*Original Article*

# Analysis of interleukin-10 gene polymorphisms and hepatitis C susceptibility in Pakistan

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**Abstract**

Introduction: Hepatitis C virus (HCV) commonly causes a chronic infection but few of patients are able to clear the virus naturally. Interleukin-10 (IL-10) is an anti-inflammatory cytokine that can suppress the immune response against HCV. Interindividual variations in IL-10 production are genetically contributed by polymorphisms within the IL-10 promoter region. This study aimed to investigate the association of the IL-10 gene promoter −1082 G/A, −819 C/T, and -592 C/A polymorphisms with HCV infection susceptibility in Pakistani individuals.

Methodology: Eighty-nine chronically infected patients and 99 controls were enrolled in the study. IL-10 (−1,082 G/A, −819 C/T, −592 C/A) genotyping was performed by amplification refractory mutation system-polymerase chain reaction (ARMS-PCR).

Results: A suggestive evidence of association with hepatitis C was obtained for the IL-10 -819 C/T (-592 C/A) (p: 0.03) promoter polymorphism at the allele level but not in genotype distribution. The IL-10 -1082 allele showed no association while positive association of GG (p: 0.001) gene and negative association for GA (0.001) gene were observed. Higher frequencies were observed for GTA (p: 0.02), ACC (p: 0.01) haplotype and GCC/GTA (p: 0.005) diplotype in HCV patients than controls while diplotype GCC/ATA showed protective effect against HCV.

Conclusions: Our findings suggest that different IL-10 gene polymorphisms may lead to an imbalance between the pro-inflammatory and anti-inflammatory cytokine responses which may in turn influence the susceptibility to HCV infection.

**Key words:** IL-10; Polymorphism; HCV

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## Introduction

Hepatitis C virus is among the foremost causes of liver diseases around the globe, inflicting around 180 million people [1]. As in the rest of the world, the morbidity and mortality ratio in Pakistan for Hepatitis C is very high. About 10 million cases of HCV have been reported [2]. HCV induced chronic liver disease is characterized by persistent liver inflammation which in extreme cases progresses to liver cirrhosis and hepatocellular carcinoma (HCC) [3]. A unique and interesting statistic about this viral infection is that, out of all the cases of HCV, 60-80% of patients develop persistent chronic infection [4] while the remaining 15% can naturally clear the virus from their systems. This fact signifies that the host genetic differences could be critical in determining the course of HCV infection and that individual will respond very differently against this virus [5]. Viral RNA can be detected in serum in about 60%-80% of HCV infected patients, suggesting persistent infection; however, there is a large ratio of individuals who are HCV antibody positive who have no sign of viral RNA in their serum [6]. Even in a homologous population, a diverse range of responses can be seen against apparently related viruses. This fact shows that it is not only the virus but the interaction between the virus and the host immune system that is important in determining the course of infection [7].

Various biochemical determinants of the human body that affect HCV infection outcomes are not yet fully understood [8]. However, there is increasing evidence concerning the contribution of genetic factors to imbalance the pro-inflammatory and pro-inflammatory cytokine profile that might affect the clinical outcome and disease severity of hepatitis C [6]. Pro- and anti-inflammatory cytokine balance may modulate the benefits of antiviral therapy, thereby influencing the outcome of the disease, such as

**Table 1.** Primers used in the study and their amplicon size

| Polymorphism/ Allele location | Primer | Sequence | Product Size (bp) |
|---|---|---|---|
| -1082 | Generic Primer (antisense) | 5'-cagtgccaactgagaatttgg-3' | 258 |
| | Primer G (sense) | 5'-ctactaaggcttctttgggag-3' | |
| | Primer A (sense) | 5'-actactaaggcttctttgggaa-3' | |
| -819*/ -592* | Generic Primer(antisense) | 5'-aggatgtgttccaggctcct-3' | 233 |
| | Primer C (sense) | 5'-cccttgtacaggtgatgtaac-3' | |
| | Primer T (sense) | 5'-acccttgtacaggtgatgtaat-3' | |
| Internal Control (Human Growth Hormone) | Primer 1 | 5'-gccttcccaaccattcectta-3' | 429 |
| | Primer 2 | 5'-tcacggatttctgttgtgtttc-3' | |

(*Polymorphism at -819 and -592 are in linkage disequilibrium with each other; *i.e.*, allele C at -819 is always present when at position -592 is allele C and allele T is always present when at position -592 is allele A)

contributing to the clearance of HCV after an acute infection or to the development of rapidly progressive liver disease [9].

Interleukin 10 (IL-10), a Th2 cytokine, is one of the many cytokines that seems to play a vital role in immune response that is generated against HCV. It shifts the Th1/Th2 balance by down regulating the Th1 responses and by suppression of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF α) and interferon gamma (IFN γ) secretion [10]. Studies have shown that cytokine gene polymorphism plays an important role in the natural clearance of HCV. Most of these polymorphisms are in the cytokine gene regulatory regions and are consequently involved directly in controlling the transcription rates of these genes [11]. There is strong evidence of genetic contribution to IL-10 production in viral clearance [12]. IL-10 possesses a highly polymorphic promoter with variations at -1082, -819 and -592 that have been extensively studied and implicated in altering the rates of IL-10 gene transcription. The -1082G, -819C and -592C (GCC) alleles have been associated with elevated levels of IL-10 production [13] while ACC and ATA haplotypes exhibit intermediate and low IL-10 gene transcription respectively [14]. We have investigated the above-mentioned three single nucleotide polymorphisms (SNP) of the IL-10 promoter in the Pakistani population to determine if they play any role in the incidence of HCV infection in Pakistan.

## Methodology

*Patients and controls*

To determine the association between IL-10 promoter polymorphisms and HCV infection, blood samples were collected from 100 ELISA positive hepatitis C patients from the NUST Center of Virology and Immunology (NCVI) Diagnostic Lab, Rawalpindi, and stored in EDTA-coated tubes until used. All the samples were subjected to polymerase chain reaction (PCR) to confirm HCV infection. Out of the 100 samples obtained, 89 were PCR positive and 11 were PCR negative. Only the 89 patients who tested PCR positive were included for further study (mean age ± SD: 40.2 ± 14.3 yr). Among these patients 58 were males (mean age ± SD: 43.4 ± 14.5 yr) and 31 were females (mean age ± SD: 35.7 ± 16.8 yr). The control group consisted of 99 healthy subjects (mean age ± SD: 38.4 ± 13.8 yr), none of whom had any history of hepatitis C infection. There were60 males (mean age ± SD: 40. 9 ± 12.3 yr) and 39 females (mean age ± SD: 32.0 ± 15.1 yr) in the healthy group. Patient and control populations were of the same ethnicity and from the same geographical area. The study was approved by the Ethical Committee of NCVI, Islamabad, Pakistan, and written consent was obtained from each participant.

*DNA extraction*

Genomic DNA from venous blood samples of both patients and healthy control subjects were extracted using a genomic DNA extraction kit (Puregene Blood Kit, Gentra, Valencia, USA) according to the manufacturer's protocol. DNA quantification was done using an Eppendorf Bio Photometer (New York, USA). DNA was stored at -20° C.

*Genetic analysis*

The amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) method was used for IL-10 promoter polymorphism genotyping as described by Perrey *et al.* [15]. For each polymorphism, two separate reactions were performed, each of which contained one of the two alleles specific for forward primers and a generic anti-sense primer (Table 1). PCR amplification was

**Table 2.** Relationship between IL-10 Polymorphic Genes, Alleles and HCV

| IL-10 locus | Control (n = 99) | Frequency (%) | Patients (n = 89 ) | Frequency (%) | P value |
|---|---|---|---|---|---|
| -1082 G/A | | | | | |
| G/G (high) | 3 | 3.03 | 15 | 16.9 | 0.001 |
| G/A (Intermediate) | 92 | 92.93 | 67 | 75.2 | 0.001 |
| A/A (Low) | 4 | 4.04 | 7 | 7.9 | NS |
| Allele Frequency | | | | | |
| G (High) | 98 | 49.49 | 97 | 54.5 | NS |
| A (Low) | 100 | 50.51 | 81 | 45.5 | |
| 819C/T (592C/A) | | | | | |
| T/T (A/A) | 15 | 15.15 | 16 | 18 | NS |
| C/T (C/A) | 81 | 81.82 | 66 | 74.1 | NS |
| C/C (C/C) | 3 | 3.03 | 7 | 7.9 | NS |
| Allele frequency | | | | | |
| T(A) | 111 | 56.06 | 80 | 44.9 | 0.031 |
| C(C) | 87 | 43.94 | 98 | 55.1 | |

performed in a 20 µl reaction volume containing 40 ng genomic DNA, 1.5 mM dNTPs, 25 mM $MgCl_2$, 1 µl of 10 pmol each primer and 0.4 units of Taq polymerase (Fermentas, Maryland, USA) in 1X Reaction Buffer with cycling conditions as follows: 95°C for 3 minutes, followed by 35 cycles at 95°C for 45 seconds, 58°C for 40 seconds, 72°C for 1 minute and finally a 7 minute extension at 72°C. To ensure PCR success, an internal control region was amplified from the human growth hormone (Details of primers and amplicon are shown in table 1). The amplified products were analyzed on 2% agarose gel.

*Statistical analysis*

Statistical analysis was performed by Study Result Software Version 1.0.4 (CreoStat HB Frolunda, Sweden). The distribution of cytokine gene polymorphisms between HCV patients and healthy controls were compared by the Fischer's exact test or $\chi^2$. P-values smaller than 0.05 were considered significant.

## Results

We found a higher frequency of GG genotype at IL-10-1082 in HCV patients than in the healthy controls (17% versus 3%). Thus a significant susceptible association was found between HCV and the −1082 GG genotype (P = 0.001), whereas IL-10-1082 genotype GA was more common in controls than in HCV patients (93% versus 75%) suggesting a possible association with the protection to HCV infection (P = 0.001). It looks as though the IL-10-1082 AA genotype occurs more frequently in patients than in controls but the difference was not statistically significant. The distribution pattern of -819 TT and CT polymorphic genotypes was not significantly different between the two study groups. The -819 CC genotype was more prevalent in patients as compared to the healthy group, but like IL-10-1082 AA, the difference was not statistically significant (−819 and −592 are in linkage disequilibrium; Table II). Our results suggest a lack of association of IL-10-1082 G and A alleles with HCV, but a significantly different distribution (P = 0.031) of −819 C and T allele polymorphism (−819 and −592 are in linkage disequilibrium) was observed between the two groups (Table II).

In this study, GTA and ACC haplotypes of IL-10 were more prevalent in HCV infected individuals than in the normal controls (9% versus 17% and 2% versus 7.3% respectively). Thus a significant positive association was found between HCV and the GTA and ACC haplotypes (P = 0.024 and 0.014 respectively). In the diplotypes (Haplotype Zygosity) distribution, the frequency of the GCC/GTA diplotypes was higher in the HCV patients when compared with the healthy controls (2% versus 12.4 %), and significant difference was observed between HCV and controls (P = 0.005). The GCC/ATA diplotypes were lower in HCV patients as compared to those in the healthy controls (76% versus 55%) and a significant difference (P = 0.003) was observed (Table 3). There was no significant association found in GTA/GTA, GCC/ACC, ACC/ATA diplotypes in controls and individuals with HCV. The results of the current study show an absence of GCC/GCC, GTA/ACC, ACC/ACC and ATA/ATA haplotypes in our local Pakistani population (Table 3).

**Table 3.** Relationship between IL-10 Haplotypes, Diplotypes and HCV

| IL-10 locus | Control (n = 99) | Frequency (%) | Patients (n = 89 ) | Frequency (%) | P value |
|---|---|---|---|---|---|
| Haplotypes | | | | | |
| GCC (high) | 83 | 41.92 | 67 | 37.6 | NS |
| GTA (high) | 18 | 9.09 | 30 | 16.9 | 0.02 |
| ACC (intermediate) | 4 | 2.02 | 13 | 7.3 | 0.01 |
| ATA (low) | 93 | 46.97 | 68 | 38.2 | NS |
| Diplotypes | | | | | |
| GCC/GCC (high) | - | - | - | - | - |
| GCC/GTA (high) | 2 | 2.02 | 11 | 12.4 | 0.005 |
| GTA/GTA (high) | 1 | 1.01 | 4 | 4.5 | NS |
| GCC/ACC (intermediate) | 3 | 3.03 | 7 | 7.9 | NS |
| GCC/ATA (intermediate) | 75 | 75.76 | 49 | 55.1 | 0.003 |
| GTA/ACC (intermediate) | - | - | - | - | - |
| GTA/ATA (intermediate) | 14 | 14.14 | 11 | 12.4 | NS |
| ACC/ACC (low) | - | - | - | - | - |
| ACC/ATA (low) | 4 | 4.04 | 6 | 6.7 | NS |
| ATA/ATA (low) | - | - | 1 | 1.1 | NS |

## Discussion

Generally, HCV infected individuals suffer from chronic liver disease with different severity, ranging minimally from hepatocytic lesions to conditions as severe as liver cirrhosis and hepatocellular carcinoma (HCC) [16]. Mounting evidence, however, suggests the crucial role of host immune responses and host genetic background in HCV infection pathogenesis and inter-individual heterogeneity of the disease outcome [6]. Cytokine production varies among individuals and this disparity is associated with certain SNPs in the coding as well as the regulatory regions of individual cytokine genes [17]. The cytokine IL-10, which is produced by monocytes and lymphocytes 18], plays a significant role in HCV pathogenesis [19], and its expression is thought to be genetically controlled [14]. Therefore, being a key player in the differential expression of this cytokine, IL-10 polymorphism determination may be crucial for predicting the probability of disease occurrence and disease progression. In this study, we have investigated the significance of IL-10 gene promoter polymorphism at 1082 G/A, 819 C/T and -592 C/A (592 C/A is in linkage disequilibrium with 819 C/T [15] and the susceptibility of HCV in Pakistani population.

In different studies around the world, analysis of IL-10 polymorphism and its association with HCV susceptibility has produced ambiguous results [6,20,21,22,23,24]. We found no significant association between IL-10 alleles *(1082 A/G, -819 C/T,* and *-592 A/C*), and HCV infection, but -819 C

was more prevalent in HCV infected people as compared to the healthy control individuals in our investigation (Table 2). However, further analysis of genotypes has shown that individuals with *-1082GG* (homozygous *G* allele) are more susceptible to HCV infection as compared to -1082 GA genotype (heterozygous). This observation was also noted in another study conducted by Reuses *et al*. which demonstrated that the IL-10-1082 GG allele produces higher levels of this cytokine which may compromise the cellular immune response to the virus [11]. Previous investigations have shown that IL-10 -1082 AG polymorphism was associated with higher HCV infection rates in the USA [20], but not in the Japan [21], Tunisia [6], Italy [22], China [23] and the Caucasian population [24]. In accordance with our results, the 1082 GA genotype was shown to be significantly higher in healthy subjects from China [23] and from Italy [25].

Investigation of IL-10-819C/T polymorphism and its association with HCV susceptibility has led us to the same conclusions as those reported by Dogra Gaurav and Chakravarti Anita (2009) from India [11]. Previous reports regarding IL-10 polymorphism association with HCV, which were based on comparison of persistence of HCV infection and individuals who self-cleared HCV, showed that IL-10-1082 GG was associated with persistent infection in African-American [5] and Caucasian [26] patients, while European-American [5] and Japanese [27] patients showed no such association. In our results, IL-10-592 A and IL-10-1082 GA were high in the control group. Similarly, another study showed that IL-10-592 A and IL-10 -1082 GA were high in self-limiting recovered HCV patients in a Caucasian population [26].

Significant variations were observed in IL-10 haplotypes GTA and ACC between patients and controls. When we analyzed the diplotypes, individuals with *IL-10/GCC: GTA* diplotype were genetically predisposed to develop HCV. *IL-10/ GTA: ATA* diplotype were more common in controls than in patients so individuals with this genetic makeup are less likely to develop HCV. Previous investigations have shown that *IL-10/GCC: GCC* or haplotypes having the G allele correlate with higher levels of IL-10 production after stimulation, which leads to the suppression of IFN-α and hence favors the development of HCV [14,28]. Contradictory to our results, IL-10 haplotypes and diplotypes were not distributed differently between healthy controls and HCV cases in different reports from Japan, Caucasian and Italy, the results may be differ due to different ethnicity of studied populations [24,26,27,29,30].

There are two previous reports from Pakistan regarding IL-10 polymorphism as a marker in HCV infection, [31,32]. These studies consisted of a small number of patients (51 and 40 respectively) and did not include healthy controls. Analogous to our results, the frequency of IL-10-1082 G/A and -819 C/T heterozygous genotypes was the highest among HCV patients. In the investigation by Abbas and Moatter [31] regarding the effect of cytokine gene polymorphism on the histological activity index in HCV infected individuals, patients with the homozygous G allele at IL-10-1082 were seen to be more prone to necro-inflammatory activity while patients with IL-10-819 TT were associated with a less severe form of liver fibrosis. According to our results, IL-10-1082 GG individuals were also more susceptible to disease while IL-10-819 TT prevalence was the same in both the diseased group and in healthy subjects.

Conflicting results between polymorphism association studies can be a reflection of one or several factors, such as sample size differences, subjects' selection for the study, genetic heterogeneity of various populations, and different gene-gene or gene-environment interactions. An alternative explanation for the discrepancies in results could be the consequence of different cultural backgrounds. As reported by Reuss *et al*., smoking and a decreasing body mass index appear to decrease IL-10 production [12].

In summary, while some of our results are in agreement with those of earlier publications, other observations from our study are contrary to previous findings on IL-10 promoter polymorphism and HCV infection in other populations. It is difficult to ascertain the magnitude of the effect of genetic polymorphisms on disease susceptibility or protection because of the existence of IL-10 homologues and different IL-10 binding receptors [33] which likely complicate the determination of levels of IL-10 expression *in vitro*. These interactions could affect the correlation between IL-10 promoter polymorphisms and the outcome of HCV infection. Further research on the functional implications of IL-10, specifically in relation to the immune response to HCV, is clearly warranted.

It could be concluded that SNPs in IL-10 may contribute to the susceptibility of HCV in the Pakistani population. Our results from this genetic polymorphism analysis study on IL-10 promoters

indicated that the distribution pattern of IL-10 polymorphism was significantly different between the control group and HCV patients. However, at present, these markers are of little clinical importance due to the small number of samples. Since this study is preliminary and based on a small sample size, we believe that our findings may stimulate further investigations on a larger scale to assess the association of these polymorphisms in HCV infected patients.

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