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# IL-28B genetic polymorphism as a Predictor for Efficacy of Treatment with Interferon in Egyptian Hepatitis C Patients.

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## ABSTRACT

Hepatitis C is a global health problem and represents a major cause of liver disease and socioeconomic burden. Effective antiviral therapy may prevent these complications, but the current treatment for patients with chronic hepatitis C virus (HCV) infection does not achieve sustained virological response (SVR) for all patients. Therefore, identification of the determinants of response to treatment is a high priority. A number of host and viral factors have been associated with treatment outcomes. Genome-wide association studies implicated IL28B single-nucleotide polymorphisms (SNPs) as the strongest genetic pretreatment predictor of SVR in HCV infection. Recently, the American Association for the Study of Liver Diseases and the European Association for the Study of the Liver included IL28B testing in their guidelines.The present study was aimed toinvestigate the possible role of the SNPs of IL-28B(rs12980275 and rs8099917) inpredicting the response to therapy in Egyptian patients infected with hepatitis C virus-4 (HCV-4).Egyptian patients were treated with Peg-IFN-α/RBV. A total of 200 HCV-4 infected patients and 100 healthy control subjects were included in the presentstudy.SNPs in the IL-28B (rs8099917 T/G andrs12980275A/G) geneswere assessed usinga simple, rapid, and inexpensive polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.The IL28B rs8099917 TT and rs12980275 AA genotypes were significantly higher in responders than in non-responder ($P$=0.011 and $P$=0.012, respectively). Significant association between IL-rs8099917 TT and rs8099975AA was observed in responder group ($P$=0.0152). No significant differences in genotype and allele frequencies of IL28B gene (rs8099917 and rs12980275) between males and females were observed ($P$> 0.05). It can be concluded that SNPs in IL-28B may be promising predictors of SVR inHCV-4 infection.

**Key words:** Chronic HCV; Interleukin-28B genes; sustained virological response.

## INTRODUCTION

Hepatitis C virus (HCV) infection is a significant problem in Egypt due to the high prevalence and so increased risk for the development of liver complications. The current standard-of-care (SOC) therapy for HCV, which is a combination of pegylated interferon-α (Peg-

IFN-α) and ribavirin (RBV), is both expensive and associated withsignificant side-effects; additionally this therapy results in only 50–70% sustained virological response *[26]*. To avoid potential adverse events in patients who do not benefit from the treatment and to reduce the cost of therapy, it is necessary to predict an individual's response before treatment *[5]*. Identification of the factors involved in the persistence of HCV infectionmay lead to the development of effective prognostic tests and hence improved treatment outcome or point to the need for development of novel antiviral agents *[12]*.

Many studies indicated that the immunity level of the host correlates with the relevant gene polymorphisms, especially in case of single nucleotide polymorphisms (SNPs) in the promoter region that regulates gene expression. Polymorphisms in the regulatory regions of the cytokine genes may influence the expression of these genes and can serve as genetic predictors of disease susceptibility or clinical outcome and are thus important for the potential individualization of the treatment regimen*[12]*. Functional gene polymorphisms have been identified in inflammatory cytokines and some reports have shown possible relationships between these genotypes and the clinical outcome of HCV related liver disease [7,14].

Interleukin 28 (IL-28) is a cytokine that exists in two isoforms, IL-28A and IL-28B, plays a role in the immune defense against viruses.These isoforms belong to the type III IFN family of cytokines (IFN-λ-1/2/3 = IL-29, IL-28A and IL-28B, respectively). IL28B encodes IFN-λ3,a cytokine distantly related to type I interferon and the IL-10 family.IL28B gene together with IL28A and IL29 genes form a cytokinegene cluster. Expression of the cytokines encoded by these three genes can be induced by RNA virus infection [4]. IFN-λ-3 andIFN-α have an additive antiviral effect as both bind to cell-surfacereceptors and activate the Janus-activated kinas - signal transducer andactivator of transcription (JAK-STAT) cell-signaling cascade leading to the induction of interferon stimulating gene factor 3 (ISGF3), whichencodes lambda or type III IFN [15]. ISGs have different antiviral properties, such as inhibition of viral replication, inhibition ofviral protein synthesis and degradation of viral RNA [23]. Multiple genome-wide association studies (GWAS) have identified SNPs near the IL28B gene to be strongly associated with both spontaneous and treatment-induced clearance of HCV infection [32,22 and 13]. IL28B polymorphisms have been also associated with treatment response tonew anti-HCV drugs, such as telaprevir [6].

## PATIENTS AND METHODS

200 chronic HCV- 4 Egyptian patients (105 males and 95 females aged 30-50 years old) were included in this study. These patients attended the national research centre (NRC), Cairo, Egypt, from Aug. 2011 till Apr.2013 to receive Peg- IFN/RBV combination therapy. None of the patients had previously received any form of IFN-based therapy or hepato-protective treatment before the study. In addition, 100 healthy volunteers were evaluated as a control group (55 males and 45 females aged 35-58 years old) with no history of liver infection and all of them had completely normal liver function tests, normal liver ultrasounds and negative serological findings for viral and autoimmune liver diseases. An informed consent for gene analysis was obtained from all subjects enrolled in this study. The criteria used to determine the hepatitis C therapy were in accordance with international guidelines. A chronic HCV infection was diagnosed by the persistence of anti-HCV and HCV RNA in serum for more than 6 months before therapy. The exclusion criteria included age below 18 years, hepatitis B virus or human immunodeficiency virus co-infection, diabetes mellitus, active schistosomiasis, hypertension, thyroid disease, a history of alcohol abuse, renal insufficiency, and autoimmune liver disease.

**Treatment regimens.** All patients with chronic HCV-4 were treated with a weekly subcutaneous injection of Peg-IFN-α-2b at a dose of 1.5 mg/kg per week in combination with a weight-adjusted dose of oral RBV (1000 mg/day for < 75 kg,

1200 mg/day for ≥75 kg) for 48 weeks. According to their response to the treatment, the patients were classified into responders, who achieved SVR, and non-responders, who did not achieve SVR. SVR is defined as the presence of an undetectable HCV RNA upon completion of treatment and 24 weeks thereafter.

**Laboratory assays.** Venous blood samples (~10 mL) were collected and the serum was separated and used to assess alanine transaminase, aspartate transaminase and alkaline phosphatase activities; the total bilirubin, direct bilirubin, HCV-RNA, and HCV specific antibody titres. Another portion of blood was collected in tubes containing citrate to separate the plasma used for the assay of albumin. A third portion of blood collected in tubes containing EDTA and The DNA was extracted from the EDTA-treated blood to genotype IL-28B (rs8099917 T/G and rs12980275A/G) genes. The viral RNA extraction was carried out by using the QIAamp® Viral RNA Kit (Qiagen/Westburg, The Netherlands). HCV genotypes were identified via direct sequencing of non-structural (NS5B) viral genes. RT-PCR was done using a one-step RT-PCR kit (QIAGEN, Inc.). The genotype of each sample was determined by comparing its sequence with those of HCV prototypes obtained from Gen Bank. The serum HCV-RNA level was measured using one-step RT-PCR kit (TaqMan assay reagents and the Ambion RNA Company (Foster City, CA, USA), USA).

**Genotyping of SNPs IL-28-rs8099917 and rs12980275.**Genomic DNA was prepared from peripheral blood lymphocytes using the QIAamp® DNA Blood Mini Kit (Qiagen, Hilden,Germany). All SNP genotyping was performed using the PCR and restriction fragment length polymorphism (RFLP) according to the manufacturer's instructions. The SNPs at the s8099917and rs12980275 were detected using primers that amplify a short fragment of DNA containing the polymorphism. For rs8099917, the primer sequence were: 5'-CCC ACT TCT GGA ACA AAT CGT CCC-3' and 5'-TCT CCT CCC CAA GTC AGG CAA CC-3'[27]. For rs12980275, the primer sequence were: 5'-GAG AGC AAG AGG AGG GAA GGA A -3', and 5'-GTG TGC CAT TAG CCA GTC AGA T -3'[33]. The PCR temperature profile comprised 94°C for 5 min; 35 cycles of 94°C for 30 s, (66°C for rs8099917 and 58°C for rs12980275) for 30 s, and 72°C for 30 s; with final extension 72°C for 5 min. The PCR products were analyzed for successful amplification on 2% agarose gel electrophoresis, where successful amplification was confirmed by the appearance of the PCR products at the expected size. The PCR products for rs8099917 and rs12980275 contained 552 and 441 base pairs, respectively. To perform RFLP assay, 10 µl of rs8099917 amplicons were digested with 10 units of fast digest BseMI (BsrDI) restriction endonuclease and 20µl of rs12980275 amplicons were digested with 5 units of fast digest BslI restriction endonuclease (Fermentas,Vilnius, Lithuania) at 37°C for 15 min.The digestion products were separated on a 3% agarose gel alongside the PhiX174 DNA/HaeIII digest molecular weight marker (Finzyme) which encompasses 11 discrete fragments (1353, 1078,872, 603, 310, 281, 271, 234, 194, 118, 72-bp) and stained with ethidium bromide for visualization using a UV trans-illuminatoras.

**Statistical analysis.** The statistical data are reported as the mean ± SD, frequencies (number) and percentages (%). A comparison of numerical variables between the study groups was performed using Student's t-test. To compare categorical data, a chi-square test was performed and Fisher exact test was used instead when the expected frequency was less than 5. Odds ratios were calculated with a 95% confidence interval. A statistical significance was considered when $P \leq 0.05$. All statistical calculations were performed using the computer program Graph pad Prism 5 for Microsoft Windows.

## RESULTS

The demographic and biochemical profile of responders and non-responders were shown in table (1). The genotype distribution and allelic frequencies were significantly different between the HCV patients and the healthy control for

both IL-28B-rs8099917 (*P*=0.0112 and *P*=0.0012, respectively) and IL-28B-rs12980275 (*P*=0.012 and *P*=0.0274, respectively), as shown in table (2).

There was a significant difference in the prevalence of SNPs and the allele frequencies in the IL-28B gene (both rs8099917 and rs12980275) between the responders and non-responders toward the INF therapy for chronic hepatitis C. In rs8099917, the prevalence of T/T was significantly higher (65%, 65/100) in responders than that observed in non-responders (45%, 45/100; *P*=0.015). In rs12980275, A/A was significantly higher (53%,53/100) in responders than in non-responders (41%, 41/100; *P*=0.019,). The prevalence of the rs8099917-T (77.5%vs.66%) and rs12980275-A (70% vs. 56%) alleles were significantly higher in responders than in non-responders (*P*= 0.014 and 0.005, respectively) as shown in table (3) and represented in figure (1) and (2).

Patients carry T allele (protective allele) in its homozygousform (TT) are 2 times more likely to develop SVR more than those carry other genotypes, (OR=2.27) and patients carry A allele (protective allele) in its homozygous form (AA) are 1.6 times more likely to develop SVR more than those carry other genotypes (OR=1.62) as shown in table (3).

No statistical significant differences in genotype and allele frequencies in IL28B gene (rs8099917 and rs12980275) between males and females were observed within responders or non-responders group (*P*> 0.05) as shown in table (4) and (5).

Significant association between IL-28B-rs8099917TT and IL-28B-rs8099975AA was observed in responder group (*P*=0.0152)where 75.47%(40/53) of rs8099975AA were carriers for rs8099917 TTand 40% (40/100) of responders were carriers for both rs8099917 TT and rs8099975AA genotypes, as shown in table (6), where no significant association between rs8099917 and rs8099975 genotypes was observed within non-responders group (*P*=0.058) as shown in table (7).

**Table (1)** Demographic and biochemical profile of responders and non-responders.

| | Responder (n=100) | Non-responder (n=100) | P-value |
|---|---|---|---|
| Mean age(years) | 38.1±8.1 | 42.18±7.8 | 0.99 |
| Sex | | | 0.157 |
| Males % | 47(47%) | 58(58%) | |
| Females % | 53(53%) | 42(42%) | |
| ALT (U/L) | 85.2±55.5 | 96.8±42.8 | 0.1 |
| AST (U/L) | 84.2±50.4 | 92.5±40.1 | 0.1 |
| ALP (U/L) | 126.3±22.8 | 100.7±27.5 | 0.1 |
| Total bilirubin (mg/dl) | 0.96±0.5 | 1.26±0.21 | 0.1 |
| Direct bilirubin (mg/dl) | 0.35±0.25 | 0.37±0.29 | 0.1 |
| Albumin (g/dl) | 4.1±1.05 | 4.0±0.19 | 0.9 |
| RNA (IU/ml) | Negative | Positive | 0.0001*** |

Data are represented as means ± SD. *Indicate statistical significance.; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase.

**Table (2)** Genotype and allele frequency of IL-28-polymorphic sites in HCV patients and healthy control.

| Genotype/ allele | HCV patients (n=200) | Healthy control (n=100) | P-value |
|---|---|---|---|
| IL-28B-rs8099917 | | | |
| T/T | 110(55%) | 40(40%) | 0.0112* |
| T/G | 67(33.5%) | 37(37%) | |
| G/G | 23(11.5%) | 23(23%) | |
| T | 287(71.75%) | 117(58.5%) | 0.0012** |
| G | 113(28.25%) | 83(41.5%) | |
| IL-28B-rs12980275 | | | |
| A/A | 94(47 %) | 30(30%) | 0.012* |
| A/G | 64(32%) | 47(47%) | |
| G/G | 42(21%) | 23(23%) | |
| A | 252(63%) | 107(53.5%) | 0.0274* |
| G | 148(37%) | 93(46.5%) | |

Results are expressed as number and percentage. *Indicates a statistical significance.IL, interleukin; rs, reference single nucleotide polymorphism (SNP) ID.

**Table (3)** Genotype and allele frequency of IL-28B-polymorphic sites in responders and non-responders hepatitis C virus (HCV) patients.

| Genotype /allele | Responders (n=100) | Non-responders (n=100) | P-value | OR |
|---|---|---|---|---|
| IL-28B- rs8099917 | | | | |
| T/T | 65(65%) | 45(45%) | 0.0154* | 2.27 |
| T/G | 25(25%) | 42(42%) | | |
| G/G | 10(10%) | 13(13%) | | |
| T | 155(77.5%) | 132(66%) | 0.0144* | |
| G | 45(22.5%) | 68(34%) | | |
| IL-28B- rs12980275 | | | | |
| A/A | 53(53%) | 41(41%) | 0.0195* | 1.62 |
| A/G | 34(34%) | 30(30%) | | |
| G/G | 13(13%) | 29(29%) | | |
| A | 140(70%) | 112(56%) | 0.0051** | |
| G | 60(30%) | 88(44%) | | |

Results are expressed as number and percentage. *Indicates a statistical significance.IL, interleukin; rs, reference single nucleotide polymorphism (SNP) ID.OR, odd ratio.

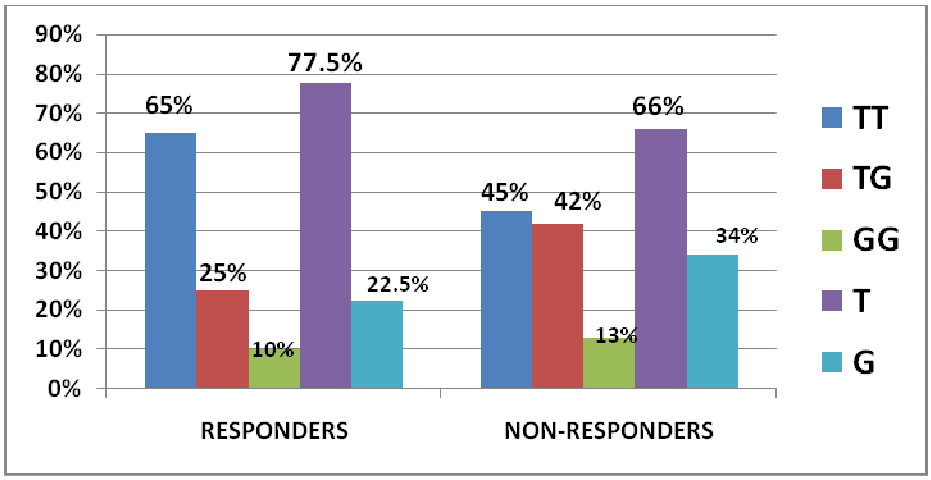


**Figure (1):** Genotype and allele frequencies of IL-28B-rs8099917in responders and non-responders

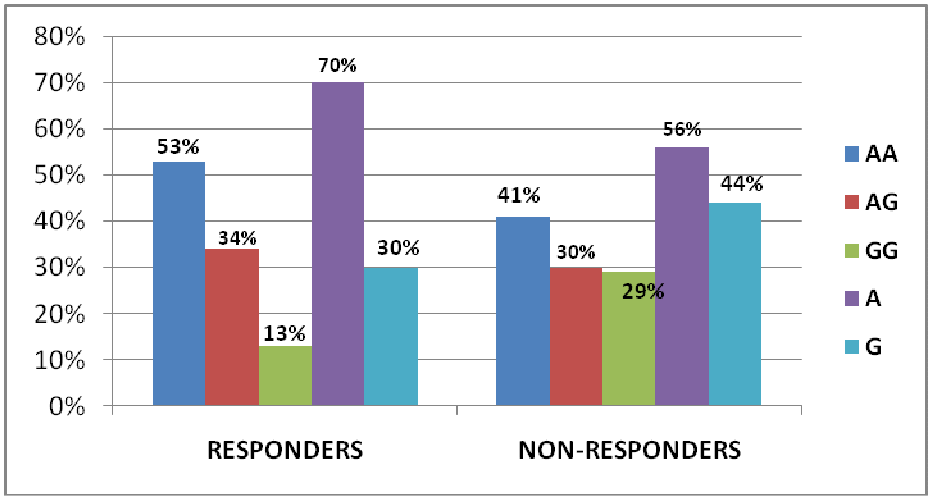


**Figure (2):** Genotype and allele frequencies of IL-28B-rs12980275in responders and non-responders

**Table (4):** Genotype and allele frequency of IL-28B-polymorphic sites in males and females in responders group.

| Genotype /allele | Male (n=47) | Female (n=53) | P-value |
|---|---|---|---|
| IL-28B- rs8099917 | | | |
| T/T | 32(68.09%) | 33(62.26%) | 0.812 |
| T/G | 11(23.40%) | 14(26.42%) | |
| G/G | 4(8.51%) | 6(11.32%) | |
| T | 75(79.79%) | 80(75.47%) | 0.501 |
| G | 19(20.21%) | 26(24.53%) | |
| IL-28B- rs12980275 | | | |
| A/A | 21(44.68%) | 33(62.264%) | 0.254 |
| A/G | 18(38.30%) | 15(28.302%) | |
| G/G | 8(17.02%) | 5(9.434%) | |

| | | | |
|---|---|---|---|
| A | 60(63.83%) | 81(76.42%) | 0.892 |
| G | 34(36.17%) | 25(23.58%) | |

Results are expressed as number and percentage. *Indicates a statistical significance.IL, interleukin; rs, reference single nucleotide polymorphism (SNP) ID.

**Table (5):** Genotype and allele frequency of IL-28B-polymorphic sites in males and females in non-responders group.

| Genotype /allele | Male (n=58) | Female(n=42) | P-value |
|---|---|---|---|
| IL-28B- rs8099917 | | | |
| T/T | 25(43.10%) | 20(47.62%) | 0.333 |
| T/G | 23(39.66%) | 19(45.24%) | |
| G/G | 10(17.24%) | 3(7.14%) | |
| T | 73(62.93%) | 59(70.24%) | 0.294 |
| G | 43(37.07%) | 25(29.76%) | |
| IL-28B- rs12980275 | | | |
| A/A | 22(37.93%) | 19(45.24%) | 0.715 |
| A/G | 19(32.76%) | 11(26.19%) | |
| G/G | 17(29.31%) | 12(28.57%) | |
| A | 63(54.31%) | 49(58.33%) | 0.665 |
| G | 53(45.69%) | 35(41.67%) | |

Results are expressed as number and percentage. *Indicates a statistical significance.IL, interleukin; rs, reference single nucleotide polymorphism (SNP) ID.

**Table (6):** Association between genotypes of IL-28B-rs8099917 and IL-28B-rs12980275 in responders group.

| | **IL-28-rs12980275** | | | P-value |
|---|---|---|---|---|
| | **A/A**(n=53) | **A/G**(n=34) | **G/G**(n=13) | |
| **IL-28B-rs8099917** | | | | |
| **T/T**(n=65) | 40(75.47%) | 19(55.88%) | 6(46.15%) | 0.0152* |
| **G/T**(n=25) | 9(16.98%) | 13(38.24%) | 3(23.08%) | |
| **G/G**(n=10) | 4(7.55%) | 2(5.88%) | 4(30.77%) | |

Results are expressed as number and percentage. *Indicates a statistical significance.IL, interleukin; rs, reference single nucleotide polymorphism (SNP) ID.

**Table (7):** Association between genotypes of IL-28B-rs8099917 and IL-28B-rs12980275 in non-responders group.

| | **IL-28-rs12980275** | | | P-value |
|---|---|---|---|---|
| | **A/A**(n=41) | **A/G**(n=30) | **G/G**(n=29) | |
| **IL-28B-rs8099917** | | | | |
| **T/T**(n=45) | 13(31.71%) | 18(60%) | 14(48.28%) | 0.058 |
| **G/T**(n=42) | 22(53.66%) | 11(36.67%) | 9(31.03%) | |
| **G/G**(n=13) | 6(14.63%) | 1(3.33%) | 6(20.69%) | |

Results are expressed as number and percentage*Indicates a statistical significance.IL, interleukin; rs, reference single nucleotide polymorphism (SNP) ID.

**DISCUSSION**

Hepatitis C virus (HCV) infection is rated by the World Health Organization (WHO) as a global health problem, based on its prevalence, the high rate (50–85%) of chronicity, the rate of severe complications such as cirrhosis and hepatocellular carcinoma, as well as the high costs of antiviral therapy and liver transplantation ***[3]***. Parameters for the prediction of SVR in patients with chronic hepatitis C (CHC) before initiation of antiviral therapy are important in order to be able to estimate the potential for treatment success. Accumulating evidences support the critical role of host genetics in interferon and ribavirin induced immune responses during HCV treatment. Consistent with previous studies [28,9], this study investigated a predictive role of IL28B polymorphisms with treatment of chronic HCV infection in Egyptian patients. Because the cytokine genetic polymorphisms and ethnicity are related, we attempted to determine the potential of the SNPs in IL-28B as predictor inHCV-4 Egyptian patients.

For IL-28B-rs8099917, this study indicating that TT genotype had a higher percentage in responders than in non-responders (65%versus 45%; *P*=0.015). For IL-28B-rs12980275 it was found that AA genotype had higher percentage in responders than in non-responders (53% versus 41%; *P*=0.019). Ourresults were agreed with [9,28,10,15,32,17,20,21 and 26]**.**

As regards allelic frequencies of IL-28B-rs8099917 (T versus G) ourstudy detected a significant difference between responders and non-responders with much higher frequency for T allele (77.5%) than that of G allele (22.5%) in responders versus 66% and 34%, respectively in non-responders (*P*=0.014). Regarding allelic frequencies of IL-28B-rs12980275 (A versus G) ourstudy detected a significant difference between responders and non-responders where much higher frequency for A allele (70%) than that of G allele (30%) in responders versus 56% and 44% ,respectively in non-responders was observed (*P*= 0.0051).

Accordingly, the T allele for IL-28B-rs8099917 and the A allele for IL-28B-rs12980275 increasing the opportunities for SVR, so they can be defined as the protective alleles while the G allele increasing the risk of chronicityand associated with non-responsiveness to treatment, so it can be defined as the risk allele. Our results were agreed with [30,22 AND 29].

Rauch et al. [22], was reporting the influence of human genetic variation on the natural control of HCV infection and showed that the minor allele IL-28B-rs8099917(GG) is associated with non–response to treatment with a stronger prediction in HCVinfected patient genotype 1and 4. This risk allele was identified in 32% of chronically infected patients who responded to therapy, and 58% who did not respond.

Li et al., [15] found that carriers of rs8099917 TT versus non-carriers had a significantly increased rate of SVR in Caucasian patients with CHC genotype 1/4 and in Asian patients with CHC genotype 1/4. Also, Shaker and Sadik [26] found that, the prevalence of IL-28B-rs8099917 T/T was significantly higher in responders (66.7%) than that observed in non-responders (13.5%); (P< 0.001) in Egyptian patients. Xie et al [33] concluded that, the rs8099917 TT is closely related to the effectiveness of peg-IFN-alpha/RBV therapy, and it is an important predictive factor before treatment in patients with chronic hepatitis C. Abdo et al., [1] showed that SVR was significantly associated with AA alleles rs12980275 (P = 0.004).

Suppiah et al., [29] concluded that, the association of IL28B genotype with therapeutic response indicates that IFN λ-3 affects viral clearance. IFN λ-3 is likely to enhance antiviral mechanisms through up-regulation of interferon stimulated genes (ISGs) in acute disease [2] but its effect may be more complicated in chronic infection, in which upregulation of ISGs in liver is associated with reduced treatment response [11].

Domagalski et al., [8] found that IL-28B polymorphisms were the strongest pretreatment predictors of response to pegylated interferon and ribavirin in Polish patients chronically infected with HCV genotype 1 and 4 where rs12980275 AA seems to be more important

than rs8099917 TT in predicting positive treatment response.

On the other hand, it was found that there was no statistical significance association between the IL28B-rs8099917 TT and IL-28B-rs12980275 AA with virologic response in genotype 2/3. Moreover, a highly significant association with higher baseline viral loads was observed in genotype 2/3 as well as in genotype 1 infected patient carrying the genotypes rs8099917 TT, and rs12980275 AA [24,12].

In the current study, the prevalence of favorable genotypes between male and female was compared, and the results showed no significant difference between male and female for both SNPs studied (P>0.05). These results were in accordance with Grebely et al.,[10] and Montes-Cano et al., [19].

During recent HCV infection, genetic variations in IL28Bregion were associated with spontaneous but not treatment-induced clearance [10]. A possible interpretation is that the effect of SNPs is attenuated by a better treatment response in patients with genotype 2 or 3 HCV as several studies have reported that IL-28B-rs8099917 TT genotype was associated with faster early viral elimination in patients with genotype 2 or 3 HCV [18,25,34 and 16] and the rate of SVR in treatment-naive subjects infected with genotype 2 or 3 is 80.6%, much higher than 48.5% in patients with HCV genotype 1 or 4.

IL28B genotype may address unresolved issues in the management of patients treated with the current standard of care. Treating patients with favorable IL28B genotype with the combination of PEG-IFN and direct acting antiviral (DAA) may allow shorter courses of therapy. The way in which SNP responder genotypes influence the outcomes of anti-viral strategies including those based upon protease and polymerase inhibition requires immediate investigation. Rather than using IL28B genotype to deny treatment to patients with an unfavorable IL28B genotype, it should be used to promote treatment in patients with favorable IL28B genotype to keep patients adherent during treatment despite adverse events or before treatment to encourage those who are hesitant to start antiviral therapy.

In summary, the IL-28B-rs8099917TT and IL-28B-rs12980275AA may be able to predict the response of peg-IFN/RBV combination therapy in Egyptian patients with chronic HCV- 4. Further studies are needed to clarify these conclusions and this information could be used to develop new treatment options.

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