



Original article

The relation between interleukin 28B gene polymorphisms (rs8099917 and rs12980275) and the response of treatment of hepatitis C virus genotype 4 patients to sofosbuvir and daclatasvir therapy

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ABSTRACT

Background: The main line of treatment of hepatitis C in Egypt is direct acting antiviral drugs (DAAs). **Objectives:** The current study aimed to explore the effect of IL28B genetic polymorphism on hepatitis C virus (HCV) infection progression and response to the new treatment in difficulty to treat individuals with HCV genotype 4. **Methods:** Blood samples were collected from 50 healthy individuals as a control and 150 HCV- patients receiving sofosbuvir/daclatasvir (SOF/DCV) with ribavirin combination. IL28B (rs8099917, rs12980275) genotyping was implemented using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method to detect its impact on HCV disease outcome. **Results :** Out of 150 patients, 141 (94%) were responders to treatment and 9 (6%) were non- responders. A total of 112 (74.7%) of patients belonged to mild to moderate stages of liver fibrosis and 38 (25.3%) had moderate to severe stages. There was a significant increase in severity of fibrosis among non-responders (p value $<0.001^*$), where all of them had late fibrosis. GG genotype of IL28B (rs8099917) was concomitant with raised susceptibility to infection while TT genotype was predominant in controls, in responders and in patients with early fibrosis (p values <0.05). while IL28B rs12980275 showed no significant difference among the study population. **Conclusion:** TT genotype of IL28B (rs8099917) may be a protective factor against infection and associated with probability of achieving sustained virologic response (SVR) to combined SOF and DCV therapy in genotype 4 HCV patients. However, GG genotype is negatively associated with HCV infection outcome. IL28B rs12980275 variants had no association with HCV infection.

Introduction

Infection by hepatitis C virus (HCV) is considered as a worldwide infection that is considered the major causes of chronic liver impairment [1]. About 55%-85% of HCV infected individuals develop chronic active hepatitis and may progress to fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [1].

The prevalence rate of HCV infection was 1.0% in 2015, with the highest prevalence (2.3%) in the middle east [2]. Egypt has one of the highest rates of HCV prevalence globally. Genotype 4 represents more than 90% of Egyptian HCV isolates [3]. The seroprevalence of HCV antibodies in Egypt has decreased from 14.7% in the Demographic Health

Survey (DHS) of 2008 to 10% in the population of same age group assessed by the Ministry of Health and Population in 2015. This significant drop of the HCV burden 30% between 2008 and 2015 may be due to aging of the affected age group or death by HCV complications [4].

In the last decade, different antiviral drugs have been established for the treatment of HCV patient and showed safety and efficacy in lowering viral loads in HCV individuals of different genotypes. One of the newly approved direct-acting antiviral (DAA) drugs are daclatasvir (DCV) and sofosbuvir (SOF) combination with or without ribavirin. Sofosbuvir is an oral pan-genotypic that cause inhibition of the HCV NS5B RNA polymerase with a very good safety profile, minimal resistance, and few drug interactions. Daclatasvir is a powerful, inhibitor of the HCV NS5A replication complex protein [5-7]. This combination was proven as safe and effective therapy for HCV genotype 4 patients with attenuation of liver fibrosis [8].

Elimination of HCV is a worldwide and national goals that influence all aspects of life. The World Health Assembly in May 2016 set goals for the elimination of viral hepatitis. These goals were reaching 90% diagnosis, 80% treatment exposure, and a 65% decrease in related mortality rates by 2030 [9]. With the application of the DAA treatment regimen (SOF plus DCV) in Egypt in 2018, treatment of more patients and disease became easier. the Egyptian government started in 2018, a vast screening and treatment program for diagnosis and treatment of all HCV-affected role to eradicate the virus in the near future [10].

Since 2009, several genetics associated studies have been carried out all over the world to identify host genes to the response to pegylated-interferon and ribavirin (PEG/RBV) therapy in chronic HCV- individuals [11-13]. Several host factors could be associated with HCV clearance or disease advancement and may predict the consequence of therapy. One of the most important host factors, IL-28B cytokine which inhibits HCV replication and helps viral clearance by Janus kinase/signal transducer and by activation of pathway of transcription. Single nucleotide polymorphisms (SNPs) in IL-28B gene has been associated with HCV response to therapy [14]. Also, the IL-28B polymorphism was associated with degree of difference in expression of intrahepatic interferon-stimulated genes (ISGs) in chronic HCV patients [15].

Interleukin-28B (IL-28B) is a gene encoding a protein interferon $\lambda 3$ (IFNL3) and recently IL-28B gene has been renamed as interferon $\lambda 3$. Interferon $\lambda 3$ is an immunomodulatory cytokine inducing an inflammatory response after viral infections [16]. The IFN- λ genes have significant roles in immunity against HCV infection. The concern of the difference in the IFNL3 (previously named IL-28B) gene region in spontaneous or treatment induced viral clearance was reported in several previous studies [11-13]. Different IL-28B gene variations were reported to be related to the natural control of HCV. IL-28B rs8099917 GG genotype is a common genetic variant negatively concomitant with the spontaneous clearance of HCV infection [17]. In chronic hepatitis C patients, a second SNP of IL28B, rs12980275, was studied and found to be highly related to sustained virologic response (SVR) [18-19]. Several Egyptian studies suggested that, SNPs in the IL-28B gene could predict the HCV viral clearance and treatment response in patients receiving interferon- α based therapy [20-22]. To date, studies associated with the impact of IL28B polymorphisms on efficacy of new DAAs to treat HCV genotype 4 patients are still scarce. Therefore, the current study aimed to investigate the distribution and effect of these variants “rs8099917 and rs12980275” on risk of infection and outcome of HCV infection amongst an Egyptian cohort study receiving the new treatment regimen approved by Egyptian Ministry of Health “(DCV and SOF) + ribavirin”.

Methods

Study population

This cohort study included 150 HCV genotype 4-patients and 50 healthy subjects as a control. All patients were treatment experienced patients attending the Outpatient Clinic of Minia Health Insurance Hospital, in Egypt, from January 2019 to July 2019. Persons with hepatitis B surface antigen positive, persons with HIV positive antibodies, undergoing liver transplantation, having malignancy, pregnancy, uncontrolled diabetes mellitus or any decompensated liver disease were excluded from the study. Demographic and clinical data was collected. The patients were scheduled to receive the new treatment regimen; sofosbuvir (400 mg) + daclatasvir (60 mg) once daily + ribavirin (600–1,000 mg) in divided doses (SOF/DCV+RIB) for 12 weeks. To identify the response to new treatment, patients who cleared HCV-RNA from serum (undetectable) at the end of treatment and for 24 weeks after the end of treatment are called responders. However, patients with serum HCV-RNA ≥ 12 IU/mL at any time during the period

of treatment or post-treatment follow-up period are called non-responders.

We assessed severity of fibrosis-by-fibrosis score 4 (FIB-4), which is a non-invasive method using Sterling equation. FIB4 was established to correlate with Ishak levels of fibrosis [23]. The protocol for research work was approved by the Research Ethics Committee (REC) of Minia University in accordance with the guidelines of human subjects' materials of Declaration of Helsinki.

Laboratory testing

Complete blood count (CBC) was determined by automated cell counter, Sysmex KX-21N (TAO Medical Incorporation, Japan). Liver function tests (bilirubin, serum alanine transferase (ALT), aspartate transferase (AST), albumin and total protein) were measured using fully automated clinical chemistry auto-analyzer system Flexor XL (Selectra, ELI Tech, France). Anti-HCV antibodies were detected by ELISA. HCV-RNA levels were measured before starting the treatment and at 6, 12, 24 and 36 weeks using quantitative reverse transcriptase polymerase chain reaction (RT-PCR) ((Qiagen, Hilden, Germany) following RNA extraction by QIAmp Viral RNA Kit (Qiagen, Santa Clarita, CA)

IL-28B genotyping

DNA was extracted from whole blood as manufacturer's instruction (QIA amp DNA Mini Kit, QIAGEN, Germany). Genotyping of SNPs rs8099917 and rs12980275 was performed by PCR and restriction fragment length polymorphism (PCR-RFLP) technique. For rs8099917, primers sequence was: 5'-CAT CCC ACT TCT GGA ACA AAT C-3' and 5'-GTA TCA ACC CCA CCT CAA ATTATC-3'. For rs12980275, primers sequence was: 5'-AAG AGG AGG GAA GGA AGT TCT G-3' and 5'-GGT CTG GTC CTA GTG GTG TTT G-3'. PCR conditions 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 [24].

Then RFLP assay was performed using restriction endonuclease enzymes. for rs8099917 genotype, after cutting with 1 U BseMI enzyme (thermo-scientific #ER1201, USA) at 55°C for 2 hours, homozygote TT was identified by one fragment (400 base pairs), GG had 2 fragments (234 and 166 base pairs) and heterozygote GT had 3 fragments (400, 234 and 166 bases). For rs12980275 genotype, after cutting the

amplified product with 1 U BseLI enzyme (thermo-scientific #ER1261, USA) at 55°C for 2 hours, AA type yielded 2 fragments (178 and 115 base pairs), GG type yielded 3 fragments (148, 115 and 30 base pairs) and AG yielded 3 fragments (178, 148, and 115 base pairs) [24]. The digested fragments for each were separated on agarose gel using 50 bp DNA ladder (QIAGEN, USA).

Statistical analysis

The statistical analysis was performed using SPSS 23 (SPSS Inc., Chicago). Quantitative variables were expressed as mean \pm SD, for normally distributed data, as median and interquartile for non-distributed data, and compared using unpaired t-student test. Mann Whitney U test for non-parametric quantitative data between two groups. Chi-square test or Fisher exact test was used for qualitative variables. The data were considered significant if P values were ≤ 0.05 ; highly significant if $P < 0.001$.

Results

Baseline characteristics of the studied subjects

One hundred -fifty patients with HCV infection were recruited. Of them 72 (48%) were males with an average age of 48 ± 7.7 years. The control group included 50 individuals, 27 (54%) of them were males with an average age (45.3 ± 6.9). According to FIB4 score, 112 (74.7%) patients had mild to moderate fibrosis "F0-1" and 38 (25.3%) patients had moderate to severe fibrosis "F2-3". The patients were subdivided according to SVR after treatment with (SOF/DCV) + RIB regimen to responders (141/150, 94%) and non- responders (9/150, 6%). The demographic and laboratory characteristics of HCV patients are shown in **table (1)**. There was a significant increase in severity of fibrosis among non-responders (p value < 0.001 *), where all of them belonged to F2-3 category (moderate to severe fibrosis).

Impact of IL-28B gene polymorphisms on susceptibility to HCV infection

The association between IL-28B genotypes and the protection from HCV infection among the study population is shown in **table (2)**. For IL-28B. rs8099917: TT genotype was significantly higher in healthy controls than HCV-patients ($p < 0.0001$). On the other hand, there was no significant difference in the frequencies of different IL-28B. rs12980275 genotypes in HCV-patients compared to healthy controls ($p > 0.05$) (**Table 2**).

IL-28B gene polymorphisms relation to fibrosis stage

For rs8099917, the distributions of the TT and TG genotypes were higher in patients with stage 1 fibrosis while the GG genotype was higher in patients with stage 2 and 3. This difference is statistically significant (P value=0.007). However, for rs12980275, the distribution of different genotypes among the different fibrosis stages was not statically significant (**Table 3**).

Impact of IL-28B gene polymorphisms on response to DAA treatment

For IL28B rs8099917, the GG genotype occurred with lower frequency in responder than in non-responders. On the other hand, TT genotype was the predominant type in responders (P value = 0.0001). However, the difference between the distributions of different rs12980275 genotypes among responders and non-responders was non-significant (P value= 0.166) (**Table 4**).

Table 1. Baseline demographic and clinical data of HCV patients.

		Responders	Non-responders	<i>P</i> value
		N=141(94%)	N=9 (6%)	
Age	<i>Range</i> <i>Mean ± SD</i>	(32-74) 48.6±7.4	(28-65) 44.2±10.4	0.176
Sex	<i>Male</i> <i>Female</i>	67(47.5%) 74(52.5%)	5(55.6%) 4(44.4%)	0.738
HCV titre, log EQ/ML	<i>Median</i> <i>IQR</i>	6.45 (4.77-7.51)	6.35 (5.55-8.55)	0.422
AFP, ng/mL	<i>Range</i> <i>Mean ± SD</i>	(12-17.5) 14.3±1.4	(12.6-16.8) 13.8±1.8	0.508
Albumin, g/dL	<i>Range</i> <i>Mean ± SD</i>	(1.2-6) 3.9±1	(2.4-5.7) 3.9±0.9	0.872
ALT, U/L	<i>Range</i> <i>Mean ± SD</i>	(34.9-68.7) 52.8±6.3	(37.3-69.7) 51.8±9.8	0.613
AST, U/L,	<i>Range</i> <i>Mean ± SD</i>	(41-67.5) 54.2±4.2	(45.6-61.2) 55.7±5.1	0.316
PC	<i>Range</i> <i>Mean ± SD</i>	(87.1-93.9) 89.9±1.8	(87.6-93.8) 89.8±2.2	0.889
Fibrosis category	<i>F0-1</i> <i>F2-3</i>	112(79.4%) 29(20.6%)	0(0%) 9(100%)	<0.001*

*: Significant level at P value < 0.05

Abbreviations: AFP, alpha fetoprotein; ALT, alanine transferase; AST, aspartate transferase; PC, platelet count; F0-1, (mild-moderate); F2-3, (moderate -severe).

Table 2. Distribution of IL28B genotypes among patients and controls.

Variable	Groups		P value	X ²
	Cases	Controls		
rs12980275:				
AA	39 (26%)	15 (30%)	0.857	0.309
GG	26 (17.3%)	8 (16%)		
AG	85 (56.7%)	27 (54%)		
rs8099917:				
TT	102 (68%)	48 (96%)	0.0001* (Fischer's exact test)	17.414
GG	6 (4%)	0		
TG	42 (28%)	2 (4%)		

Table 3. Distribution of IL28Brs12980275 and IL28B rs8099917 genotypes in different fibrosis stages.

Gene polymorphism	Stage 0	Stage 1	Stage 2	Stage 3	P value	X ²
rs12980275:						
AA (39)	3 (7.7%)	24(61.5%)	11 (28.2%)	1 (2.6%)	0.133	82492
GG (26)	0	18(69.2%)	8 (30.8%)	0		
AG (85)	1 (1.2%)	66(77.6%)	18 (21.2%)	0		
rs8099917:						
TT (102)	3 (2.9%)	73(71.6%)	26 (25.5%)	0	0.007*	15.980
GG (6)	0	1 (16.7%)	4 (66.7%)	1(16.7%)		
TG (42)	1 (2.4%)	34 (81%)	7 (16.7%)	0		

Table 4. Distribution of IL28B rs12980275 and IL28B rs8099917 genotypes in both responders' and non-responders' patients.

Gene polymorphism	Responders	Non-responders	P value	X ²
IL28Brs12980275				
AA	34(24.5%)	5(55.6%)	0.166	3.657
GG	25(17.7%)	1(11.1%)		
AG	82(58.2 %)	3(33.3%)		
IL28Brs8099917				
TT	99(70.2%)	3(33.3%)	*0.0001	25.717
GG	1(0.7%)	5(55.6%)		
TG	41(29.1%)	1(11.1%)		

Discussion

New treatment regimen by generic SOF+DCV (with or without RBV) became the main line of therapy in the Egyptian national program for HCV control from November 2015, due to its efficacy and safety on a large scale of patients. Several publications compared the efficacy of brand and low-cost generic SOF with DAA molecules drugs produced in Egypt and found it safe and effective [25]. In 2018, Egypt started a massive HCV screening and treatment program by the new regimen “(SOF/DCV) +RIB”. Hepatitis C virus seroprevalence decreased due to the new treatment regimen with DAA when compared with HCV seroprevalence in the 2015 that proved the high efficacy of the new treatment [10]. In this study, out of 150 difficulty to treat HCV-patients with previous interferon-based treatment experience, 141 (94%) were responders to (SOF/DCV) + RIB regimen. Only 9 patients were resistant to the treatment (6%). This percent was slightly higher than previous studies on treatment experienced patients. **Abdel-Moneim et al.** found SVR clearance rate; 95% in the easy-to treat group receiving SOF and DCV and a 92% in the difficult-to-treat group receiving the same treatment in cohort of 946 Egyptian patients with chronic HCV infections [26]. In another study, with combined DCV and SOF therapy, out of 250 HCV genotype 3 patients, 219 (87.6%) patients achieved SVR, whereas 19 (7.6%) relapsed and 12 (4.8%) failed to respond the therapy. The results of the previous studies were less than our result because the patients recruited in these studies were belonging to different genotypes that may be more difficult in treatment than genotype 4. Moreover, adding ribavirin increases the SVR rate in treatment-experienced and cirrhotic patients [27]. Fibrosis 4 (FIB-4) index is one of the most widely used methods for non-invasive assessment of liver fibrosis. Directly acting antivirals had shown to improve FIB-4 index and APRI [28,29]. In the current study, there was a significant increase in severity of fibrosis among non-responders (*p value* <0.001*), where all non-responder patients belonged to F2-3 category (moderate to severe fibrosis). This finding indicated the association between therapeutic failure and stage of fibrosis. In line with these results, many studies also reported low response rate of cirrhotic patients with HCV infection to interferon-based therapy or protease-inhibitor containing regimen [30]. The distribution of IL28B SNPs in population and its confusing role in HCV susceptibility were challenged in this study. To the best of our knowledge, this is 1st study of the impact of

IL28B gene polymorphism (rs12980275 and rs8099917) on response to DAAs therapy in Egyptian patients with HCV genotype 4. All previous studies were done to investigate IL28B gene polymorphisms (rs12980275 and rs8099917) in response to old treatment (interferon and ribavirin) [20-22]. However, IL28B gene polymorphism (rs12979860) was recently studied as predictors of new DAA in Egypt in 2020 [31].

There is an association between IFNL3(IL28B) and DAAs treatment response, but the underlying mechanism of IL28B in relation to treatment response with DAAs is still unknown. It may be like the IFN-based treatment response mechanism in which IL28B signals through the IFN- λ receptor complex induces expression of IFN-stimulated genes (ISGs) via JAKSTAT signaling and has antiviral properties. If the patient is carrying the favorable allele of IL28B gene, basal levels of IFN- λ and ISGs will be relatively low and so treatment with DAAs can induce a potent antiviral state leading to viral clearance and respond to treatment. However, if patient is carrying the unfavorable allele of IL28B, the basal levels of IFN- λ and ISGs will be high which can lead to unresponsiveness to the DAAs treatment [32-34].

In the current study, the distribution of the rs8099917 TT genotype among the HCV patients and control groups were 68% and 96%, respectively. On the other hand, the frequency of the GG allele among the HCV patients and control groups were 4% and 0%, respectively. While the TG distribution were 28% and 4% respectively with significant P value of 0.0001*, indicating that the TT type may be a protective genotype and T is a favorable allele while those with GG type are more susceptible to HCV infection. Considering that no information on IL28B SNPs genotype distribution was known for response to new treatment (DAA) in Egypt, we were interested in evaluating the frequency of rs12980275 and rs8099917 in response to new regimen “(SOF) and (DCV) combination with ribavirin” in difficulty to treat HCV genotype 4. In our study, the distribution of the TT, GG, and TG of IL28B rs8099917 genotypes among responders were 70.2%, 0.7% and 29.1% respectively while among non-responders were 33.30%, 55.60% and 11.10 % respectively with significant P value = 0.0001. These findings agree with the previous studies that found that rs8099917 minor allele (G) was associated with both progression to chronic hepatitis C and failure to respond to treatment, with the strongest effects in patients infected with genotypes 1 and 4 [30-33]. Also, it

agrees with a study by **Khan et al.** who demonstrated that TT genotype of IL28 (rs8099917) is associated with a higher SVR, to combined SOF+DCV therapy in genotype 3-infected HCV patients [34].

This study is the only one that specifically examined the genotype frequencies of IL-28B rs12980275 polymorphisms in Egyptian patients with chronic HCV-4 infection with new DAAs. Our results showed that there was no significant difference in the genotype frequencies between responders and non-responders. This result comes in line with a study examined the frequencies of IL-28B rs12980275 polymorphisms in Egyptian patients with chronic HCV-4 infection with the old regimen of treatment (IFN- α based therapy) [20].

In conclusion, our results indicate that the new regimen by DCV and SOF with ribavirin had high efficacy and was well tolerated in difficult to treat HCV genotype 4 patients. These findings indicated that there is association between therapeutic failure and stage of fibrosis. Also, our results suggested that GG genotype of IFNL3 (rs8099917) is associated with an increased susceptibility to infection and TT genotype may be a protective gene and is associated with likelihood of achieving SVR to combined DCV and SOF therapy in genotype 4 infected HCV patients. Moreover, no statistically significant difference was observed between the different genotypes of IL28B rs12980275 between responders and non-responders.

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Author contributions

Concept and design of study: RMMK, SSH, MAE, HAA, MS. Experiments: RMMK, SSH and MS. The manuscript was drafted by RMMK, SSH, MAE, SSA and revised by all authors.

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