

Detection of IL28B-rs12979860 Polymorphism and Estimation of IL28B Serum Level in Iraqi Hepatitis C Infected Patients

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Abstract: *The genetic polymorphism of the IL-28B gene was determined at a position rs12979860 for 67 Iraqi Arabs hepatitis C infected patients and 53 controls. Testing for Hardy-Weinberg (H-W) equilibrium revealed that HCV patients showed no significant variation in the distribution of IL-28B rs-12979860 genotypes ($P > 0.05$). Comparing patients with controls revealed that IL-28B rs-12979860 CC genotype was significantly decreased in hepatitis C patients (35.8 vs. 56.6 %; $P = 0.027$), and the EF of such difference was 0.32. However, in terms of allele frequencies, the C alleles was significantly decreased (59 vs. 72.6%, $P = 0.03$) in patients than controls, while allele T was significantly increased (41 vs. 27.4%, $P = 0.03$) in patients compared to controls. Also, the heterozygous genotype CT and heterozygote genotype TT of IL-28B rs-12979860 have positively associated with HCV infection in Iraqi patients while the homozygote IL-28B rs-12979860 CC genotype have a significant negative association with HCV infection in Iraqi patients and protected against it. In this study serum level of IL-28B was not significantly decreased in HCV patients compared to control and IL-28B showed different serum levels in its three IL-28B rs-12979860 genotypes. The present study designed to determine the IL-28B gene polymorphism defined by real time PCR at a rs12979860 SNP in HCV Iraqi Arabs infected patients and its association with IL-28B level in serum due to its role in HCV infection clearance.*

Keywords: Hepatitis C infection, IL-28B polymorphism, IL-28B serum level, Odds ratio

1. Introduction

Hepatitis C virus (HCV) is one of the most important globally cause of morbidity and death. The worldwide estimations showed a raise in HCV seroprevalence more than the previous decade to 2.8%, corresponding to > 185 million infections [1]. Chronic infection of it's commonly related with the progress of liver cirrhosis, hepatocellular cancer, liver failure, and death. It has been expected in the developed world, while the frequency of HCV infection seems to decrease, mortality secondary related to infection with HCV will continue to increase over the next twenty years. So, while much information suggest that HCV infection might be eliminated in the next 15-20 years by paying attention therapeutic strategies, a high-quality understanding of HCV infections should be essential to develop strategies to put off new infections [2].

Independent genome-wide association studies have yielded impressive results by recognizing single nucleotide polymorphisms near the IL28B gene (encoding IFN- λ 3), which are related strongly with spontaneous induced clearance of HCV infection and treatment [3]. Later, these results were confirmed. In fact, HCV genotype 1 patients with good quality response IL28B genotype (e.g., rs12979860 CC vs. CT/ TT) reach Sustained Viral Response (SVR) rates following PEG-IFN- α /ribavirin therapy close to persons which can be achieved in the 'easy to treat' HCV genotype 2 and 3 patients in general [4].

HCV infection clearance is also connected to host genetic factors in infected individuals. One of the greatest described factors is interferon lambda 3 (IL-28B), a constituent assumed to cause a huge response against HCV infection and is situated on chromosome 19 of the IL-28B gene. IFN- λ 3 controls T reg. cells and augments adaptive cellular

immunity. The IL-28B cytokine acts as an antiviral by induction of interferon- stimulated genes through JAK-STAT pathway [5].

2. Patients and Methods

A total of 120 Iraqi Arab individuals were included in this study, they were referred for diagnosis and treatment to the Gastroenterology and Hepatology Teaching Hospital in Baghdad during the period November 2015 to April 2016. 67 individuals of them had hepatitis C infection (patients group); Anti -HCV antibody was detected in their sera by ELISA technique and confirmed by real time PCR analysis to detect the viral genetic material. The patients were 37 males and 30 females with age range 4 -71 years. While the others 53 apparently healthy subjects were the control group, 27 males and 26 females with age range similar with patients group.

2.1 Specimens Collection

Patients and controls, blood was collected and distributed into two aliquots. The first aliquot was placed in plain disposable tubes, then left to stand at room temperature (18-25°C) to clot. Sera were separated by centrifugation for 5 minutes at 3000 rpm. The second aliquot was transferred to EDTA tube and both aliquots were kept at -20°C until assayed.

2.2 Detection of IL28B-rs12979860

2.2.1. DNA Extraction

The Blood Genomic DNA ExiPrep™ Plus Kit and the ExiPrep™ 16 Plus, automatic nucleic acid purification tool, (Bioneer, Korea) was used for genomic DNA extraction from whole blood, according to manufacturer protocol.

2.2.2 Detection of IL28B-rs12979860 genotypes

The real-time PCR mutation detection / allelic discrimination kit (Primerdesign, UK) was used to detect IL28B -rs12979860 genotyping by using double-dye hydrolysis probes. Each genotyping primer/probe mix contains two labeled probes homologous to the two genotypes under investigation. During real-time PCR amplification of the target DNA the probes will compete for binding across the variant region. The probe that is 100% homologous to the DNA binding site will preferentially bind and give a fluorescent signal as PCR proceeds. It follows that the wild-type sequence will give a strong amplification plot through one channel whilst giving a very weak signal through the alternative channel. Homozygous variant samples will give an exactly inverse result. Heterozygote samples contain both probe binding sites on each of the two alleles and therefore give an intermediate single through both channels. The three possible genotypes can be resolved by comparing end point fluorescence.

A complete genotyping reaction mix was prepared for each primer and probe mix as in table 1. Sufficient reactions for all samples were prepared include 2 extra reactions for the wild type and variant positive controls and 1 extra sample as a no template negative control.

Table 1: A complete genotype reaction mix for one sample

Components	Volume
Oasig™ 2XPCR mastermix (after preparation as in 2.3.a)	10µl
IL-28B- rs12979860 primer/probe mix (after preparation as in 2.3.b)	1 µl
RNAse/DNAse free water	4 µl
Final volume	15 µl

Fluorogenic data should be collected through the ROX and VIC channels. The wild-type probe is labeled to read through the ROX channel whilst the mutant probe is labelled to read through the VIC channel. On wild-type sequences the ROX channel will give a strong amplification plot and the VIC channel none or very low detection. The signals are reversed on mutant samples. Heterozygote samples will give an intermediate signal through both ROX and VIC channels. The genotype of each sample is calculated by comparing the ratio of signals between the two channels (ROX and VIC).

2.3 Assessment of Interleukin-28B serum levels

Sera of patients and controls were assessed for the level of IL-28B by using Elabscience human IL-28B ELISA kit (Elabscience, USA) according to manufacturer protocol.

2.4 Statistical analysis

All statistical analysis was performed using statistical package for social science (SPSS) program version 17 for windows (SPSS INC., Chicago IL, USA). Results were

expressed as mean ± SD. Comparisons between two groups were performed using T test for categorical data. P value of <0.05 were considered to indicate statistical significance.

Genotypes of IL-28Brs-12979860 were presented as percentage frequencies, and significant differences between their distributions in hepatitis C patients and controls were assessed by two-tailed Fisher's exact probability (P). In addition, odd ratio (OR), etiological fraction (EF) and preventive fraction (PF) were also estimated to define the association between a genotype with the disease. The OR value can range from less than one (negative association) to more than one (positive association). If the association was positive, the EF was calculated, while if it was negative, the PF was given [6]. These estimations were calculated by using the WINPEPI computer programs for epidemiologists. The latest version of the WINPEPI package (including the programs and their manuals) is available free online at <http://www.brixtonhealth.com>.

Allele frequencies of IL-28Brs-12979860 genes were calculated by direct gene counting methods, while a significant departure from Hardy-Weinberg (H-W) equilibrium was estimated using H-W calculator for two alleles, which is available free online at <http://www.had2know.com/academics/hardy-weinbergequilibrium-calculator-3-ale;es.html>.

Hardy-Weinberg equilibrium is the expected frequencies of genotypes if mating is non – assortative and there are no mutations from one allele to another. When there are two alleles for a particular gene; A and B, and their respective population frequencies are p and q, then the expected frequencies of the genotypes AA, AB, BB are p², 2pq and q², respectively. Significant differences between the observed and expected frequencies are assessed by Pearson's Chi-square test [6].

3. Results and Discussion

3.1 Genetic polymorphism of IL-28B

The genetic polymorphism of the IL-28B gene was determined at a position rs12979860, which were presented with three genotypes (CC, CT, and TT) in HCV patients and controls.

Testing for Hardy-Weinberg (H-W) equilibrium revealed that HCV patients showed not significant variation in the distribution of IL-28B rs-12979860 genotypes (P> 0.05). There is no difference between the observed and expected frequencies of CC, CT and TT genotypes in patients and control samples, in which the observed and expected genotype frequencies were in a good agreement with H-W equilibrium, as shown in table 2.

Table 2: Observed and expected numbers and percentage frequencies and Hardy-Weinberg (H-W) equilibrium of IL-28B rs-12979860 genotypes and alleles in Hepatitis C patients and controls.

Studied groups			IL-28B rs-12979860 genotypes or allele					H-W P ≤
			CC	CT	TT	C	T	
Hepatitis C patients	Observed	No.	24	31	12	79	55	Not significant
		%	35.8	46.3	17.9	59	41	
	Expected	No.	23.3	32.4	11.3	Not estimated		
		%	34.8	48.4	16.8	Not estimated		
Control	Observed	No.	30	17	6	77	29	Not significant
		%	56.6	32.1	11.3	72.6	27.4	
	Expected	No.	28	21.1	4	Not estimated		
		%	52.8	39.8	5.5	Not estimated		

Comparing patients with controls, IL-28B rs-12979860 CC genotype was significantly decreased in hepatitis C patients (35.8 vs. 56.6 %; P = 0.027), and the EF of such difference was 0.32. However, in terms of allele frequencies, the C

alleles was significant decreased (59 vs. 72.6%, P= 0.03) in patients than controls, while allele T was significantly increased (41 vs. 27.4%, P=0.03) in patients compared to controls, as shown in table 3.

Table 3: Statistical evaluations of associations between IL-28B rs-12979860 genotypes or allele and hepatitis C infection

IL-28B rs-12979860 Genotypes or Allele	Patients (67)		Controls (53)		OR	Etiological or Preventive Fraction	Fishers Exact Probability	95%CI
	N	%	N	%				
Genotypes								
CC	24	35.8	30	56.6	0.43	0.32	0.027	0.21-0.89
CT	31	46.3	17	32.1	1.82	0.20	0.135	0.87-3.84
TT	12	17.9	6	11.3	1.71	0.07	0.441	0.60-4.86
Alleles	(134)		(106)					
C	79	59	77	72.6	0.54	0.33	0.030	0.31- 0.93
T	55	41	29	27.4	1.85	0.18	0.030	1.07-3.19

OR= odds ratio; 95%CI = 95% confidence interval

There are numerous studies in HCV infected patients in Europe, United State, Australia and Iran showed that the most common rs12979860 genotype was CT, CC and TT consequently [7-9] and this similar to present result.

In Iranian patients, the distribution of IL-28B rs12979860 genotypes as well as alleles in patients with hepatitis C and healthy individuals were reported and the distribution of CT and CC genotypes in IL-28B rs12979860 SNPs was higher than other type in patients with hepatitis C and healthy individuals respectively. The differences in the distribution of IL-28B genotypes between patients with hepatitis C and healthy individuals related to rs12979860 were not statistically significant. The distribution of the C allele in IL-28B rs12979860 SNPs was higher than the T allele in patients with hepatitis C and healthy individuals respectively. The differences in the distribution of IL-28B rs12979860 alleles between patients with hepatitis C and healthy individuals were statistically significant [10]. This is similar to the present study in case of HCV patients while difference in control because the distribution of genotype CC is higher than CT in control group and CC genotype was significantly decreased in HCV patients.

In Egypt, Hameed, [11] reported that the distribution of IL-28B genotypes of rs12979860 in chronic HCV patients was CT 54%, CC 30% and TT 16%. In Pakistan, Aziz *et al.*, [12] found that the frequency of CC genotype of rs12979860 was 54.3%, CT 37.1% and TT 8.6% genotypes in HCV patients which is not in agreement with present result. Clark *et al.*, [13] observed that different ethnic groups have marked differential distribution of IL-28B polymorphisms. The favorable CC allele of rs12979860 is least frequent in African- American and most frequent in Asian. Allele

frequencies differ between ethnic groups, largely explaining the observed differences in response rate between Caucasians, African Americans and Asians. Asselah *et al.*, [14] reported differential frequencies of the C allele of rs12979860 in HCV infected patients among three ethnic groups in their study, i.e. 61.4%, 54.7% and 31.0% for Egyptian, European and sub-Saharan African origin patients, respectively.

The results pointed out that genotypes or alleles of IL-28B rs-12979860 gene were associated with increased risk to promote HCV infection in humans or protection against it, and recorded OR, EF and PF values are in favor of such conclusion. The heterozygous genotype CT and heterozygote genotype TT of IL-28B rs-12979860 which scored OR higher than one value (1.82 and 1.17, respectively) and EF value (0.2 and 0.07, respectively) in HCV patients that may regard those genotype IL-28B rs-12979860 CT and TT as positively associated with HCV infection in Iraqi patients while this association were not significant. The homozygote IL-28B rs-12979860 CC genotype scored OR value less than one (0.43) and PF value (0.32) that may regard that this genotype have a significant negative association with HCV infection in Iraqi patient and protected against it.

In study subjected in China, 896 Chinese subjects were involved, including 529 subjects with persistent HCV infection, 196 subjects who cleared the infection and 171 healthy controls. Shi *et al.*, [15] found that the distribution of the alleles of rs12979860 was in accordance with the Hardy-Weinberg equilibrium in this study (P=0.51). They confirmed that the rs12979860 C allele frequency in this healthy Chinese population was 95%. The C allele was

lower significantly in the persistent infection group (95.3%) than in the clearance group (97.7%). However, the frequency of the T allele was greater among individuals of the persistent infection group than those of the clearance group (4.7% vs 2.3%; $P=0.04$) and Spontaneous HCV clearance was more common in subjects carrying the CC genotype than CT/TT genotype ($P=0.04$, $OR=2.12$, 95% $CI=1.01-4.42$). Also khubaib *et al.*, [16] reported that the CC genotype of *IL-28B* SNP rs12979860 is an independent predictive factor for SVR in chronic HCV-infected patients in Pakistan. The later findings are in a good agreement with the present study results. These results and irrespective of their different observed associations, may highlight the role of *IL-28B* gene polymorphism in conferring susceptibility or resistant against the progression HCV infection, and the significant departure from H-W equilibrium may favor such conclusion.

3.2 Serum level of IL-28B

Serum level of *IL-28B* was not significantly ($P > 0.05$) decreased in HCV patients (145.578 ± 235.520 pg/ml) compared to control (235.418 ± 173.181 pg/ml) as shown in table 4.

Table 4: Serum level of *IL-28B* in hepatitis C patients and controls

Parameter	Controls Mean \pm SD	Patients Mean \pm SD
Levels of <i>IL-28B</i> pg/ml	235.418 \pm 173.181	145.578 \pm 235.520
P Value	> 0.05	

This result was agreed with Alborzi *et al.*, [17] who found that *IL-28B* serum level was also significantly higher in healthy group compared with the chronic hepatitis C and with Shi *et al.*, [15] results who compared the *IL-28B* levels in chronic HCV infection, clearance and healthy control groups by ELISA. The serum levels of *IL-28B* were obviously reduced and significantly lower mRNA and serum levels of *IL-28B* in the chronic HCV infected group compared to the spontaneous clearance and healthy control groups.

Table 5: Serum level of *IL-28B* in hepatitis C patients and controls distributed by *IL-28B* rs-12979860 genotypes.

	<i>IL-28B</i> rs-12979860 genotypes					
	Patients			Controls		
	CC	CT	TT	CC	CT	TT
Levels of <i>IL-28B</i> pg/ml	149.86 \pm 195.38	104.96 \pm 118.45	260.07 \pm 471.91	240.44 \pm 178.19	256.60 \pm 198.64	170.73 \pm 85.21
P value	Significant differences ($P=0.035$) in <i>IL-28B</i> levels between CT genotype					

For advance understanding of cytokines gene polymorphisms role in HCV infection, the impact of *IL-28B* gene polymorphism on serum level of *IL-28B* was evaluated in patients and control, because Jin *et al.*, [26] reported that it has been well documented that cytokines gene polymorphism have a functional importance and might be associated with high or low production of the corresponding cytokines.

From present data the *IL-28B* TT genotype might have a role in the increase production of *IL-28B* in HCV infected patients but not in the control. Allele expression studies showed that in HCV infected patients, with respect to

There is inconsistent evidence for the up-regulation of *IFN- λ* in chronically HCV infected liver. Some investigations have shown up-regulated serum levels of *IFN- λ* in chronic HCV infected patients when compared to serum levels of patients with either non-viral liver disease or control with livers non-diseased [18, 19], whereas others have revealed lower *IFN- λ* serum levels in HCV chronic livers compared to non-diseased livers [20]. During investigation of *IFN- λ* transcripts in liver biopsies, Mihm *et al.* [21] showed no difference between HCV diseased liver and non-viral liver disease while Lee *et al.*, [22] showed an increase in *IFN- λ* transcripts when HCV-infected livers were compared to healthy livers. *In vitro* studies revealed that HCV infection causes *IFN- λ* transcription in PHH, FHH, and the human hepatocyte cell line PH5CH8 [23, 22].

Primary human hepatocytes, *in vivo* studies have shown that HCV induces primarily an *IFN- λ* response rather than *IFN- α* or $-\beta$. *IFN- λ* can then act in an autocrine fashion, stimulating the expression of *IFN*-stimulated genes (ISGs) [24]. Exogenous addition of *IFN- λ* to hepatocytes inhibits replication of both HCV replicons [25]. Concomitant with a reduction in HCV replication, *IFN- λ* also suppresses the microRNA miR-122. The addition of both *IFN- λ* and the miR-122 inhibitor (miRIDIAN) increased the suppression of HCV replication [22].

3.3 *IL-28B* rs-12979860 genotype impact on *IL-28B* levels

The impact of *IL-28B* rs-12979860 gene polymorphism on serum level was determined in patients and control (table 5). *IL-28B* rs-12979860 showed different levels in its three genotypes. In patients group, TT genotype showed the highest level (260.07 ± 471.91 pg/ml) followed by genotype CC with 149.86 ± 195.38 pg/ml and CT with 104.96 ± 118.45 pg/ml. however, an opposite was made in controls, in which the CT genotype showed highest level (256.60 ± 198.64 pg/ml) followed by CC genotype with 240.44 ± 178.19 pg/ml and TT genotype 170.73 ± 85.21 pg/ml, and the differences in CT genotypes was significant.

rs12979860 genotypes, *IL-28B* levels were significantly higher in CC homozygous than TT/TC homo or heterozygous ($P=0.02$) but not in the healthy control [15]. It has demonstrated that serum *IL-29* and *IL-28* corresponded with TT rs12979860 genotypes irrespective of the treatment response. However, they also showed correlation of *IL-28B* serum level with the C allele of rs12979860 SNP [20] while no relationship was noticed between the serum level of *IL-28* and the different rs12979860 genotypes in HCV Egyptian studied patients [27].

The results of this study at variance with the results obtained by Al-Qahtani *et al.*, [28] found that in HCV patients from

Saudi Arabia, rs8099917 was the only SNP that significantly correlated with the IL28B serum levels, while the other four SNPs, including rs12979860, failed to show any correlation with serum IL28B levels. These differences could be correlated with the virus strain in addition to racial variation may also have its impact as these polymorphisms show different frequencies in different ethnic communities [29].

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