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RESEARCH ARTICLE

Interleukin 28B polymorphism predicts treatment outcome among Egyptian patients infected with HCV genotype 4.

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Abstract

Background/ Aims: Egyptian patients with HCV genotype 4 respond less successfully to treatment with pegylated interferon regimen.The Aim of this study was to investigate the associations between IL-28B single nucleotide polymorphisms (SNPs) and treatment outcome among them . **METHODS**: The HCV patients classified into (I) patients with sustained virological responders (SVRs) (N=87) &(II) Non responder patients (NRs) (N=135)where they were genotyped as CC, CT or TT at the polymorphic site rs12979860.

RESULTS: The frequencies of the IL-28B genotypes were as follows:The proportions of rs12979860 CC, CT, and TT genotypes were 59.7%, 34.5%, and 5.8% among patients with SVR, versus 7.4 %, 50.37 % and 42.23 % among those with treatment failure (NRs) (P <0.001). By using univariate regression analysis, the minor allele of IL28B (p <0.0001), high serum level of HCV-RNA (p = 0.035) and advanced fibrosis (p = 0.02) were associated with (NRs)(Odds ratio =3.75 with 95% confidence interval (2.308 - 6.1067),. While, in multivariate logistic regression analysis, rs12979860 CC genotype was the strongest predictive of SVR (OR = 20.83, 95%CI = 11.63–37.04, p <0.0001).

CONCLUSION: The IL28B rs12979860 SNP is the strongest predictor of an SVR among Egyptian patients infected with HCV -4.

INTRODUCTION

Hepatitis C is a major health problem which affects about 3% of the total population (Munir et al 2010). Egypt has the highest prevalence of hepatitis C virus (HCV) infection estimated to be about 15% of the population(with a seroprevalence ranging from 10% in children to 45% in adults (Sievert et al 2011). genotype 4 is the most prevalent one in Egypt [about 90%] (Sievert et al 2011). Several immunological factors have been implicated in determining disease outcomes in HCV infections (Rehermann , 2009). Only 30% of exposed population clear the infection naturally, whereas the remaining 70% develop chronic hepatitis C which may progress to end stage liver disease even hepatocellular carcinoma (HCC) can develop on top of cirrhosis (Morgan , 2011).

The current standard-of-care for chronic HCV infection comprises weekly injections of Peg-interferon-α (peg-IFN α) in combination with daily oral ribavirin for 24-48 weeks (Hadziyannis et al 2004 ; Dahari et al 2007). Approximately 45 % of patients infected with the most common form of HCV (genotype 1) achieve a sustained viral response (SVR) with this treatment. The effectiveness of this combination therapy has two main phases: a rapid first phase decline, referring to the viral decline during the initial day(s) after onset of treatment, and a slower second phase decline, which is usually defined as the reduction of HCV RNA levels from the second to the fourth week (Herrmann et al 2003). These phases are assumed to reflect, respectively, the antiviral action of interferon (first phase) and the loss of infected hepatocytes (second phase) (Lagging et al 2009).

On the other hand the high cost of the current standard of care for chronic hepatitis C (CHC) using pegylated (Peg) IFN plus ribavirin with a variable response & many unpleasant side effects among the patients is triggering for understanding of the difference in host resistance to HCV infection and in response to treatment & this will be of course clinically important and may lead to novel therapeutic interventions (Strader et al 2004).

Several Predictors of response to treatment have been investigated in CHC patients including viral factors (Shire et al 2006), host factors (Backus et al 2007), metabolic factors (Persico et al 2007), histopathological factors (Myers et al 2003), types of regimen (Strader et al 2004), and duration of infection (Lin et al 1996). Among these factors, viral kinetics following antiviral therapy has been thoroughly investigated & recognized as one of the most widely accepted predictors of response to IFN-based treatment (Fried et al 2008).

Recently, several genome wide association studies (GWAs) have revealed that single nucleotide polymorphisms (SNPs) within or adjacent to IL28B predicts spontaneous clearance of HCV (Rauch et al 2010 ; Thomas et al 2009) as well as the likelihood of SVR following therapy for chronic hepatitis C in different Western populations (Ge et al 2009; Suppiah et al 2009; Tanaka et al 2009). The IL28B gene encodes for interferon-λ3 (IFN- λ3), which constitutes the IFN- λ family together with IFN- λ 1 (encoded by IL29) and IFN- λ 2 (encoded by IL28A). The IL28A, IL28B, and IL29 genes are located on chromosome 19 (19q13) (Lange et al 2011). Several SNPs have been identified in the promoter region of IL-28B, some of these markers had highly predictive of SVR in HCV genotype 1 infected patients, i.e. rs12979860 and rs 8099917 markers. The present study was designed to investigate the association between IL28B-related SNP, rs12979860, as predictive marker for treatment outcome of HCV in Egyptian patients (Gomma et al 2015, Matsuuraet al 2014)

Patient Selection

This study is a part of the project on the liver disease Funded by the Mansoura university post graduate & research affairs in which 222 consecutive adult patients with chronic hepatitis C genotype 4 were included. The study was performed among patient attending outpatient clinic of Tropical Medicine Department receiving pegylated interferon therapy and ribavirin for 48 weeks .The study conducted between 2009 and 2012 in Mansoura University Hospital. The inclusion criteria were as follows: patients above the age of 18 years with positive HCV RNA in serum and elevated alanine aminotransferase (ALT) levels at least 6 months before the inclusion, chronic hepatitis confirmed by histological examination, body mass index (BMI) below 30 kg/m^2. The exclusion criteria were as follows: decompensated liver cirrhosis, autoimmune liver disease, uncontrolled thyroid disease, alcohol abuse, liver cancer, hepatitis B virus or HIV coinfection, any severe chronic disease, hemochromatosis, and immunosuppressive therapy. Patients were treated for 48 weeks with standard of care medication: PegIFN α-2a 180 μg subcutaneously once a week (Hoffmann-La Roche, Basel, Switzerland); or PegIFN α-2b 1.5 μg/kg subcutaneously once a week (Schering-Plough Co, MSD, United States) plus oral ribavirin (1,000 mg/ day for patients with body weight < 75 kg; 1,200 mg/day for patients with body weight > 75 kg) for 48 weeks. Patients were classified into the following two groups based on treatment outcome:(I) sustained virological responders (SVRs) and(II) non- responders (NRs). SVRs had no evidence of viraemia at 24 weeks after completion of IFN therapy, whereas NRs were still viraemic at this stage. All subjects in the present study received a detailed explanation, and all signed a written informed consent. This study was approved by the local Ethical Committee, and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Efficacy Assessments

Serum HCV RNA levels were measured by real time polymerase chain reaction assay (ABI 7300 ; limit of detection 15 IU/mL) at weeks 12, 24 and 48 during treatment and at week 72 after treatment. The primary end point was SVR, defined as undetectable HCV RNA at week 72 (Herrmann et al 2003).

Quantitative PCR

Total cellular and viral RNA was isolated using RNeasy Mini columns (QIAGEN) followed by one step RT-PCR (Applied Biosystems; Foster City, CA), then quantited by 7300 ABI real time PCR using the DyNAmo HS SYBR Green qPCR Kit). The viral load was quantited against standard curve.

HCV Genotyping

Because the currently recommended treatment durations and ribavirin doses depend on the HCV genotype, HCV genotyping is recommended for patients considering antiviral therapy (Bowden 2006). HCV genotyping was determined by VERSANT HCV Genotype Assay (LiPA), (Bayer Corporation, Tarrytown, NY, USA). The Amplicor HCV kit and the LiPA were performed according to manufactures′ instructions.

IL28B Genotyping.

Only 222 patients have been genotyped for IL-28B rs12979860 polymorphism. The genomic region associated with HCV response (Suppiah et al 2009) contains several highly correlated SNPs around the IL28B gene. We selected the most strongly associated SNP, rs12979860, located upstream of this gene for genotyping in our cohort using restriction fragment length polymorphism (RFLP) procedure (Fabris et al 2011) with some modification. DNA from peripheral blood mononuclear cells was isolated using the QIAamp DNA mini kit (Qiagen) and quantified on a GENWAY 6105 spectrophotometer. DNA samples were then genotyped for the IL28B rs12979860 polymorphism. PCR primers were designed to amplify products of exactly 151 pb using Primer3 (v. 0.4.0) (http://frodo.wi.mit.edu/) software to generate more appropriate allele-discriminating DNA fragments. A DNA fragment of 151 bp containing the C/T polymorphism of IL28B rs12979860 was amplified using the forward primer 5'-GGTCGTGCCTGTCGTGTACT-3' and reverse primer 5'-AGGCTCAGGGTCAATCACAG-3' to generate 151 bp.
We carried out PCR using 1 ng DNA, 0.2 mM of each primer, 1X reaction buffer, 200 mM dNTPs, 2.5 mM MgCl2, and 1 unit of Taq DNA polymerase in 25-ul reactions. PCR amplification was done using a block thermal cycler (DNA Engine, MJ Research) programmed 35 cycles of 94 C for 1.0 min, 59 C for 30 s and 72 C for 30 s following a 10 min Taq activation step.
The PCR products (5 ul) were then electrophorsed using 1.5 agrose gel stained with ethidium bromide to check a PCR reaction. Ten microliter of remaining PCR products were digested using 10x NE buffer and 2.0 U of appropriate restriction enzymes (Hpy166II). The reaction mixture was incubated at 37°C for 4 hours according to manufacturer's instructions (New England Biolabs, Beverly, MA, USA). This enzyme will cut PCR product at T allele.
The resolved PCR digest was then visualized by staining with ethedium bromide on 2% agarose gels and examination under ultra violet light. The 100 bp ladder was used as molecular size markers. The T allele (wild allele) was identified by the presence of two fragments 105 and 30 bps. Whereas C allele (rare allele) was identified by the presence of one fragment of size 135 bps (figure 1). (Panel A) . Real time allelic discrimination PCR was done using the following validated probes (Invitrogen™ Inc, UK): FAM- TGGTTCGCGCCTTC-MGB ; VIC -CTGGTTCACGCCTTC-MGB for the genotyping of C,T alleles respectively (figure 1) (Panel B).

Liver biopsy:

Liver biopsies were obtained with an 18-gauge or larger needle with a minimum of five portal tracts and were routinely stained with hematoxylin-eosin stain. Biopsies were interpreted according to the scoring schema developed by the METAVIR group. Needle liver biopsy specimens were examined by a pathologist unaware of the laboratory results. METAVIR score was used to stage fibrosis (F0–F4). Fibrosis was scored on a 5-point scale: F0, no fibrosis; F1, portal fibrosis alone; F2, portal fibrosis with rare septae; F3, portal fibrosis with many septae; F4, cirrhosis. (Poynard et al 1997).

Statistical analysis was performed using the standard statistical software SPSS 10.0 (Chicago, IL). application program. Frequency tables were calculated to obtain information about the occurrence of the different risk factors and polymorphic variants between the three groups. The Hardy-Weinberg equilibrium was calculated to describe the relationship between gene frequency and genotype frequency. . Genotypes were compared by using the Chi-square test or Fisher's exact test for categorical data and one-way analysis of variance (ANOVA) nonparametric test for continuous data. P values less than 0.05 were considered statistically significant.

Results:

Descriptive Results.

We performed genotyping for IL28B rs12979860 in a total of 222 Egyptian with chronic active hepatitis C treated with combined peginterferon alpha and ribavirin . Our Cohort included 87 patients responded to treatment, and 135

non responders. The clinical characteristics of our study cohort of Egyptian patients with chronic active hepatitis C are summarized in Table 1. Eighty-seven patients (39.2%) responded successfully to HCV treatment (i.e. achieved SVR). The stage of fibrosis was not significantly associated with treatment failure.

IL28B rs12979860 Polymorphism:

We successfully genotyped 222 patients with CHC for IL28B rs12979860 Polymorphism using RFLP analysis procedure (figure 1).

The genotype frequencies were in Hardy–Weinberg equilibrium in both groups (P > 0.05), and the polymorphism information content were 29% and 34% in SVRs and NRs groups respectively. The homozygosity was 66.7% and 49.6% among SVRs and NRs groups respectively (Table 2).

The proportions of rs12979860 CC, CT, and TT genotypes were 59.7%, 34.5%, and 5.8% among patients with SVRs, versus 7.4 %, 50.37 % and 42.23 % among those with treatment failure (NRs). The frequency of wild type allele C was 77.6 % in SVR while allele frequency of rare allele T in NRS group was 67.4%.

The association of rs12979860 SNP genotype with the HCV peg-IFN-α/RBV treatment response has been evaluated. Overall, allele T carriers had a significantly higher risk of treatment failure than patients carrying the C genotype, Odds ratio (OR) 3.75 (CI, 95% 2.3- 6.1) and P <0.0001 (Table 3). This suggests that this rs12979860 SNP may predict treatment failure before peg-IFN-α/RBV therapy. The odds and the risk of carriage for each genotype were also significantly different in SVRs group compared to NRs group (Table 3). p values were also significant after Bonferroni correction.

By univariate analysis, the minor T allele of IL28B (p <0.0001), high serum level of HCV-RNA (p = 0.035), and advanced fibrosis (p = 0.02) were associated with NRs. By multivariate analysis, the minor T allele of IL28B (OR = 3.7546, 95%CI = 2.3084-6.1067, p <0.0001) was associated with NRs independent of other covariates.

Table (1): Clinical and laboratory characteristics of two groups of patients with HCV.

	SVRs (n=87)	NRs (n=135)	P value
Mean age (SD)	42 (15.4)	44(13.4)	>0.05
Gender n (%) male	66 (75.9)	108 (80%)	>0.05
ALT IU/L (SD)	65 (9.5)	62 (11.5)	>0.05
AST IU/L (SD)	55 (7.8)	59 (10.4)	>0.05
HCV viral Load IU/ml(SD)	127,200	101,200	<0.05
Fibrosis stage n(%) 2 3	 51 (58.6) 36 (41.4)	 71 (52.6) 64 (47.4)	>0.05

SVRs = sustained virological responders NRS = Non responders

Table (2): distributions of the IL28B rs12979860 polymorphism among HCV patients.

	SVRs	NRs
Wild type (CC)	52 (59.77)	10 (7.40)
Heterozygous (CT)	30 (34.48)	68 (50.37)
Rare allele (TT)	5 (5.75)	57 (42.23)
Allele frequencies C T	 77.6% 22.4 %	 32.6 % 67.4 %
PIC	0.29	0.34
Homozygosty	66.7 %	49.6%
PD	0.51	0.56
Hardy-Weinberg x2 P	 0.059 0.81	 2.89 0.089

PIC =polymorphism information content
PD=Power of Discrimination

Table (3): Association between IL28 rs12979860 SNP and interferon response in HCV patients

	OR	95 % CI lower	high	Chi-square	P
Overall T	3.7546	2.3084	6.1067	28.51	<.0001
CT	1.9284	1.1058	3.3629	4.79	0.028625
TT	11.9846	4.5645	31.4667	33.18	<.0001

OR= Odds ratio

Figure 1: Ethidium bromide stained agarose patients with different genotypes of IL-28B rs12979860
1= HAEIII DNA marker, 2, 4, 5 =CT, 3,6=TT, 7=CC.

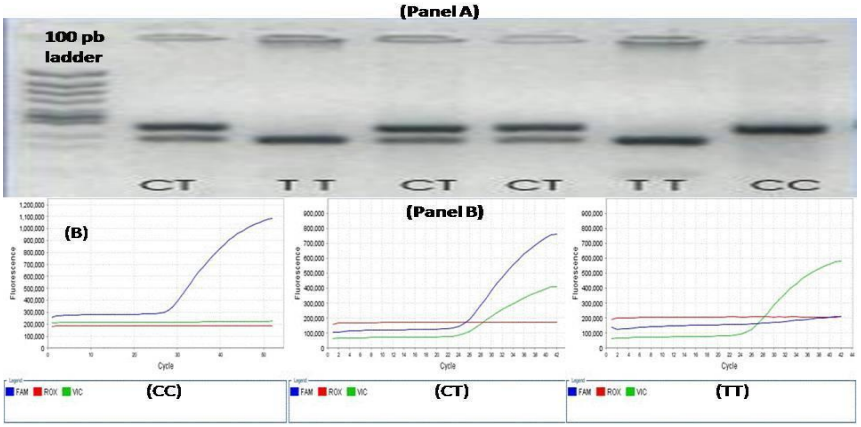

Figure 1: (Panel A) Ethidium bromide stained agarose gel for patients with different genotypes of IL28B rs 12979860
(Panel B) Real time PCR charts for different genotypes of IL28B rs 12979860

DISCUSSION:

Various predictors of response (whether viral or host factors) to IFN plus ribavirin –based treatment can determine the outcome of therapy (Akuta et al 2007).Genetic factors can be observed in ethnic differences in the treatment response to IFN and in the rate of spontaneous clearance in chronic hepatitis C (Welzel et al 2009). Several SNPs have been identified in the promoter region of IL-28B, some of these markers had highly predictive of SVR in HCV genotype 1 infected patients (Rauch et al 2010 ; Thomas et al 2009). The current study was conducted to explore the impact of the IL28B variations (rs12979860) on the therapeutic outcome in the Egyptian population infected with HCV genotype 4.

Previous studies have revealed a substantial impact of genetic polymorphisms near the IL28B gene, including rs12979860 on the outcome of treatment with PEG-IFN-a and ribavirin in patients with chronic hepatitis C as well as on the outcome of acute hepatitis C in genotype 1 in Caucasians. Ge D et al 2009 reported that among Caucasian patients with G1, the rs12979860 (3 kilobases upstream of the IL-28B gene) wild CC genotype was an independent predictor favoring SVR (Ge et al 2009).
Also, McCarthy et al. demonstrated a similar finding with respect to off-treatment viral loads in Caucasian patients with G1 (McCarthy et al 2010).

In the current study, we provide evidence for association of IL28B gene with the response of Egyptian HCV patients to treatment with Peg-IFN-α and ribavirin. In our data, the frequency distribution of rs 12979860 SNP in patients with chronic HCV who achieved SVR was 59.7%, 34.5% and 5.8% for CC genotype, CT and TT genotypes respectively. However, in NRs, the frequency distribution of rs 12979860 SNP was 42.2% , 50.4% and 7.4% for TT genotype, CT and CC genotypes. The frequency of T allele (rs12979860) was significantly higher among individuals not responding to treatment than those with SVRs. The C allele had higher SVR rates while the carrying one or two T alleles had about 4 fold risk of failure to PEG-IFN-α and ribavirin treatment. This is in accordance with several global studies revealed that the C/C genotype is strongly associated with viral clearance, while both the C/T and T/T genotypes have less chance to clear the virus (Liao et al 2011; Ruiz-Extremera et al 2011; Shaikh et al 2015).

There is no established mechanism by which polymorphisms within this locus affect the response to treatment. It was suggested that one of the polymorphisms in strong linkage disequilibrium with the two SNPs is a missense substitution within the IL-28B coding region. Recently, Urban et al.2010 using an HCV replicon system suggested that there was no difference in antiviral potency between wild-type IL-28B and amino-acid substituted variant in vitro. On contradiction it has been found that genetic variation in the IL-28B locus is associated with expression levels of IL-28B (plus IL-28A) IFN in peripheral blood mononuclear cells (Suppiah et al 2009; Tanaka et al 2009), however findings reported by Ge et al. (2009) are contradictory (Ge et al 2009). Further researches are needed to prove these points.

The rs12979860 allele is 3 kb upstream from the IL28B locus which also contains several genes, including IL28A and IL-29 (Sheppard et al 2003). It is likely that the SNP may also affect the function of other genes in the locus. Actually, it has been found that this variant is associated with increased serum IL-29 and IL-28A/B levels and the resolution of HCV infection (Rauch et al 2010 ; Langhans et al 2011). These results suggest that the genetic polymorphism of IL28B gene may have an important role on the expression and production of all IFNs, which may elucidate, at least in part, their strong link with the outcomes of HCV infection. (Matsuuraet al 2014)
Meanwhile, the IL-28B variant has been used also as a predictor of response to treatment in HCV patients after liver transplantation (Charlton et al 2011, Fukuhara et al 2010). These findings suggest that the IL-28B polymorphism may be associated with innate as well as adaptive immunity (Gomma et al 2015).
Potentially, this genotype could be associated with a weaker antibody response and a bias toward both innate and adaptive cell mediated immunity (Knapp et al 2011). Interestingly, Zhang et al (2012) demonstrate that IL28B inhibits HCV replication in three independent HCV models (Zhang et al 2011). Further studies suggest that the inhibition of HCV by IL28B is predominantly mediated by the JAK–STAT pathway. Therefore, the mechanism by which the gene variants regulate the expression of IFNs in HCV infection must be clearly identified.

In conclusion, the data from our present study confirmed the association of C allele of rs12979860 in Egyptian patients with HCV and response to treatment with peginterferon and ribavirin combination therapy. Further studies will be needed to investigate the other SNPs closed to rs12979860 in promoter region of IL-28B gene in larger cohort (s). This will allow investigating whether this association is true or due to population stratification.

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Conflict of interest

The authors who have taken part in this study declare that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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