Haplotype Analysis of Interleukin-10 Gene Promoter Polymorphisms in Chronic Hepatitis C Infection: A Case Control Study

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Abstract

High prevalence of hepatitis c virus (HCV) infection in some areas necessitates more investigations of the causative factors. Genetic factors that cause disruption in operation or secretion of interleukin 10 (IL-10), an anti-inflammatory cytokine, may play a role in the intensity of the disease. The aim of this study was to evaluate genetic variants of *IL-10* gene polymorphisms in HCV patients and their relationship with HCV disease.

Fifty HCV patients and the same number of healthy individuals who were referred to hepatitis clinic in Mashhad, northeast of Iran, were recruited. Genomic DNA was extracted from whole blood. Genotyping for *IL-10* gene promoter polymorphisms in three positions (–1082 G>A, –819 C>T and –592 C>A) was conducted by amplification refractory mutation system-polymerase chain reaction. Haplotype analysis was performed using PHASE software.

In a recessive analysis model of the –1082 position (GG vs. AA + AG), GG genotype was more common in patients (adjusted $p=0.02$; OR = 4.66 [95% CI 1.31–16.35]). Also, ATA haplotype was more prevalent in HCV patients (adjusted $p=0.061$; OR = 1.87 [95% CI 0.97–3.61]). Also, ATC/GCA diplotypes were more common in controls (adjusted $p=0.002$; adjusted OR = 0.27 [95% CI 0.11–0.63]). Although we found a possible association between *IL-10* promoter polymorphisms and HCV infection, certain genotypes or diplotypes may confer a higher risk or susceptibility for developing HCV infection.

Introduction

HEPATITIS C VIRUS (HCV) infection is a major cause of chronic hepatitis (7) and approximately 170 million people worldwide are infected with HCV (2,6). Infection of HCV stimulates a wide range of innate and acquired immune responses. Some 70–80% of patients with chronic HCV infection may also develop hepatic chronic disease, including fibrosis, hepatic cirrhosis, and hepatocellular carcinoma (24–26). Dysfunction of several factors such as cytokines has an important role in immune system deficiency. In addition, it is more evident that there is a close relationship between cytokine genes polymorphisms and HCV infection (12,20). Interleukin 10 (IL-10) is a potent anti-inflammatory and immune regulatory cytokine that is produced in liver cells, including hepatic, cooper, sinusoidal endothelial, hepatic satellite cells, monocytes, and macrophages (9,13,22,23,33). Chronic HCV infection is associated with fluctuation of

alanine aminotransferase (ALT) levels, an indicator of liver disease in infected patients (12,19). IL-10, which plays an important role in the immune system against viral infection, is polymorphic and its allele frequencies have varied in different studies (4,8,32). Due to the importance of *IL-10* in immune pathway, any defects in this gene can possibly make a person more susceptible to HCV infection. Therefore, the study of genetic changes, including mutations in the *IL-10* gene, may help to dissect the genetic susceptibility of HCV infection (16). The *IL-10* gene contains five exons on chromosomes 1q31 and q32, and several polymorphic sites have also been identified in the promoter region (14,15,29). Predominantly, single nucleotide polymorphisms (SNPs) at positions –1082 G>A, –819 C>T, and –592 C>A are the most common polymorphisms in the *IL-10* gene promoter (29). Several studies on functional characteristics of these three SNPs of *IL-10* gene showed their association with the production of different cytokines (11,18). A “G to A”

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nucleotide change at the -1082 position in the *IL-10* gene may directly affect the production of *IL-10*. Additionally, HCV patients with polymorphisms (-1082 G>A, -819 C>T, and -592 C>A) and high production of *IL-10* appear to have a poor response to interferon-alpha therapy (15,31). It is believed that genetic background has an important role in infection and thus progression of HCV. Since the polymorphisms may differ depending on the racial background, we tried to evaluate this hypothesis in our study. In the present study, we analyzed the genotype and haplotype distribution of the *IL-10* promoter SNPs (-1082 G>A, -819 C>T, and -592 C>A) in HCV patients compared to a control group.

Materials and Methods

Study population

After ethical committee approval and obtaining informed written consent from the participants, 10 mL blood samples were collected from all participants, including patients who were suffering from chronic HCV ($n=50$) and 50 healthy control individuals whose test for HCV infection with enzyme-linked immunosorbent assay kits (Delaware Biotech, Inc., Wilmington, DE) was negative. HCV viral load test was carried out by real time polymerase chain reaction (PCR).

All factors that could affect the risk of HCV infection, including drug and alcohol addiction, transfusion, HIV infection, and tattoos, were considered to be included in the final analysis, when results were adjusted for confounders (Table 1).

Biochemical assay

ALT levels were evaluated for both groups (patients and controls) with the Pars Azmoon kit (Pars Azmoon, Inc., Tehran, Iran). According to the kit instructions, ALT levels at 37°C of <31 U/L for women and <41 U/L for men were considered normal.

DNA extraction

Ten mL of whole blood was collected in EDTA tubes. Genomic DNA was extracted from the blood using a genomic DNA extraction Kit (GeNet Bio, Daejeon, Korea).

Genotyping technique

Genotyping for *IL-10* gene promoter polymorphisms in three positions (-1082 G>A, -819 C>T, and -592

C>A) was conducted using the amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) method. For this purpose, two allele-specific forward and one generic reverse primer were designed for each position. Forward allele specific primers for *IL-10* -1082 G (5'-CTA CTA AGG CTT CTT TGG GAG-3') and for *IL-10* -1082 A (5'-ACT ACT AAG GCT TCT TTG GGA A-3') were separately mixed to a reverse primer (5'-CAG TGC CAA CTG AGA ATT TGG-3'), and the PCR experiment was performed under the following conditions: 95°C for 3 min, followed by 35 cycles at 95°C for 45 sec, 58°C for 40 sec, 72°C for 1 min, and finally a 7 min extension at 72°C. PCR (96-well thermal cycler; Applied Biosystems) amplification for the *IL-10* -1082 G>A position was performed in a 20 μ L reaction volume containing 40 ng genomic DNA, 1.5 mM each dNTPs, 2.5 mM MgCl₂, 1 μ L of 10 pmol each primer, and 0.4 units of *Taq* polymerase (GeNet Bio) in 1 $\times$ reaction buffer. The product size for the *IL-10* -1082 G>A position was 258 bp. For the *IL-10* -819 C and -592 C polymorphism positions, product sizes were 233 bp. Sequences of specific primers for these SNPs were as follows: for the *IL-10* -819 T forward primer: 5'-CCC TTG TAC AGG TGA TGT AAC-3'; for the *IL-10* position -592 A forward primer: 5'-ACC CTT GTA CAG GTG ATGT AAT-3'; and for the reverse primer for the two positions: 5'-AGG ATG TGT TCC AGG CTC CT-3'). All conditions including PCR program set-up for *IL-10* -819 C>T and -592 C>A polymorphisms were performed with similar condition to the PCR program for the -1082 position. Finally, the amplified products were analyzed on 2% agarose gel.

HCV genotyping

HCV genotyping was performed according to the method described by Afshari *et al.* and Ohno *et al.* (3,17).

Statistical analysis

Statistical analysis was conducted using IBM SPSS Statistics for Windows v21 (IBM Corp., Armonk, NY). For determination of normality, data were assessed with the Kolmogorov-Smirnov test. Allele frequency and polymorphism distribution in the three positions of the *IL-10* gene for patients and healthy controls were analyzed using the chi-square test. PHASE software v2.1 (28) was used for haplotype and diplotype analysis. A p -value of <0.05 was considered significant.

Results

Demographic data showed that in the patients group, 84% were males, with an age range of 16–61 years. In the control group, 36% were male, with an age range of 18–65 years. The mean age of patients and controls was 41.49 ± 10.61 years ($\pm SD$) and 36.87 ± 13.11 years ($\pm SD$) respectively (Table 1).

All samples were tested for Hardy-Weinberg equilibrium at each position. Genotypes of cases followed the Hardy-Weinberg equilibrium ($p>0.05$). However, a deviation was observed in controls. To rule out any genotyping errors, 5% of samples were re-genotyped and no technical issues were found. Positions -592 and -819 were tightly in linkage disequilibrium (LD); "A" at position -592 with "T" at position

TABLE 1. DEMOGRAPHIC AND RISK FACTORS IN HEPATITIS C VIRUS PATIENTS AND HEALTHY GROUPS

Characteristics	Patients	Controls	p-Value
M:F	42:8	18:33	<0.05
Age (years; mean $\pm$ SD)	41.49 ± 10.61	36.87 ± 13.11	0.06
Addiction	21 (42%)	—	<0.05
Alcohol	19 (38%)	1 (2%)	<0.05
Transfusion	14 (28%)	9 (17%)	0.06
Tattoo	11 (22%)	—	<0.05
ALT (U/L, mean $\pm$ SD)	33.18 ± 35.54	23.66 ± 16.10	0.7
Increased ALT*	12 (24%)	11 (22%)	0.3

ALT, alanine aminotransferase; U/L, unit per liter. * >41 U/L for men and >31 U/L for women.

TABLE 2. LINKAGE DISEQUILIBRIUM MAP OF *IL-10* GENE MARKERS

	1082	592	819
1082		0.011	0.011
592	0.011		1.000
819	0.011	1.000	

–819; Table 2). The genotypes and allele frequencies for the three SNPs are shown in Table 3.

Genotype and allele frequency

We found higher frequencies of G allele and GG genotypes at position –1082 *IL-10* genes in patients (G 50%; GG 30%) compared to healthy controls (G 49%; GG 10%; Table 3). On the other hand, genotype GA was dominant in the control group (78% for controls and 40% for patients; $p=0.02$; OR=5.38 [95% CI 1.28–22.57]). After adjusting for confounders, including transfusion, tattoo, and addiction (drug and alcohol), which might influence the risk of HCV infection, there was still a significant difference between cases and controls (adjusted $p=0.02$; OR=5.38 [95% CI 1.28–22.57]).

In a recessive model analysis (GG vs. AA+GA), GG was significantly higher in patients (30%) compared to controls (10%; $p=0.04$; OR=1.06–7.81 [95% CI 1.06–7.81]). This difference remained significant even after adjustment for confounders (adjusted $p=0.02$; OR=4.66 [95% CI 1.31–16.35]).

The distribution pattern of –592 and –819 CC genotypes was not significant for either HCV patients or controls (10% vs. 11.8% respectively). In addition, TT and AA genotypes at –592, –819 loci respectively were more frequent in patients compared to controls (32% vs. 19.6%). CT and CA were more frequent in controls (58% vs. 68.6% respectively). However, these differences were not statistically significant ($p>0.05$; Table 3).

Haplotype and diplotype distribution

In the present study, haplotype and diplotype analysis in three positions of the *IL-10* gene (–592, –819, –1082) were conducted using PHASE software (Table 4). The results showed that the ATA haplotype had a higher frequency in patients compared to controls (27.3% vs. 16.7%). After adjusting for confounders, this difference remained non-significant (adjusted $p=0.061$; OR=1.87 [95% CI 0.97–3.61]). In addition, we analyzed the diplotype frequencies in patients and controls (Table 4). The ATC/GCA diplotype with a frequency of 48.1% in controls and 20% in patients was significantly different, and remained significant after adjusting for confounders (adjusted $p=0.002$; OR=0.27 [95% CI 0.11–0.63]).

HCV genotypes

Genotypes of the HCV were only available for 60% of studied patients. HCV genotype frequencies were as follows: 1a: 37.5%, 3a: 34.44%, 1a/1b: 17.24%/3.44% of patients had type 2 virus genotypes. Figure 1 shows the relationship between genotypes of hepatitis virus and some risk factors.

All participants in the present study were tested for HIV infection in which the results were negative for both patients and healthy controls. In the patients group, 42% of participants were drug addicted, while no addiction background was found in healthy controls. There was 38% alcohol addiction in the patients group compared to controls, which had only one case (2%). Twenty-eight percent of patients and 17% of controls had a history of blood transfusion. Other risk factors are listed in Table 1.

Biochemical results (ALT assay)

Biochemical test results showed an elevated level of ALT up to 33.18 ± 35.54 U/L in patients, while in controls it was

TABLE 3. DISTRIBUTION OF GENOTYPES FREQUENCY IN THREE POSITIONS OF *IL-10* GENE POLYMORPHISMS

	<i>Control</i> (n=50) N (%)	<i>Patients</i> (n=50) N (%)	<i>p-Value</i> <i>crude</i>	<i>OR crude</i> [95% CI]	<i>p-Value</i> <i>adjusted*</i>	<i>OR adjusted*</i> [95% CI]	
IL-10 locus 1082 G/A							
AA	6 (12)	15 (30)			Ref		
GG	5 (10)	15 (30)	0.80	0.83 [0.23–3.33]		0.8	0.83 [0.23–3.33]
GA	39 (78)	20 (40)	0.04	4.87 [1.64–14.49]		0.02	5.38 [1.28–22.57]
GG	5 (10)	15 (30)	0.04	2.88 [1.06–7.81]		0.02	4.66 [1.31–16.35]
AA/GA	45 (90)	35 (70)					
<i>Allele frequency</i>							
G	49 (49)	50 (50)	0.89	0.96 [0.55–1.67]	0.89	0.96 [0.55–1.67]	
A	51 (51)	50 (50)					
IL-10 locus –819 C/T(–592 C/A)							
TT(AA)	10 (19.6)	16 (32)			Ref		
CC	6 (11.8)	5 (10)	0.37	1.92 [0.46–7.99]		0.37	1.92 [0.46–7.99]
CT(CA)	35 (68.6)	29 (58)	0.17	1.93 [0.76–4.90]		0.17	1.93 [0.76–4.90]
TT(AA)	10 (19.6)	16 (35)	0.16	1.93 [0.78–4.80]		0.16	1.93 [0.78–4.80]
CC+CT(CA)	41 (80.4)	34 (68)					
<i>Allele frequency</i>							
T(A)	55 (55)	61 (61)	0.31	1.34 [0.76–2.34]	0.31	1.34 [0.76–2.34]	
C	47 (47)	39 (39)					

*Adjusted based on transfusion, tattoo, and addiction.

TABLE 4. DISTRIBUTION OF HAPLOTYPE AND DIPLTYPE FREQUENCIES IN PATIENT AND CONTROL GROUPS

Locus	Controls (n=50) N (%)	Patients (n=50) N (%)	p-Value adjusted*	OR adjusted*	95% CI
Haplotypes					
ATA	18 (16.7)	30 (27.3)	0.061	1.87	[0.97–3.61]
ATC	43 (39.8)	40 (36.4)	0.6	0.86	[0.5–1.49]
GCC	10 (9.3)	15 (13.6)	0.31	1.55	[0.66–3.61]
GCA	37 (34.3)	25 (22.7)	0.61	0.56	[0.31–1.03]
Diploypes					
ATA/ATA	1 (1.9)	5 (9.1)	0.13	5.3	[0.59–46.95]
ATA/ATC	12 (22.2)	10 (18.2)	0.60	0.78	[0.30–1.99]
ATA/GCA	4 (7.4)	10 (18.2)	0.10	2.77	[0.81–9.47]
ATC/GCC	5 (9.3)	9 (16.4)	0.27	1.91	[0.59–6.14]
ATC/GCA	26 (48.1)	11 (20)	0.002	0.27	[0.11–0.63]
GCA/GCC	5 (9.3)	4 (7.3)	0.71	0.77	[0.19–3.03]

*Adjusted based on transfusion, tattoo, and addiction.

23.66 ± 16.10 U/L. However, the difference was not statistically significant. About 76% of patients and 78% of controls had ALT levels in the normal range. Figure 2 shows the proportion of risk factors in individuals (patients and controls) who had abnormal ALT levels.

Discussion

Many studies on HCV infections have been conducted. The results of these studies indicate that genetic polymorphisms may influence hepatitis. Therefore, it is important to dissect the genetic backgrounds that may infer susceptibility to HCV infection or its outcome (4,7,16,27). Our results showed that the ATA haplotype was more common in patients. So there might be an association between this genetic variation and susceptibility to HCV infection. Nevertheless, this needs to be confirmed in a larger sample size.

Many studies with cytokine gene polymorphism have been conducted. For example, Kusumoto *et al.* studied *IL-10* gene polymorphism in HCV patients, and their results showed that at three different positions of the *IL-10* gene (–1082, –592, and –819), genotypes AA, CC, and CC were more common respectively (2). Part of our results was in agreement with

their study, and we showed that the AA genotype in the –1082 position of *IL-10* had a higher frequency in patients, but it was different for positions –592 and –819 of *IL-10*, where AA and TT genotypes were more prevalent in our patients. However, these differences were not significant.

Some studies showed similar results (4,10,21). For example, in two studies performed by Barrett *et al.* and Tso *et al.* (30) on *IL-10* gene polymorphisms at these three loci, it was found that the most common diplotype in their studied patients was GCC/GCC. In addition, Tso *et al.* found that only 5% of cases had the GCC haplotype, whereas in our patients, the frequency of the GCC haplotype was 13.6%. Yee *et al.* divided patients into two groups of virologically sustained responders and nonresponders (NR), but we did not divide our patients on such a basis. They showed that the most frequent genotypes at positions –819 and –592 were CC and CA respectively.

Bouzzgarrou *et al.* reported that among 100 patients with HCV infection, genotype GA at position –1082 had the highest frequency (43%) (5), but we did not find this in our patients. Abbas *et al.* worked with HCV patients with type 3 virus genotype and studied the relationship between three different SNPs at *IL-10* gene positions (–1082, –592, and

FIG. 1. Relationship between virus genotype and common risk factors (alcohol, drug addiction, transfusion, and tattoo) in hepatitis C virus patients.

FIG. 2. ALT levels in presence of common risk factors. ALT, alanine aminotransferase.

–819), and reported that GA in the –1082 position and AC in positions –592 and –819 were more common in their patients (1). Hence, the results were different from our study.

Our findings suggested a possible association between HCV infection and interleukin-10 polymorphisms (–1082 G>A, –819 C>T, and –592 C>A; $p<0.05$), when the results were analyzed in a recessive model (Table 2). On the other hand, the existence of the ATA haplotype with a higher frequency in HCV individuals (adjusted $p=0.061$) showed that people with this haplotype could be more at risk of HCV infection. In control groups, ATC/GCA diplotype (adjusted $p=0.002$) with a higher frequency may suggest that individuals with such genotype (diplotype) could have some degree of protection against HCV infection. The results remained significant even after adjusting for confounders (Table 3). However, a larger sample size and replication in other populations are required to confirm such association.

We acknowledge that, predictably, a notable number of patients had drug and alcohol addictions. However, we took this on board and adjusted our results for confounders. We also found that patients with history of transfusion were infected dominantly with 3a virus genotype (20.68%). In contrast, alcoholism and addiction in patients with type 1a virus were more prevalent. Considering that the required sample size was obtained using standard statistical formulas, we conducted our study on the recruited patients. However, it is suggested that future studies use a larger sample size, as this will help with better interpretation of the data and provide more reliable results.

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Author Disclosure Statement

No competing financial interests exist.

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