11/4%) moderate (stage 3&4) and 27 (38.6%) sever (stage 5&6) fibrosis. For necroinflammatory activity 48(68.6 %) mild (grade 0-4&4-8), 21(30%) moderate (grade 8-13) and 1(1.4%) had sever (grade 13-18) necroinflammatory activity. There were no statistically significant differences between the three IL28B subgroups in any of the two polymorphisms regarding the fibrosis severity (P = 0.75 for rs12979860 & P=0.55 for rs8099917) , or necroinflamatory activity (P = 0.49 for rs12979860 & P=0.06 for rs8099917) by Chi-square test. Also there were no significant differences in another analysis of histology of liver in the two favorable (CC of rs12979860 & TT of rs8099917) versus the unfavorable (non CC of rs12979860 & non TT of rs8099917) genotypes of both polymorphisms by Mann-Whitney test. (P= 0.76 for stage & P= 0.17 for grade in rs12979860 & P= 0.8 for stage & P= 0.53 for grade in rs8099917)

Table 1&2 summarize patients characteristics in each IL28B genotype consisting of serum Alt level, gender distribution, baseline viral load and liver histology.

Table1. Patient characteristics and genotypic frequencies of rs12979860 of IL28B in HCV patients.

		Frequency Of TT%(n)	Frequency Of CT%(n)	Frequency Of CC%(n)	P- Value	Total

sex	male	12.6(15)	73.1(87)	14.3(17)		119
	female	20.7(6)	69(20)	10.3(3)		29
Viral load	>400000	22.7(10)	70.5(31)	6.8(3)	0.036	44
	<=400000	7(4)	75.4(43)	17.5(10)		57
Stage of Histology*	Mild	8.6(3)	74.3(26)	17.1(6)		35
	Moderate	12.5(1)	87.5(7)	0(0)	0.75	8
	Sever	7.4(2)	66.7(18)	25/9(7)		27
Grade of Histology**	Mild	6.25(3)	70.8(34)	22.9(11)		48
	Moderate	14.3(3)	76.2(16)	9.5(2)	0.49	21
	Sever	0(0)	100(1)	0(0)		1

*Staging was done according to Modified HAI - scoring system.
**Grading was done according to Modified HAI - scoring system.

Table 2. Patient characteristics and genotypic frequencies of rs8099917 of IL28B in HCV patients.

		Frequency Of TT%(n)	Frequency Of TG%(n)	Frequency Of GG%(n)	P- Value	Total
sex	male	58(69)	38.7(46)	3.4(4)		119
	female	58.6(17)	37.9(11)	3.4(1)		29
Viral load	>400000	50 (22)	43.2(19)	6.8(3)	0.1	44
	<=400000	61.4(35)	38.6(22)	0(0)		57
Stage of Histology*	Mild	65.7(23)	28.6(10)	5.7(2)		35
	Moderate	87.5(7)	0(0)	12.5(1)	0.55	8
	Sever	63(17)	37(10)	0(0)		27
Grade of Histology**	Mild	75(33)	25(15)	0(0)		48
	Moderate	61.9(13)	23.8(5)	14.3(3)	0.06	21
	Sever	100(1)	0(0)	0(0)		1

*Staging was done according to Modified HAI - scoring system.
**Grading was done according to Modified HAI - scoring system.

5. Discussion.

Hepatitis C affects many people in all part of the world. Therefore, all factors influencing its natural course or differences in its response to treatment are of great importance. One of these most important factors is variation of IL28B polymorphisms as a predictor of HCV outcomes.

In our study which was conducted in Mashhad, Iran, in the Southwest of Asia with Caucasian ethnicity, we genotyped two IL28B polymorphisms in 148 HCV patients, using a Tetra-arms PCR method.

Similar to the former studies carried on Caucasian patients in Europe, United State, Australia and Iran, the most common rs12979860 genotype was CT. [20, 21] Moreover, similar to the results of Li meta-analysis of different countries, the prevalence of TT genotype of rs12979860 was significantly lower in Caucasian, compared to African-Americans. [22]

We also determined polymorphisms of IL28B rs8099917 with the same method and obtained the following results: the most prevalent genotype was TT, followed by GT and GG. The results of genotyping of rs8099917 were similar to other studies on Caucasian patients from other parts of the world with similar frequencies of each polymorphism and very low frequencies (less than 5 percent) of the unfavorable GG genotype in all of them. [22-25]

As we know, the rs12979860 C allele and rs8099917 T allele are the favorable alleles for the spontaneous clearance of HCV and the prediction of response rate to Peg-IFN and RBV in patients infected with genotype 1 or 4. [26]
Similarities between frequencies of rs8099917 genotype among Caucasian patients in different studies were more than genotypes of rs12979860. [22-25]
This interesting finding may indicate the importance of rs8099917 polymorphisms in determining HCV outcomes in Caucasian patients, compared to rs12979860.
An interesting and previously unreported observation in our study was the low frequency of association of the two unfavorable genotypes that were seen in only 2.7% of Iranian patients, which might be the cause of Iranian's good response to the treatment. [27]
In this study, we designed a simple, inexpensive, and reproducible T-ARMS-PCR method for the detection of rs8099917 and rs12979860 IL28B polymorphisms which can be used for routine assays**.**
There are some logical reasons to use a simple and rapid method for genotyping IL 28B polymorphisms even in the era of IFN- free approved regimens for the treatment of HCV.

The first reason is that highly potent DAA drugs in the IFN- free regimens which are recommended for first-line treatment of HCV in new guidelines are of high cost, [28] therefore, they are not available to many patients, especially the ones in low-income countries of Asia.
Regarding the high prevalence of favorable genotypes of IL28B polymorphisms with good response to IFN _based regimens, it seems that treatments with IFN_ based regimens are superior to waiting with no treatment in patients with favorable genotypes of IL28B with a high probability of progression to fibrosis and cirrhosis .

The second reason is that according to some researches, it has been shown that rs12979860 polymorphisms are relevant to treatment responses to IFN-free regimens. For example, in the INFORM-1 study, the slope of the viral decline was steeper in patients with a good response IL28B genotype. [29]The SOUND-C2 study also suggested that IL28B genotype may remain relevant to IFN-free treatment regimens, while also pointing to critical roles of HCV subtype and RBV. [30]
Regarding the treatment with Sofosbuvir and Simeprevir, The NEUTRINO trial reported that non-CC IL28B genotype patients were strongly associated with reduced response (92%, CC-genotype vs 87%, non-CC genotype). However, the SVR rate was still found to be high. [31] Even a recent study from Japan has shown that IL28B genotype had an impact on SVR rates in patients, even in LDV/SOF regimen. [32] It seems that more studies are required to determine the association of host IL28B genotype and SVR in patients across different geographical population subgroups. Thus, the clinical relevance of *IL28B* genotyping in the setting of the new interferon-free HCV regimens might be diminished, although it is potentially useful in shortening the duration and choice of treatment regimens.

The third reason is the results of several studies that have pointed out the association of genotypes of two IL28 B polymorphisms with the natural course of HCV, especially regarding the fibrosis progression and liver inflammation.
For example, using transient elastography (TE), Barreiro et al. reported the association of the CC genotype of rs12979860 with liver fibrosis in co -infected HIV/HCV patients.[33]
Rembeck et al. and Ydreborg et al. showed the enhancement of portal inflammation and steatosis by the CC genotype in HCV genotype 3 patients. [34]
In the study of Noureddin et al. the IL28B rs12979860 CC genotype, was associated with hepatic inflammation and worse clinical outcomes. Also, the rs8099917 GG genotype was associated with slower fibrosis progression compared to the TT genotype in Caucasian patients. [15] The results of some other studies were conflicting. That includes the study conducted by Nobuharo Tamaki on fibrosis progression rate of patients who did not achieve sustained virological response by interferon_ based therapy. He showed that IL28B TG/GG was significantly associated with the increased liver fibrosis progression rate. [14] Most of these studies, except one, shows that the rate of necroinflammatory activity and fibrosis are more rapid in favorable genotypes of IL28B. [35]Thus it is important to initiate with the most available treatment, even PEG- IFN regimens instead of waiting for the availability of more potent drugs.
Similar to these studies, we also analyzed serum ALT level and histological findings of our patients, in order to detect any association of the outcome predictors with IL28B polymorphisms. The results of our study differ from the previously mentioned studies and there was not any correlation between serum Alt

level, necroinflammatory activity or the stage of fibrosis and IL28B genotypes in our study. The cause of differences between the results of liver histology in our study and other studies might have been the small sample size of patients that had liver biopsy in our study. Another cause might have been the fact that our study was cross- sectional and it was not possible for us to evaluating fibrosis and necroinflammatory activity during the research by repeated evaluation, as in some other studies.

As another aspect of our study, we evaluated the association of both polymorphisms of IL28B with the baseline viral load of HCV in blood samples of the patients.

Surprisingly, the high viral load of HCV showed a meaningful correlation with the unfavourable genotype of TT rs12979860. However, there was not any association of the viral load with rs8099917 polymorphisms.

In literature, the data on this association are conflicting. But most of them showed a paradoxical relationship between favorable polymorphisms and the viral load in that the 'responder' allele of rs12979860 was statistically associated with a higher baseline viral load, inconsistent with the clinical observation that higher baseline viral load is typically associated with a poorer treatment response. As an example, Grebely et al. highlighted the role of the IL28B genotype in HCV-RNA levels as an important factor affecting the progression of fibrosis. [36] Another study showed that Patients with the TT/CC genotype for rs12979860/rs8099917 had a significant correlation with higher HCVRNA levels. [37]

Our results did not confirm this paradoxical association, but it shows that as we expect a better response to treatment, favorable polymorphisms of rs12979860 are associated with a lower base line viral load. These conflicting data show that the role of IL28B in influencing the baseline viral load is still poorly understood.

Conclusion

As a conclusion, the data on the association of IL28B and outcomes of HCV are insufficient and require more work. Our method of genotyping can be used, as a rapid and accurate way, for widespread clinical studies in this field.

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