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Original Article

Impact of *IL28B* gene polymorphisms rs8099917 and rs12980275 on response to pegylated interferon-α/ribavirin therapy in chronic hepatitis C genotype 4 patients

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Abstract

Host genetic factors may predict the outcome and treatment response in hepatitis C virus (HCV) infection. One of these factors is the single nucleotide polymorphisms of the interleukin 28B (*IL28B*) gene. We sought to evaluate the outcome of pegylated interferon and ribavirin therapy in association with *IL-28B* rs8099917 and rs12980275 in patients infected with HCV genotype 4. A total of 180 patients with chronic hepatitis C were selected from Egyptians who have received combined therapy with pegylated interferon and ribavirin for 6 months and their response was evaluated after follow-up at 0, 6, 12, 24 and 48 weeks from the beginning of the therapy. Blood samples were collected from responders and non-responders. Genomic DNA was extracted from whole blood and genotyping was carried out by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). Our results showed that TT genotype of rs8099917 was associated with higher sustained viral response (SVR) rates and G allele represented a risk factor for failure of response (OR=3.7, CI=1.8:7.64) while rs12980275 was not significantly associated with SVR in genotype 4 Egyptian patients. The determination of *IL-28B* SNPs may be useful in enhancing correct prediction of SVR achievement in treating this group of genotype 4 patients.

Keywords: IL-28B polymorphism, hepatitis C virus, sustained viral response, genotype

Introduction

Hepatitis C virus (HCV) infection is one of the main causes of chronic liver disease worldwide[1]. Currently, there are 7 major HCV genotypes, which have different geographical distributions and susceptibilities to interferon-α treatment[2]. In Egypt, more than 90% of cases belong to HCV genotype 4[3]. The combination of pegylated interferon and ribavirin is still the most effective therapy in HCV-4[4], and the success rate of current therapy chronic hepatitis C is related to a variety of host and viral factors that can be used as response predictors[5-6]. Identifying predictors of response to current therapy, particularly in patients infected with genotype 1 and 4, remains as one of the main objectives of research[7]. Recent genome-wide association studies (GWAS) have shown that human genetic variations (single-nucleotide polymorphisms, SNPs) of the gene for interleukin 28B (IL-28B) may explain differences in treatment outcomes of adults chronically infected with HCV and that they can be useful as therapy response markers[8-9,10-11]. Among them, the most important ones seem to be rs12979860, rs8099917, and rs12980275[7]. Previously, it was reported that the

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favorable *IL-28B* genotypes were independent strong pretreatment predictors of treatment response in European patients[12], and IL28B polymorphism rs12979860 has a marked differential distribution between racial groups, being least frequent in African Americans, most frequent in Asians, and with an intermediate frequency in Hispanics and Caucasians[8,13].

There are few data so far regarding the role of *IL28B* polymorphism in HCV-4 Egyptian patients with respect to response to antiviral therapy or fibrosis progression[14]. The current study, aimed to determine the relationship between SNPs rs12980275, and rs8099917 and response to pegylated interferon and ribavirin combination therapy in Egyptian patients infected with HCV genotype 4. This information may be important for better prediction of response to therapy, and could allow for a better selection of patients for further treatment.

Patients and methods

Patient selection

A total of 180 patients with chronic hepatitis C from Liver Unit of Minia Healthy Insurance Hospital who were scheduled to receive combined pegylated interferon-α and ribavirin for 6 months were chosen. All patients volunteered to participate in the study and their responses were evaluated after follow-up at 0, 6, 12, 24 and 48 weeks from the beginning of the therapy. Available complete data of patients were included in the study. Patients were subjected to thorough history taking, clinical examination and routine pre-treatment work up, including serum transaminases (aspartate aminotransferase, AST; alanine aminotransferase, ALT), total bilirubin, albumin, alkaline phosphatase (ALP) and hepatitis B surface antigen (HBsAg), alpha fetoprotein (AFP), and quantitative real time- RT-PCR.

Exclusion criteria included hepatitis B or human immunodeficiency virus (HIV) co-infection, coexistent autoimmune liver diseases, hemochromatosis, or other coexistent chronic liver diseases. Blood samples from these patients were used for genotyping. All patients were subjected to quantitative RT-PCR repetitively to detect viral load and determine the response to treatment with combined pegylated interferon-α/ribavirin therapy. The study protocol was approved by the local institutional review board at the authors' affiliated institution and consent was obtained from all the study participants.

Study design

Patients were divided according to their response to pegylated interferon-α and ribavirin therapy into 2 groups. Group I: 90 non-responders (NR), including those who had detectable HCV RNA at week 12, 24 or 48. Group II: 90 patients with sustained virological response (SVR) (responders) with undetectable HCV-RNA 6 months after stopping treatment.

DNA extraction and IL-28B genotyping

Five mL blood samples were collected from all subjects in sterile tubes containing ethylene diamine tetraacetic acid. Immediately after collection, whole blood was stored at -20°C until use. Genomic DNA was extracted from whole blood using the established protocol as described by Medrano *et al.*[15].

Genotyping of SNPs rs12980275 and rs8099917 was carried out by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) assay. For rs8099917, oligonucleotide primers were: 5'-CAT CCC ACT TCT GGA ACA AAT C-3' (sense) and 5'-GTATCA ACC CCA CCT CAA ATTATC-3' (antisense). For rs12980275, primer sequences were: 5'-AAG AGG AGG GAA GGA AGT TCT G-3' (sense) and 5'-GGT CTG GTC CTA GTG GTG TTT G-3' (antisense). PCR conditions (25 μL) were: initial denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 25 seconds, and extension at 72°C for 40 seconds. PCR products for rs8099917 and rs12980275 were of 400 and 293 base pairs, respectively[7].

To perform RFLP assay for rs8099917 genotype, 50 μL amplicons were digested with 1 U *BseM*I restriction endonuclease (Fermentas, Vilnius, Lithuania) at 55°C for 12 hours. *BseM*I digestion of allele TT yields fragments of 400 base pairs, whereas DNA containing allele GG polymorphism yields fragments of 234 and 166 base pairs. For RFLP assay for rs12980275 genotype, 50 μL amplicons were digested with 2 U *BseL*I restriction endonuclease (Fermentas) at 55°C for 12 hours. *BseL*I digestion of allele AA yields fragments of 178 and 115 base pairs, whereas DNA containing allele GG polymorphism yields fragments of 148, 115, and 30 base pairs[7]. Restriction digestion products for each were separated on agarose gel stained with ethidium bromide for visualization on a UV transilluminator.

Serum IL28 quantification

Serum IL28 was assessed by enzyme-linked immunosorbent assay (ELISA) using ELISA kit for interleukin 28B (IL28B, USCN Life Science inc., Wuhan, China). The color was developed and the concentration of IL28 was measured.

Statistical analysis

The statistical program SPSS for windows (Version 20) had been used in data entry and analysis. Statistical significance was determined at the 95% confidence interval (CI) level. The lowest accepted level of significance was 0.05.

Descriptive analysis was performed to compare genotype frequencies using the chi-square test for qualitative data and mean ± SD for quantitative data. Genotypic and allelic frequencies were assessed using Hardy-Weinberg equilibrium. We estimated adjusted odds ratios (ORs) and 95% confidence interval (95%CI) for association between SVR and genetic polymorphism using logistic regression models, and dominant model of inheritance was applied.

Results

Patient demographic and baseline characteristics

Baseline characteristics of the 180 patients are shown in ***Table 1***. The demographic features showed no statistically significant difference in age and gender between the two groups. The mean age was 43.1 ± 7.3 years in responders and 44.63 ± 6.5 years in non-responders, with male pre-dominance in both groups. α-Fetoprotein (AFP) was significantly higher in non-responders compared to responders. Meanwhile, no significant difference was observed in AST, ALT, albumin, hemoglobin and bilirubin between the two groups. In addition, our results showed a significantly higher viral load in non-responders than that in responders (P=0.001).

Table 1 **Demographic and laboratory data of the study groups.**

Features	Responders (n=90)	Non-responders (n=90)	P value
- Age (years)	43.1 ± 7.3	44.63 ± 6.5	0.33
- Gender			
Male	62 (68.9%)	58 (64.4%)	0.4
Females	28 (31.1%)	32 (35.6%)	0.16
-ALT (IU/L)	54.3 ± 5.2	56.8 ± 4.3	0.22
-AST (IU/L)	48.6 ± 4.7	51.4 ± 3.8	0.08
-Albumin (gm/dL)	4.31 ± 0.36	3.94 ± 0.4	0.33
-Bilirubin(mg/dL)	0.58 ± 0.22	0.61 ± 0.18	0.51
-AFP (ng/dL)	2.3 ± 1.9	4.2 ± 2.5	0.02[a]
-ALP (IU/L)	123 ± 66	131 ± 52	0.64
-Hemoglobin (gm/dL)	14.2 ± 1.8	13.87 ± 2.2	0.51
-Hepatitis C Virus RNA (IU/mL)	65732 ± 1448	723 327 ± 7521	0.001[b]

[a]P≤0.05, [b]P≤0.01

Table 2 **Relation between treatment response and different *IL–28B* gene allele.**

	Responders (n=90)	Non-responders (n=90)	X^2	P value
rs8099917				
TT	72 (80%)	30 (33.33%)	6.3	0.001[a]
TG	12 (13.33%)	54 (60%)	6.5	0.001[a]
GG	6 (6.67%)	6 (6.67%)	0	0.5
rs12980275				
AA	75 (83.33%)	69 (76.67%)	1.11	0.13
AG	3 (3.34%)	6 (6.67%)	1.02	0.15
GG	12 (13.33%)	15 (16.66%)	0.6	0.26

[a]P≤0.01

Genotyping analysis

The frequency of "TT type of rs8099917" was 80% in responders and 33.33% in non-responders while the frequency of "TG type of rs8099917" and GG type was 20% among responders and 66.67% in patients who did not respond to combined treatment showing a significant difference in these polymorphisms (P=0.001, ***Table 2***) with (OR=8; 95%CI=4.06-15.7, P=0.001) (***Table 3***). For G carrier, there was a significant increase (P=0.001) in the frequency of heterozygous polymorphism TG among non-responders, while there was no significant difference in the homozygous allele GG (P=0.5). Generally G allele represents risk factor (OR 3.7; 95%CI=1.8-7.64, P=0.001) (***Table 3***).

For the frequency of rs12980275, there was no significant difference in all alleles between responders and non-responders, showing that the polymorphism in this site may not be a dependent predictor of SVR

Table 3 **Association between interferon response and *IL–28B* gene polymorphisms.**

	Responders (n=90)	Non-responders (n=90)	OR (95% CI)	P value
rs8099917				
TT (ref)	72 (80%)	30 (33.33%)		
TG	12 (13.33%)	54 (60%)	8 (4.06-15.7)	
GG	6 (6.67%)	6 (6.67%)		0.001[a]
T allele	156 (86.67 %)	114 (63.33 %)	3.7 (1.8-7.64)	
G allele	24 (13.33%)	66 (36.67%)		0.001[a]
rs12980275				
AA (ref)	75 (83.33%)	69 (76.67%)		
AG	3 (3.34%)	6 (6.67%)	1.5 (0.72-3.18)	
GG	12 (13.33%)	15 (16.66%)		0.3
A allele	153 (85 %)	144 (80 %)	0.7 (0.33-1.4)	
G allele	27 (15 %)	36 (20 %)		0.18

[a]P≤0.05. CI: confidence interval; OR: odds ratio.

in patients with chronic HCV (P=0.3) Generally G allele doesn't represents risk factor (OR 0.7; 95%CI=0.33-1.47, P=0.18) (***Table 3***).

Serum IL-28 level

There were no significant differences in serum IL-28 level between responders and non-responders and in comparing serum level among genetic alleles, it was non-significantly lower in patients carrying G allele of rs8099917, especially TG allele, while in rs12980275, the level of serum IL-28 was almost the same in different polymorphisms (***Fig. 1***).

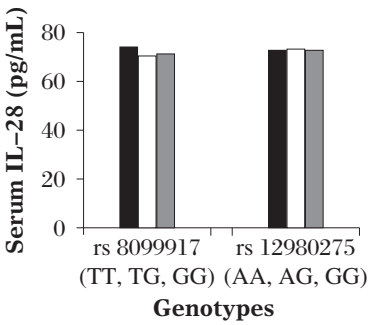

Fig. 1 **Serum IL–28 level in different genotypes of the study population.**

Discussion

HCV genotype 4 is the most frequent cause of chronic hepatitis C in the Middle East and North Africa[16]. Sustained virological response in genotype 4 for combination of pegylated interferon and ribavirin therapy ranges between 43%-70%[17-18]. High treatment cost is a serious economic challenge in developing countries, including Egypt that requires more extensive studies on predictors of treatment response such as viral factors and host genetic factors such as *IL28* gene polymorphisms. Although many studies have reported an association between several SNPs in the *IL28* locus and the response of polyethylene glycol-ribavirin combination therapy for genotype 1[11,13], only few studies have investigated the role of these SNPs in the treatment of other genotypes, especially genotype 4.

IL28B SNP rs8099917 is extensively investigated in Asia, while in the USA and Europe, rs12979860 is determined to predict response to therapy[19]. The role of *IL28B* gene SNP rs12979860 in response to treatment in genotype 4 has been recently studied in Egypt; therefore, our study aimed to evaluate the genotype and allele frequencies of IL-28B rs8099917 and rs12980275, as well as its association with the outcome of HCV infection in a group of Egyptian patients treated with pegylated interferon and ribavirin.

Several studies demonstrated that AFP was a strong predictor of failure to achieve SVR either in genotype 4[20] or genotype 1[21], which is in agreement with our results showing that serum AFP was higher in non-responders compared to responders, confirming the suggestion that serum AFP should be added to the list of predictive factors of treatment response in CHC[22].

In a previous study, the genotype frequency for the polymorphisms in *IL-28B* gene (for rs8099917) was associated with spontaneous viral clearance[23] as the TT genotype corresponds to a positive predictive value of 89% for spontaneous viral clearance. In addition, patients infected with HCV genotype 1 homozygous with T allele of rs8099917 have a 2- to 3-fold higher chance of eradicating the virus under treatment with polyethylene glycol/ribavirin than patients carrying G allele[19]. This is in agreement with our results as the OR of both TG and GG *vs.* TT alleles was 8 (CI=4.06-15.7, P=0.001), indicating that this allele is associated with poor response to combined treatment of HCV genotype 4. Another study revealed a high prevalence of rs8099917 G allele in HCV/HIV-1-coinfected patients and its strong association with treatment failure in HCV genotype 1-infected patients[24].

IL28B encodes for interferon-λ3 (IFNL3), which belongs, together with IFNL1 (IL29) and IFNL2 (IL28A), to the family of type III interferons[25]. The molecular mechanisms whereby IL28B influences treatment outcome are still unknown; one possible answer might be that the SNPs of IL28B linked with SVR were strongly associated with lower hepatic expression of interferon-stimulated genes (ISGs)[26-27].

It was demonstrated that *IFN-γ* gene polymorphisms influence its expression, changing the levels of the cytokine, which may lead to different outcomes of the viral infection. Low levels of IFN-γ were associated with persistent HCV infection[28]. This may explain the lower level of serum IL28B found in patients carrying G allele; although it was non-significant, the level was lower in patients with TG and GG allele. However, previous study revealed that subjects carrying rs8099917-GG genotype had higher serum level of IL28B than those with GT or TT genotypes[29].

There is a paucity of literatures concerning the effect of SNP of IL-28B rs12980275 on treatment response. To the best of our knowledge, this study is one of the few studies that specifically examined the genotype frequencies of *IL-28B* rs12980275 polymorphisms in Egyptian patients with chronic HCV-4 infection. Our results showed that there was no significant difference in the genotype frequencies between responders and non-responders, and the carrier of G allele of rs12980275 did not represent any risk factors for the response failure of chronically infected patients to com-

bined treatment. This is in the context with a previous study showing no significant differences in children and adolescents chronically infected with HCV genotypes 1 and 4[7]; however, in another study of the rs12980275 genotype, homozygous AA was found in 22 of 50 cases with SVR, *vs.* 4 of 49 in non-responders patients ($P<0.0001$).

Our results also disagreed with Venegas *et al.*[30], who found that the proportion of patients with rs12980275 AA, AG and GG genotypes was 44%, 46% and 10% in those with SVR, respectively. In non-responders, this proportion was 8.2%, 69.4% and 22.4%, respectively, indicating that this genetic polymorphism is strongly associated with response to treatment with pegylated interferon/ribavirin in Chilean patients infected with HCV genotype 1.

A possible hypothesis for these seemingly contradictory findings is that carriage of the favorable allele A at rs12980275, being associated with a slightly diminished baseline activation of ISGs, is beneficial for clearance of all HCV genotypes as reflected by the association with a greater first phase decline in HCV RNA during therapy[31]. However, continuous re-exposure to a variety of HCV genotypes following a possible initial spontaneous clearance of HCV, as is often the case among intravenous drug users in addition to the lack of a lasting protective immune response[32], and this skewness will exert selective pressure and over time lead to an under-representation of these favorable alleles among genotype 4 patients as in genotype 1[31].

Finally, our findings showed that polymorphism in rs8099917 of IL-28B was related to the outcome of combined treatment response of HCV and may be useful to be considered as pre-treatment predictor while rs12980275 did not predict treatment response.

There may be a debate if genetic markers are still needed if the upcoming new treatments become available, although recent data have shown that the IL28B genotype remains one of the most important predictors of SVR with sofosbuvir-based triple therapy for HCV genotype 1 patients[33]. However, in many countries, these treatments will not be available for foreseeable future. In these countries, interferon-based therapies will be used for many years. Thus, well-known pre-treatment predictors for non-responders like high baseline viral load, older age, advanced fibrosis stage and host genetic factors should further be considered prior to initiation of anti-viral therapy[19], this might reduce the cost and side effects associated with longer term treatment[34].

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