The Role of rs12979860 Single Nucleotide Polymorphism of IL 28B Gene in Early Virological Response in Egyptian Patients with Hepatitis C Virus Genotype 4

Mohsen M. Maher[1], Mohamed A. Mohamed[1], Hanaa A. Amer[2], Tarek M. Yosef[1], Moataz M. Sayed[1], Maha M. Hussein[1], Sara H. Abou Agwa[3], Mahmod A. Hewaidy[1].

Department of Gastroenterology and Hepatology[1]- Ain Shams University. Department of Clinical Pathology[2]- Ain Shams University. Medical Research Center[3]- Ain Shams University.
tarekyosef31@yahoo.com

Abstract: Recently, single nucleotide polymorphisms (SNPs) of IL28B and host response to pegylated interferon α (PEG-IFNα) and ribavirin (RBV) were shown to be strongly associated. This study aimed to investigate the relation between SNP at rs12979860 allele of IL28B gene and early virological response (EVR) in Egyptian patients with HCV genotype 4 (HCV-4). The HCV RNA level of 27 patients with HCV-4 receiving pegylated interferon and ribavirin therapy (P-INF/RBV) was assessed by quantitative measurement at baseline and 24 weeks after start of treatment. Genotyping of IL28B was done by PCR Amplification followed by SNPs of rs12979860 by Direct Sequencing using Automated Sequencer (ABI system), for all patients during treatment. The results showed that The CC genotype of rs12979860 was identified in 11 (40.74%) patients, 10 of them (90.9%) achieved EVR, while the CT heterozygous was detected in 6 (22.22%) patients, 4 of them (66.7%) achieved EVR and the TT was found in 10 (37.04%) patients and none of them (0%) was responder at 12 weeks. The EVR was significantly associated with CC genotypes compared to other genotypes (p<0.001), the TT genotype was associated with failure to achieve EVR. These results suggest that IL28B genotyping can be used to predict EVR in patients with chronic hepatitis C genotype 4.

Key words: single nucleotide, Hepatitis C Virus, IL28B gene, Genotype.

1. Introduction

Infection with hepatitis C virus (HCV) is a major global health issue. Only a small proportion of patients clear the virus spontaneously and the majorities develop chronic hepatitis C infection [1]. About 30% of individuals with persistent infection develop chronic liver disease, including cirrhosis and hepatocellular carcinoma [2]. The facts that the current standard treatment, consisting of pegylated-interferon and ribavirin (P-INF/RBV), is suboptimal, together with its high cost and significant toxicity led to increasing need to identify accurate predictors of treatment outcome to facilitate treatment decision-making [1].

Recently, genome-wide association studies (GWAS) have identified host genetic variation to be critical for predicting treatment response and spontaneous clearance in patients infected with hepatitis C virus (HCV) [3]. Single-nucleotide polymorphisms (SNP) in the region of the IL28B gene on chromosome 19, coding for the interferon (IFN)-λ-3 or IL28B gene, are strongly associated with sustained virological response (SVR) and with natural viral clearance [3,4]. The SNP variants occur in the minor allele rs8099917 and the proximate polymorphism rs12979860 [4].

The available data imply that an important interaction between HCV infection and IL28B is critical for viral persistence or clearance and the outcome of IFN based therapy in patients with genotype 1 chronic hepatitis C infection is strongly related to IL28B genotype [5]. Several reports suggested that polymorphism of CC genotype at rs12979860 and the TT genotype at rs8099917 allele of IL28B gene are associated with significantly higher response rate to INF based therapy in patients with chronic hepatitis C genotype 1 (HCV-1) and they can independently predict the SVR with high sensitivity and specificity [6, 7, 8].

Haflon, 2011 confirmed that SNPs rs8099917 and rs12979860 used alone may be useful for predicting the outcome of HCV treatment. He found that the highest predictive SVR associated with rs12979860 CC compared with the rs8099917 TT (respective positive predictive value: 72% vs. 63% respectively). So, he recommended rs12979860 determination alone for predicting interferon response [9].

Egypt has the highest prevalence of HCV worldwide, with more than 90% of infections due to genotype 4. The features of hepatitis C genotype 4 (HCV-4) infection and the appropriate therapeutic regimen have not been well characterized [10]. The

ongoing interest in host genetic factors, particularly as regard the role of IL28B gene polymorphism, in prediction of virological response and the paucity of reports in this regards for patients infected with HCV-4 made the necessity to explore this point.

Aim of the study:

The aim was to investigate the predictive power of the rs12979860 IL28B SNP for early virological response (EVR) to pegylated interferon and ribavirin (P-INF/RBV) therapy in chronic hepatitis C genotype 4 (HCV-4) patients.

2. Patients and Methods:

This study included 27 patients with chronic hepatitis C genotype 4 (HCV-4) who were receiving pegylated interferon and ribavirin therapy at Yasin Abd-Elghafar Liver Center. Patients were asked to provide a blood sample for genotyping of rs12979860 SNP of IL28B gene during their follow up visits. The aim and the design of the study were explained to all patients and they all provided a written consent for enrollment. The study protocol was approved by the ethical committee of Ain Shams University Hospital and Yasin Abd-Elghafar Liver Center.

Inclusion criteria:

Treatment naïve adult patients (age 18-60 years) with HCV-4 who received full dose of pegylated interferon and ribavirin therapy for at least 12 weeks

Patients base line data and follow up:

Before the start of treatment patients were evaluated for combined (P-INF/ RBV) treatment according to Yasin Abd-Elghafar Liver Center protocol by liver function tests, complete blood counts, serum creatinine, prothrombin concentration, INR, thyroid functions, HBs Ag, HBc Ab and alpha fetoprotein. In addition electrocardiogram (ECG), abdominal ultrasound and fundus examination were performed to all patients. Patients were all eligible for treatment after evaluation.

HCV-RNA levels were analyzed by real time polymerase chain reaction at 0 and 12weeks of starting treatment, using a QIA amp RNA minikit supplied by Qiagen. Extraction steps were carried on a Biosafety cabinet level II under complete sterile condition according to the manufacturer's instructions. Work was done using 7500 fast Real time PCR machine supplied by Applied Biosystem. HCV genotyping was done using INNO-LiPA III (Line Immunoprobe assay) provided by Innogenetics, Ghent, Belgium.

All patients received Pegylated interferon-alpha 2a (Roche) 180 mcg per week in combination with Ribavirin (Roche) 15mg/kg per day divided into 2 doses. Early virological response (EVR) defined as HCV RNA either undetectable (complete EVR) or decrease by ≥2 log but still detectable (partial EVR) after 12 weeks of the start of treatment.

Patients were followed by liver function tests, complete blood counts and serum creatinine every 4 weeks.

None of the included patients had co-infection with HBV.

(rs12979860) SNP of IL28B genotyping:

- DNA collection and extraction:
 Blood was collected into EDTA tubes following standard procedures. Genomic DNA was extracted from peripheral blood mononuclear cells using DNA extraction kit supplied by Qiagen Co., work was done in Biosafety level II cabinet under complete aseptic conditions. Extracted DNA was stored at   -70°C until submitted to sequencing procedure.

- Rs(12979860) SNP genotyping :
 SNP genotyping was performed by PCR amplification followed by direct PCR Sequencing using automated sequencer ABI system.
 The oligonucleotides used for PCR were ks (5'GTGCATATGTTTGTGAC-3') and (5'-GAGGCCCCTCACCCATGC-3') antisense.
 The PCR amplification mixture 5 ul of DNA genomic DNA solution, 10 pmol of each nucleotides, 12.5Ul AmpliTaq Gold 360 master mix (Applied Biosystems) under the following thermal cycler conditions : stage 1; 94 $\overset{\circ}{}$ C for 5 minutes, stage 2 ; 94 ^{o}C for 30 secs, 72 ^{o}C for 45 secs, for a total of 35 cycles and stage 3 ; 72 ^{o}C for 7 min.
 Sequencing: 1.0 Ul of the PCR products was incubated with the Big Dye Terminator v.3.1 cycle sequencing kit (Applied Biosystems)

3. Results:

Patients were divided into 3 groups according to IL28B genetic pleomorphism (rs12979860). Patients' groups (Figure 1) were:

- Group 1 CC type included 11 (40.74%) patients.
- Group 2 CT type included 6 (22.22 %) patients.
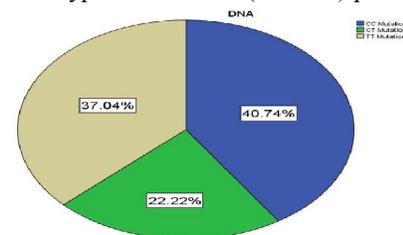- Group 3 TT type included 10 (37.04%) patients.

Figure (1): Distribution of different IL28B genotypes in studied patients.

Table (1) summarizes the demographic and laboratory data of all patients before start of

treatment. All patients in different groups were comparable at base line as regard age, sex and laboratory functions. It was noticed that patients in group 3 who had the TT variant of IL 28B gene showed slightly higher serum HCV RNA level before treatment than other patients however, it had no statistical significance.

Table 1: Demographic and laboratory data of all patients before treatment

Variables	Group 1 CC (n=11)		Group 2 CT (n=6)		Group 3 TT (n=10)		*P* value
Demography							
Gender							
Males (n:21)	8/11 (72.7%)		4/6(66.7%)		9/10(90%)		0.483
Females (n:6)	3/11 (27.3%)		2/6(33.3%)		1/10(10%)		
	Mean	SD	Mean	SD	Mean	SD	
Age (years)	47.54	5.69	42.66	7.86	47.6	4.351	0.208
Laboratory data							
ALT (u/l)	53.36	40.01	42.83	13.54	45.00	18.49	0.715
AST (u/l)	40.45	24.54	53.83	25.24	49.70	14.55	0.424
Total bilirubin mg/dl	1.12	0.57	1.25	0.4	1.18	0.63	0.898
Direct bilirubin mg/dl	0.354	0.39	0.40	0.26	0.466	0.393	0.788
Albumin(gm/dl)	4.01	0.402	3.53	0.484	3.59	0.486	0.057
INR	1.115	0.0618	1.146	0.104	1.203	0.136	0.176
AFP (ng/ml)	5.33	4.43	8.98	9.07	13.23	12.89	0.177
HGB (gm/dl)	13.418	1.47	13.416	1.47	12.91	1.456	0.691
WBC 1000/mm^3	10.37	18.48	6.21	2.27	5.47	1.73	0.619
PLT 1000/mm^3	168.9	50.24	126.5	88.49	108.7	41.00	0.069
HCV RNA (IU/ml)	585.19	559.59	549.8	123.36	733.6	445.24	. 821

The response to combined (P-INF/ RBV) therapy was evaluated after 12 weeks of treatment by HCV RNA level to detect early virological response (EVR). A total of 13 (48.15%) patients achieved complete EVR (cEVR) and one patient (3.7%) had partial EVR (pEVR). The remaining 13 (48.15%) patients were non responders. Response to antiviral treatment at 12 weeks is shown in table (2) and figure (2).

Table 2: Response to antiviral treatment at 12 weeks.

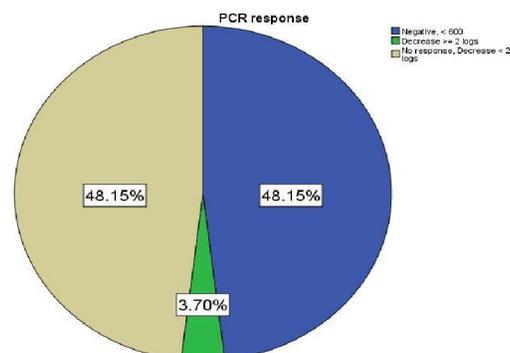| Response type		Number	Percentage	Total
Responders	cEVR	13	48.15%	14 (51.85%)
	pEVR	1	3.7%	
Non responders		13	48.15%	13 (48.15%)
Total		27	100%	27 (100%)

Figure (2): Response to treatment at 12 weeks

Response to treatment was analyzed in different groups of patients in table (3); ten (91%) patients with CC type SNP rs12979860 of IL28B gene achieved EVR (9 of them were cEVR and one patient was pEVR), four patients (66.7%) with CT genotype had EVR and none of the patients with TT genotype achieved EVR. All patients (100%) with TT genotype were non responders to (P-INF/ RBV) therapy at 12 weeks. The difference between the groups in response to treatment at 12 weeks was highly significant (*P* value < 0.001).

Table 3: Response to treatment in different groups

Variables	Group 1	Group 2	Group 3	*P* value
	CC (n=11)	CT (n=6)	TT (n=10)	
EVR (n=14)	10/11 (90.9%)	4/6(66.7%)	0/10(0%)	<0.001
Non responders (n=13)	1/11(9.1%)	2/6(33.3%)	10/10(100%)	

In patients who achieved EVR (total 14 patients), there were 71.43% had the CC genotype of IL28B and 28.57% had the CT genotype. None of the responders at 12 weeks had the TT genotype at the rs12979860 allele of IL28B. The percentage of non responders (total 13 patients) in different genotypes was 76.92%, 15.38% and 7.7% for TT, CT and CC genotypes respectively (Table 4).

Table 4: Response to treatment according to IL28 genotype

IL28B genotypes	**Responders** (n=14)	**Non responders** (n=13)	*P* value
CC (n=11)	10/14 (71.43%)	1/13 (7.7%)	<0.001
CT (n=6)	4/14 (28.57%)	2/13 (15.38%)	
TT (n=10)	0/14 (0%)	10/13 (76.92%)	
Total (n=27)	14/27 (51.85%)	13/27 (49 .15%)	

4. Discussion:

Hepatitis C infection is a common infection with significant morbidity and mortality [11]. Epidemiological, viral and host factors have been associated with the differences in HCV clearance or persistence, and studies have demonstrated that a strong host immune response against HCV favors viral clearance [2]. Recently, all interest has been turned towards demonstration of host polymorphism located upstream of IL-28B gene which is associated with sustained virological response to treatment with Pegylated interferon α in combination with Ribavirin [12] in genotype 1 hepatitis C virus (HCV) patients [13]. There are minimal data for patients with genotype 4 HCV. This study was designed to investigate SNP of IL28B gene at rs12979860 allele as a predictor of EVR in HCV-4 patients.

In this study, the frequencies of the IL-28B genotypes were as follows: CC, 40.74%; CT, 22.22%; and TT, 37.04%. Overall 51.85% of patients achieved EVR; 71.43% of them had CC genotype, 28.57% had the CT genotype and none of patients who had TT genotype achieved EVR. Analysis of EVR of different groups showed that EVR occurred in (90.9%, 66.7% and 0.0%) of patients with CC, CT and TT genotypes of IL28B respectively with significant difference. These findings suggest an association between achievement of EVR and different IL28B genotypes.

It was found that patients with EVR achieve high SVR rates with the current treatment duration of 48 weeks in genotype 1 and 4 patients [14]. So based on our findings, we can conclude that IL28B genotyping can potentially predict SVR because of its significant association with EVR in HCV-4 patients.

This conclusion is supported by **Hendy *et al.*** They studied a cohort of patients infected with HCV-4 and found that SVR is related to SNPs in IL28B gene. In their study patients with CC genotype of rs12979860 allele and TT genotype of rs8099917 allele of IL28B gene had a significantly higher SVR rates than patients with other genetic patterns [15]. Other investigators also reported that rs12979860 SNP genotype CC was highly associated with treatment success in HCV genotype 4-infected patients [16, 17].

The effect of IL28B genotype on virological response of HCV to standard therapy appears to be similar in different HCV genotypes and in different ethnic groups. **Bochud *et al.*** showed that the T allele of rs12979860 was an independent risk factor for a less pronounced first phase HCV RNA decline [18]. They also showed that in genotype 2/3 patients, the T (rs12979860) was associated with a reduced first phase decline in viral load. They concluded that polymorphisms in IL28B are strongly associated with the first phase viral decline during Peginterferon-α/Ribavirin therapy of chronic HCV infection, irrespective of HCV genotype [18]. Several other

reports supported the role of IL28B gene in response of treatment in different HCV genotypes [9, 13, 19-22].

The mechanism that underlies this association is not fully understood. Some studies found that IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes (ISGs), a new reported determinant viral factor, in patients with chronic hepatitis C [23, 24]. Furthermore, serum IL-28A/B levels were significantly higher in patients with chronic hepatitis C with good allele of IL28B genotype [8, 25]. These evidences indicate that both direct antiviral effect and immune-mediated effect of IL28B could be affected by these polymorphisms. However, detailed mechanistic understanding needs further investigation [8].

Concerning the relationship between baseline viral load and SNPs of IL28B, the results from previous reports were not consistent. Some reports claimed the baseline viral load was correlated with the genotype of the SNP but others did not [6, 8, 26-28]. Although in our study, patients with TT genotype had higher mean viral load than the other two groups, our data showed no significant relationship between baseline viral load and the SNPs.

Currently, many decisions for the treatment of hepatitis C virus (HCV) are based on genotype, which is the most significant baseline predictor of response to therapy. However, it has become increasingly apparent that fixed treatment durations might not be appropriate for all patients infected with HCV [14]. This study suggests that SNPs at rs12979860 allele of IL28B gene may be useful for predicting the EVR of HCV-4 patients. This may guide to tailor treatment duration in the light of the new baseline host genetics predictors of response and the emerging concept of response-guided therapy especially in genotype 1 and 4 patients.

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