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Interleukin 28B Genotyping as a Predictor Marker for Responsiveness of HCV Patients to Therapy

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Abstract: ***Single nucleotide polymorphism (SNPs) in the IL-28B gene, namely rs12979860 and rs8099917 could predict response to pegylated interferon and ribavirin therapy in hepatitis C virus patients. The current study investigated 50 Iraqi HCV infected patients with HCV genotype1 and 4 compared to 40 healthy individuals. For rs12979860, the homozygous C allele genotype (CC) showed high rate of viral response (75%) compared to other genotypes CT and TT (66.6%, 45,4% respectively). For rs8099917, interestingly the homozygous G allele genotype (GG) showed no response (0%) while other genotypes TG and TT showed (58.6%, 63.1%) respectively. Our findings highlight an association of IL28B rs12979860 and rs8099917 genotypes, and HCV genotyping which is useful in clinical practice for patients risk stratification based on interferon responsiveness.***

Keywords: HCV, Interleukin 28B

1. Introduction

Hepatitis C virus is a major public health problem causing acute and chronic infection which might develop to liver cirrhosis and/or liver cancer that often results in liver failure eventually lead to liver transplant (1). The combinational therapy of pegylated interferon (peg-IFN) and ribavarin in chronic HCV patients yield a sustained virological response (SVR) rate of 45% to 50% and has many unpleasant adverse effect therefore identifying predictive factors of therapeutic response in patients with HCV is important (2)

Epidemiological, viral, and host factors have been associated with the differences in HCV clearance or persistence, and it has been demonstrated that a strong host immune response against HCV favors viral clearance. Thus, variation in genes involved in the immune response may contribute to the ability to clear the virus (3).

Interleukin 28B is a member of a family of pleiotropic cytokines that exhibit potent antiviral, anti proliferative, apoptotic, and immune regulatory activities. These activities are controlled, in part, by a set of cellular genes that are rapidly induced upon binding of IL-28B to specific membrane-bound receptors (4). Genome-wide association studies concurrently provided the overwhelming evidence that single nucleotide polymorphisms (SNPs) of IL-28B on chromosome 19q13contribute to IFN treatment response and spontaneous HCV clearance in HCV infection (5).

Two of these SNPs, rs12979860 (located *3 kb upstream of IL-28B) and rs8099917 (located *8 kb upstream of IL-28B) were identified as the variant most strongly associated with SVR (6)

The purpose of this study is to underline the relevance of SNP in the IL28B gene region rs12979860 and rs8099917 in cohort of Iraqi HCV patient who were treated with Pegylated interferon and ribavirin.

2. Patients and Methods

This study was carried out from March 2015 to November 2015. Diagnosed HCV patients referred to GIT hospital at Baghdad governorate were included. Fifty patients, 25 male and 25 Female, with age ranging from 31 to 60 years infected with HCV genotypes 1 and 4 and began antiviral peg-IFN-ribavarin dual therapy. All of the patients were followed up for 3 and 6 months after starting therapy by measuring HCV RNA load at time zero, 3 months and 6 months of therapy. In addition, forty age- and sex- matched healthy control were included in this study. The exclusion criteria were patients with history of irregular antiviral combined therapy, HBV co-infected patients, liver transplant, and children patients. Written consent from patients was pursued and ethical approval from College of Medicine, Alnahrain University was granted. According to response to therapy, description of response was categorized as good (median log zero after 6 months of therapy), moderate (median log declines more than 2 log but not zero after 6 months of therapy), poor (median log don't decline or decline less than 2 log after 6 months of therapy). Positivity of HCV Ab in patients and negativity in the control were confirmed by commercially available third –generation enzyme linked immunosorbant assay (plasmatic, UK)

Extraction of hepatitis C virus RNA level

Viral RNA was extracted from patients plasma using QIAamp viral RNA Minikit (Cat No.52906,

Qiagen,Germany). The protocol used was recommended by the kit manufacturer. The sample is first lysed under denaturating condition to inactivate RNase and molecular grade buffering solutions were used to provide optimum binding of RNA to the QIA amp membrane. Samples were loaded onto the QIA amp Mini spin column. The RNA binds to the membrane and contaminants are efficiently washed in two steps using two different wash buffers, then high- quality RNA was eluted in a special RNase- free buffer ready for use or safe storage.

Viral load detection

Viral load of HCV was measured by Artus HCV RG-RT-PCR (Ref no.4518233,Qiagen,Germany). HCV RNA titer of samples were determined using Rotor-Q- gene thermo cycler (Qiagen, Germany)

Twenty µl of extracted HCV-RNA were used for Real-time qPCR amplification step using HCV virus-RGRT-PCR (Ref No.4518233. Qiagen, Germany). The protocol of technique was according to the manufacturer guidelines. The Hepatitis C virus RG consist of Master A and B contain reagents and enzymes for the reverse transcription and specific amplification of a 240 bp region of the HCV genome and for the direct detection of the specific amplicon in fluorescence channel cycling Green of the Rotor- Q-Gene in addition, a second amplification system to identify possible PCR inhibition and detected as an internal control (IC) in fluorescence channel cycling orange of the Rotor- Q-Gene. External positive controls are supplied which allow the determination of the amount of viral RNA, as shown in table (1):

Table 1: Thermal cycling program of real-time qPCR for measurement of HCV RNA load

Temperature (ºc)	Time	Nº cycle
50	30 min	1
95	15 min	2
95 50 72	30 sec 60 sec 30 sec	50

The standard curve was constructed by adding 20µl of serial dilutions of HCV standard RNA from 10 IU/µL to10000 IU/ µL. Standard curve was also used for calculating PCR run efficiency which was above 96%. Internal control was added through extraction steps, PCR water grade used as negative control. After the run is finished, the data via was analyzed signal fluorescence detection FAM for positive samples, ROX for internal control:

- Any samples not give signal in ROX channel neglected and repeated.
- If signal not detected in FAM but appear in ROX the sample consider negative or not detected.
- If signal is detected in FAM channel and detected in ROX channel the sample consider positive and value obtained enter equation below to change IU/ µl to IU/ml following equation to convert the values determined using the standard curve into IU/ml of sample material:

HCV RNA (IU/ml) = Result(IU/µl)× Elution volume(µl)
Sample volume (ml)

Interleukin 28B polymorphism genotyping

Human genomic DNA was extracted from whole blood using QIA amp DNA Blood Minikit, Ref No. 148023901, Qiagen, Germany). The sample was first lysed by proteinase K and then mixed with lyses buffer. Ethanol (96-100%) used to lyse lipid component of the cell. Mixture were applied to QIA amp Mini spin column. Two different wash buffers in two steps were used to remove contaminant. Human genomic DNA was eluted in a special eluted buffer and incubated at room temperature for 1 minute, and then centrifuged at 3200 g.

Detection of IL 28B rs12979860 / rs8099917 was achieved by Real TM-PCR (Ref R-05-100 FRT(Sacace Biotechnologies, Italy). The test was performed by the detection of the genetic variants within the IL28B gene using competitive allele-specific amplification in Real-Time PCR. Kit contains one primer PCR mix tube for detection of rs8099917 polymorphism and one primer PCR mix tube for the detection of rs12979860 polymorphism. Internal Control (IC) serves as an amplification control for each individually processed specimen and to identify possible inhibition reaction, as shown in table (2):

Table 2: Fluorophore channel for IL-28B allele and internal control

Reaction Mix	rs8099917	rs12979860
Fluorophore channel	Detected nucleotide	
FAM	T	T
JOE	G	C
ROX	IC	IC

Real TM-PCR were carried out in a total volume 25µl with 10 µl of extracted DNA sample, 5 µl of PCR-mix2 FRT, 0.5 µl of Taq polymerase, 10 µl of PCRmix1FRT IL28B specific primers,

Reaction volume was prepared respectively for each polymorphism rs80999917/ rs12979860. Results were interpreted as follows: if ROX (orange) Ct is present and less than 25, results were evaluated as shown:

Only FAM (green) Ct is present= TT/rs80999917/ TT rs12979860
Only JOE (yellow) Ct is present= GG rs80999917/ CC rs12979860
Both FAM and JOE Ct present:
FAM Ct less JOE Ct = TT rs80999917/ TT rs12979860
FAM Ct larger JOE Ct = TG rs80999917/ CT rs12979860

Statistical analysis

Data were processed using SPSS software version 12.1.1.3. percentages were used for observational data. Anova test and Mann-Whitney tests were used for parametric and non-parametric data, respectively. P values less than 0.05 were considered significant. Moreover, for qualitative assessment of response to therapy, patients with two log reduction were considered responders.

3. Results

Distribution of rs12979860 and rs8099917- based SNPs IL-28B genotypes and alleles in the population of the study

Interleukin 28B rs12979860 and rs8099917genotypes as well as alleles showed no significant difference between patients and controls (p> 0.05) as shown in table (3).

Association between SNPs rs12979860- and rs8099917- based IL-28B genotypes and alleles with stage of liver fibrosis in hepatitis patients

Concerning association between rs12979860 and rs8099917 based IL28B genotypes and alleles with stages of liver fibrosis, a significant association (p<0.05) was found between stages of liver fibrosis in hepatitis patients and rs12979860 genotypes but not rs12979860 alleles or rs80999917 genotypes and alleles as summarized in table (4).

Association between SNPs rs12979860- and rs8099917- based IL-28B genotypes and alleles and Hepatitis C virus genotypes

Distribution of rs12979860 genotypes and alleles were significantly associated with HCV genotypes (P<0.05); however, rs8099917 genotypes and alleles were not associated with HCV genotypes (P>0.05), as shown in table (5). Regarding the distribution of rs12979860 genotypes in patients with HCV genotype 1, TT genotype was the highest, then CT, and zero at CC, while in HCV genotype 4, the highest number of patients was in CT, then in TT and least in CC (P<0.05). For alleles' distribution, HCV genotype 1 was significantly associated, 78.3%, with allele T versus only 21.7% with allele C while HCV genotype 4 was not found to be strongly associated with certain allele (P<0.05).

Association between patients' response to therapy and their SNPs rs12979860- and rs8099917- based IL-28B genotypes and alleles

Response of hepatitis patients to dual therapy was associated with patients' own IL-28B SNP rs12979860 and SNP rs8099917 genotypes (P<0.05). For rs12979860, TT genotype was inferior to CC or CT genotypes in term of responsiveness to therapy. Median of HCV load in blood decreased only one log in the first 3 months of therapy versus 3 logs and 5 logs for CT and CC, respectively. However, after 6 months of treatment, the median log of viral load in CC and CT genotypes was zero versus 2 log in TT genotype. For rs8099917, interestingly, patients with genotype GG showed no response at all after 6 months of dual therapy, while patients with TT genotype showed the best response, 5 log reduction of median HCV load just after 3 months of therapy and then patients with TG genotype who showed 3 log reduction after 3 months and further 2 log reduction after 6 months of therapy (P<0.05) as summarized in table (6).

In the qualitative assessment of association, it was found that no significant association between response to therapy and rs12979860 genotypes. However, response of patients was much better in CC genotype, 75% responders to therapy, versus CT and TT group, only 56.52% responders to therapy (P>0.05).

For rs8099917, the response of patients to therapy was borderline associated with rs8099917 genotypes being GG genotype significantly the worst group of patients in response to therapy, 100% no response (P<0.05). as illustrated in table (7)

Table 3: Distribution of rs12979860- and rs8099917- based SNPs IL-28B genotypes and alleles in the population of the study.

			Study groups		Total	P value
			Hepatitis patients	Control		
rs12979860 genotypes	CC	Count	4	0	4	0.11
		%	8.00	0.00	4.40	
	CT	Count	24	26	50	
		%	48.00	65.00	55.60	
	TT	Count	22	14	36	
		%	44.00	35.00	40.00	
Total		Count	50	40	90	
		%	100.00	100.00	100.00	
			Study groups		Total	P value
			Hepatitis	Control		
rs12979860 Alleles	C	Count	32	26	58	0.535 OR:0.977 CI: (0.52-1.8)
		%	32.00	32.50	32.20	
	T	Count	68	54	122	
		%	68.00	67.50	67.80	
Total		Count	100	80	180	
		%	100.00	100.00	100.00	

			Study groups		Total	P value
			Hepatitis Patients	Control		
rs 8099917	GG	Count	2	2	4	0.949

genotypes		%	4.00	5.00	4.40	
	TG	Count	29	23	52	
		%	58.00	57.50	57.80	
	TT	Count	19	15	34	
		%	38.00	37.50	37.80	
Total		Count	50	40	90	
		%	100	100.00	100.00	
			Study groups		Total	P value
			Hepatitis	Control		
rs 8099917 Alleles	T	Count	67	53	120	0.521 OR:1.03 CI: (0.55-1.9)
		%	67.00	66.20	66.70	
	G	Count	33	27	60	
		%	33.00	33.80	33.30	
Total		Count	100	80	180	
		%	100.00	100.00	100.00	

Table 4: Association between SNPs rs12979860- and rs8099917- based IL-28B genotypes and alleles with stage of liver fibrosis in hepatitis patients

			Fibrosis stage of liver				Total	p value
			1	2	3	4		
rs12979860 genotypes	CC	Count	0	0	2	0	2	0.021
		%	0.00	0.00	100.00	0.00	100.00	
	CT	Count	2	1	2	3	8	
		%	25.00	12.50	25.00	37.50	100.00	
	TT	Count	1	4	3	1	9	
		%	11.10	44.40	33.30	11.10	100.00	
Total		Count	3	5	7	4	19	
		%	15.80	26.30	36.80	21.10	100.00	
			Fibrosis stage of liver				Total	p value
			1	2	3	4		
rs12979860 Alleles	Allele C	Count	2	1	6	3	12	0.374
		%	16.70	8.30	50.00	25.00	100.00	
	Allele T	Count	4	9	8	5	26	
		%	15.40	34.60	30.80	19.20	100.00	
Total		Count	6	10	14	8	38	
		%	15.80	26.30	36.80	21.10	100.00	

			Fibrosis				Total	p value
			1	2	3	4		
rs8099917 Genotypes	GG	Count	0	0	1	0	1	0.212
		%	0.00	0.00	100.00	0.00	100.00	
	TG	Count	1	3	5	2	11	
		%	9.10	27.30	45.50	18.20	100.00	
	TT	Count	2	2	1	2	7	
		%	28.60	28.60	14.30	28.60	100.00	
Total		Count	3	5	7	4	19	
		%	15.80	26.30	36.80	21.10	100.00	
			Fibrosis				Total	p value
			1	2	3	4		
rs8099917 Alleles	Allele G	Count	1	3	7	2	13	0.432
		%	7.70	23.10	53.80	15.40	100.00	
	Allele T	Count	5	7	7	6	25	
		%	20.00	28.00	28.00	24.00	100.00	
Total		Count	6	10	14	8	38	
		%	15.80	26.30	36.80	21.10	100.00	

Table 5: Association between SNPs rs 12979860 and rs 8099917- based IL-28B genotypes and alleles and Hepatitis C virus genotypes

	rs 12979860 Genotype			Total	rs 12979860 Allele		Total
	CC	CT	TT		Allele C	Allele T	
Genotype 1	0	10	13	23	10	36	46
%	0.00	43.50	56.50	100	21.70	78.30	100

Genotype 4	4	14	9	27	22	32	54
%	14.80	51.90	33.30	100	40.70	59.30	100
P value	0.006*				0.042*		

	rs 8099917 Genotype			Total	rs 8099917Allele		Total
	GG	TG	TT		Allele G	Allele T	
Genotype 1	1	15	7	23	17	29	46
%	4.30	65.20	30.40	100	37.00	63.00	100
Genotype 4	1	14	12	27	16	38	54
%	3.70	51.90	44.40	100	29.60	70.40	100
P value	0.355 NS				0.437 NS		

Table 6: Quantitative association between patients' response to therapy and their SNPs rs12979860 and rs 8099917 based IL-28B genotypes

		rs12979860 genotypes		
		CC	CT	TT
HCV viral load before treatment (IU /ml blood)	Median	**2.00E+05**	**4.00E+05**	**4.00E+05**
	25-75 CI	(9.E+04-4.E+05)	(2.E+05 - 2.E+06)	(4.E+04 - 1.E+06)
HCV viral load 3 months after treatment (IU/ml blood)	Median	**1.00E+00**	**0.00E+02**	**2.00E+04**
	25-75 CI	(6.E+02 - 1.E+04)	(0.E+00 - 1.E+04)	(0.E+00 - 1.E+06)
HCV viral load 6 months after treatment (IU/ml blood)	Median	**0.00E+00**	**0.00E+00**	**0.00E+02**
	25-75 CI	(0.E+00 - 5.E+04)	(0.E+00 - 3.E+02)	(0.E+00 - 4.E+04)
base line-after 3 months		<0.001	<0.001	0.002
base line-after 6 months		0.002	<0.001	<0.001
3 months- 6 months		0.26	0.003	<0.001
P value 0.042				
		rs8099917Genotypes		
		GG	TG	TT
Base line	Median	**2.00E+06**	**4.00E+05**	**6.00E+05**
	25-75 CI	(2.E+05-3.E+06)	(4.E+04-1.E+06)	(1.E+05-2.E+06)
After 3 months	Median	**1.00E+06**	**4.00E+02**	**0.00E+00**
	25-75 CI	(2.E+05-2.E+06)	(0.E+00-3.E+04)	(0.E+00-1.E+05)
After 6 months	Median	**1.00E+06**	**0.00E+00**	**0.00E+00**
	25-75 CI	(5.E+05-2.E+06)	(0.E+00-0.E+00)	(0.E+00-9.E+04)
base line-after 3 months		0.343	<0.001	<0.001
base line-after 6 months		1	<0.001	<0.001
3 months- 6 months		1	<0.001	0.487
P value 0.02				

Table 7: Qualitative assessment of IL28B association with response to therapy

		Responsiveness				Total	p value
		R	%	NR	%		
rs12979860 genotype	CC	3	75.00	1	25.00	4	0.072
	CT	16	66.67	8	33.33	24	
	TT	10	45.45	12	54.55	22	
Total		29	58.00	21	42.00	50	
		Responsiveness				Total	p value
		R	%	NR	%		
Rs12979860 genotype	CC+CT	19	67.86	9	32.14	28	0.3098
	TT	10	45.45	12	54.55	22	
Total		29	58.00	21	42.00	50	
		Responsiveness				Total	p value
		R	%	NR	%		
rs12979860 genotype	CC	3	75.00	1	25.00	4	0.0243
	TT+CT	26	56.52	20	43.48	46	
Total		29	58.00	21	42.00	50	
		Responsiveness				Total	p value
		R	%	NR	%		
rs 12979860 Allele	Allele C	22	68.75	5	15.63	32	0.192
	Allele T	36	52.94	32	47.06	68	
Total		58	58.00	42	42.00	100	

		Responsiveness				Total	p value
		R	%	NR	%		
rs 8099917 Genotype	GG	0	0.00	2	100.00	2	0.051
	TG	17	58.62	12	41.38	29	
	TT	12	63.16	7	36.84	19	
Total		29	58.00	21	42.00	50	
		Responsiveness				Total	p value
		R	%	NR	%		
rs8099917 Genotype	GG+TG	17	54.84	14	45.16	31	0.4133
	TT	12	63.16	7	36.84	19	
Total		29	58.00	21	42.00	50	
		Responsiveness				Total	p value
		R	%	NR	%		
rs8099917 Genotype	GG	0	0.00	2	100.00	2	0.0165
	TT+TG	29	60.42	19	39.58	48	
Total		29	58.00	21	42.00	50	
		Responsiveness				Total	p value
		R	%	NR	%		
rs 8099917 Allele	Allele G	17	51.52	16	48.48	33	0.239
	Allele T	41	61.19	26	38.81	67	
Total		58	58.00	42	42.00	100	

4. Discussion

The current study found no significant difference in the frequency of rs12979860 and rs8099917 genotypes of IL28B between HCV patients and healthy individuals, the control group; this is consistent with data of other studies done by Seyed et al and Sharafi et al in Iran on 147 positive HCV patients between 2012-2013; they found there was no difference in the frequency of rs12979860 and rs8099917 genotypes between total hepatitis C patients and the healthy individuals (7,8). This finding indicates that IL28B polymorphism has nothing to do with the susceptibility of individuals to contract HCV infection.

The different distributions of IL-28B genotypes among Asians, Europeans, and Africans might explain the different rates of SVR in these populations; therefore the determination of the IL-28B SNPs distribution in any community seems useful (9, 10). Several studies in HCV patients in Europe, United State, Australia and Iran showed that the most common rs12979860 genotype was CT, CC and TT consequently (7,5,11). Furthermore, the most prevalent rs8099917 genotype was shown to be TT, GT and GG consequently (7, 12). Accordingly, the distribution of rs12979860-based genotypes of IL28B shown in the current study is different from most of previous studies worldwide. Our findings: CT is the most dominant, then TT, and CC was the least dominant genotype. The interesting notion of the distribution of rs12979860 genotypes discovered in Iraqi HCV patients is that it is different even from the distribution found in neighboring countries like Iran.

The present study reveals that TT genotype of IL28B was two folds associated with advanced stage of liver fibrosis when compared to the early stages while CC and CT genotypes showed no marked preference to either stage of liver fibrosis. This outcome seems to come along with the known association of rs12979860 TT genotype of IL28B with bad response to therapy (13,14,15,16). On the other hand, regarding IL-28B rs8099917 genotypes, one possible reason for the lack of significant relationship with stage of liver fibrosis could be the high frequency of the TG genotype which neutralizes the effect of other two genotypes.

The results of the current study were compared with previous reports studying the differential association between HCV genotypes and IL28B genotypes. A study conducted in 2014 in Turkey by Aygen et al on HCV genotypes1 and 4, found the highest rate of rs12979860 genotypes were CT (52.7%), TT (24.7%), and CC (22.6%) in both HCV genotypes 1 and 4, without any variation (17). Their results are similar to the results of the current study in term of HCV genotype 4 but not in term of HCV genotype 1. Another study conducted on HCV genotype 4 cases in Egypt in 2012, they found that CT and CC genotypes were present in 34.8% of patients, while TT genotype was present in 30.2% of cases (18). Another study also in Egypt published by Olfat shaker et al was done on HCV genotype 4, they found that CC, CT, and TT genotypes were present in 54, 34, and 12% of cases (19).

The finding of this study indicated that rs12979860 CC genotype had the best percentage of responders, 75%, then CT 66.7%, and least TT45.4%. For rs8099917, TT genotype showed highest percentage of responders 63.1%, then TG 58.6%, and no response at all at GG, 0%. These findings came in line with another study (20) which found that rate of response to HCV therapy is significantly higher in CC genotype (58.6%) compared to CT/TT (20.3%). In addition, another study (19) found that CC genotype of rs12979860 is associated with a better rate of SVR vs TT genotypes, and they also found that TT genotype of rs8099917 has higher prevalence (68.3 vs 15%) among responders than non responders. Another study by Sheppard et al and Fried et al (21,22) also found that TT genotypes of rs8099917 was significantly associated with better rapid viral response and SVR rates, beside many authors found that TT genotypes was an important predictor for SVR (23,24).

Additionally the current findings about GG genotype agree with a study done by Olfat Shaker et al who found higher

prevalence of GG genotype in non responder than responder (32% vs 5%), respectively (19).

Accordingly, GG genotype of rs8099917 is shown by the present study and previous studies as an important predictor for response failure. Rauch etal (5) agreed with these finding who found several SNPs near IL-28B locus that were associated with chronic HCV infection at a genome wide significance level. The strongest association with treatment failure was found with rs8099917, individuals carrying one or two copies of the rs8099917 risk G allele which was shown to have higher risks of treatment failure compared to individuals carrying commoner genotype TT.

Interestingly, several studies on HCV genotype 2, but not genotype 1 or 4, infected Asian patients revealed that IL28B genotypes are not associated with SVR of chronic HCV infection (25,5). This might give a clue on the variable effect of IL28B polymorphism on the response to therapy depending on the genotype of HCV infection.

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