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Impact of IL-10 (-1082) Promoter–Single Nucleotide Polymorphism on the Outcome of Hepatitis C Virus Genotype 4 Infection

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ABSTRACT: Immunoregulatory cytokines may influence the hepatitis C virus (HCV) infection outcome. This study aimed to determine the genotypic and allelic frequencies of the interleukin (IL)-10 (-1082) G/A polymorphism, and its association with chronicity or resolution of HCV genotype 4 infection in Egypt. The frequencies of different dimorphic polymorphisms based on single nucleotide substitution in chronic HCV patients (50) and resolved HCV patients (50) were: IL-10 (-1082) G/G 22 (44%) and 18 (36%), G/A 19 (38%) and 24 (48%), and A/A 9 (18%), and 8 (16%), respectively. In the sustained virologic response (SVR) (36) and spontaneously resolved subjects (14) groups, the frequencies were: IL-10 (-1082) G/G 11 (30.6%) and 7 (50%) G/A 18 (50%) and 6 (42.9%), A/A 7 (19.4%) and 1 (7.1%), respectively. An association between male gender and chronic hepatitis C outcome (*P* value 0.041) was found. However, no significant gender difference was found when we compared females versus males with elevated alanine transaminase (ALT) levels in the chronic HCV patient group (*P* value = 1).

CONCLUSION: No significant difference in the frequency of IL-10 single nucleotide polymorphism (SNP) at position 1082 was found between chronic and resolved HCV subjects.

KEYWORDS: interleukin-10, single nucleotide polymorphism, chronic hepatitis C, resolved hepatitis C, HCV genotype 4

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Introduction

Hepatitis C virus (HCV) is the most common cause of acute and chronic liver disease worldwide. Infection with the HCV is characterized by a broad spectrum of possible outcomes. Infection is self-limited in a fortunate minority, while the majority of patients develop persistent (chronic) infection.[1] Similarly, the outcome of interferon-based anti-HCV therapies varies, with only about 15% achieving sustained virologic remission with interferon monotherapy[2] and 40% achieving sustained response rates with combination therapy including ribavirin.[3] Cytokines play a key role in the modulation of immune responses. Two distinct patterns of cytokines production may occur.[4] Type 1 responses are characterized by the production of interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ), which are prime, maintain antigen-specific cellular immunity,[5,6] and are important in defense against viruses. Type 2 responses are characterized by IL-4, IL-5, and IL-10, which promote humoral immune responses. An imbalance in helper T-cell type 1 (Th1) and type 2 (Th2) cytokines is suggested to play an important role in the pathogenesis of chronic hepatitis C.[7] IL-10 is an immunoregulatory cytokine produced by T-helper 2 (Th2) cells, monocytes/macrophages, and regulatory T cells. IL-10 is also produced by various cell types in the liver, including hepatocytes, sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells, and liver-associated lymphocytes. It has anti-inflammatory activity through the inhibition of the synthesis of cytokines such as IL-6, IL-8, IL-12, and TNF-α by

activated macrophage and interferon γ by T cells.[8] Intrahepatic IFN-γ and IL-2 mRNA expression is upregulated in chronic hepatitis C, while the expression of IL-10, a Th2-like cytokine, is downregulated.[7] Moreover, IL-10 may influence HCV infection susceptibility as well as spontaneous and treatment-induced HCV eradication.[9] Its production is regulated at the transcriptional, posttranscriptional, and translational levels. In patients chronically infected with HCV, the production of inappropriate amounts of cytokines, such as IL-10, were reported to be associated with HCV clearance, fibrogenesis, and even resistance to interferon therapy.[10,11]

However, there is compelling evidence that IL-10 plays a role in HCV disease pathogenesis; contradictory reports exist as to the exact effect of the IL-10 promoter polymorphisms on the natural outcome of HCV infection. We set out to analyze the role of IL-10 (-1082) promoter polymorphism in HCV infection outcome.

Methods

Patients. A total of 100 HCV patients were enrolled in the study, with ages ranging from 30–65 years; 86 of the participants were males and 14 were females. Patients were grouped according to the outcome of HCV infection as follows: (1) 50 HCV RNA^+ (chronic non-responder HCV) patients, having circulating HCV RNA detected by Toyobo RNA-direct real-time PCR on light cycler-LG Lifescience, Korea, and (2) 50 HCV RNA^- (resolved HCV) cases, that were divided into (i) 36 HCV RNA^- sustained responder patients who had a negative HCV RNA six months after completing 48 weeks therapy. Patients were treated by either pegylated or short-acting interferon along with ribavirin,[12] (ii) 14 HCV RNA^- spontaneously resolved patients (self-limiting HCV infection), patients had at least three negative HCV RNA PCR, six months apart with positive HCV-Ab on many occasions.[13] The resolved HCV cases were identified as being negative for circulating HCV RNA and positive for the confirmatory test for the presence of anti-HCV antibodies by use of enzyme linked immunosorbent assay (ELISA) 3rd generation (CTK-Biotech, USA) according to the manufacturer's protocols. The participants were recruited from the Gastroenterology and Hepatology Clinic at the National Research Service Unit over an 18-month period. All patients gave their written, informed consent to participate in the research protocol, which had been approved by the National Research Centre Ethical Committee. All enrolled participants were subjected to full history taking, clinical examination, and abdominal ultrasonography, and information about the state of HCV infection, type of treatment, its duration, and response were recorded. All cases infected with human immunodeficiency virus (HIV), hepatitis B virus (HBV), and schistosoma were excluded from the start of the study. All chronic HCV participants with immune hepatitis were also excluded.

Laboratory investigations. Hepatitis B surface antigen (HBsAg), hepatitis B core antibody (HBcAb), HBeAg, HIV, and schistosoma IgG were detected by commercially available ELISA kits to exclude the positive cases. Anti-nuclear antibody (ANA), anti-smooth muscle antibody (ASMA), anti-mitochondrial (AMA), and liver-kidney microsomal antibody (LKM) were also measured to exclude immune hepatitis.

The presence of HCV antibodies was determined by third-generation ELISA (CTK-Biotech, USA). Liver function tests including alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and albumin were assayed using Olympus autoanalyser AU400 (Olympus Diagnostica, Japan).

Detection of HCV RNA by real-time PCR and HCV genotyping. Viral RNA was extracted from patient's plasma using the QIAamp Viral RNA Kit (Qiagen Hilden, Germany, Cat no. 52904) according to the manufacturer's protocol. For detection of the presence of hepatitis C viral particles, HCV RNA was detected by commercially available Toyobo RNA-direct real-time PCR kit on SLAN Real-Time PCR Detection System, LG Lifescience, Korea.

For genotyping of HCV, a reverse line probe assay, GEN-C Reverse Hybridization Strip Assay (NLM diagnostic, Cat. no. AC004, Italy) was used, in which amplicons obtained through RT-PCR of HCV RNA 5'-UTR region isolated from the patient's plasma and a volume of denaturing solution equal to that of the amplicon to be used for typing (30 μL) were mixed with hybridization buffer prewarmed to 45°C. After the addition of the conjugate solution and color developer, all positive lines for each strip were read on the decoder table to determine the corresponding HCV genotype.

TaqMan genotyping assay of IL-10 gene promoter at -1082. Prior to the real-time PCR, genomic DNA was extracted from peripheral blood mononuclear cells using the QIAamp DNA minikit (Qiagen, Germany) according to the manufacturer's instructions. Real-time PCR allelic discrimination assay was designed using Taq-Man SNP Genotyping assays (Applied Biosystems, Foster City, CA) to perform genotyping of the G→A -1082 (dbSNP ID: rs1800896, TaqMan SNP Genotyping Assays ID: C_1747360_10) SNP in the IL-10 promoter region. SNP in the IL-10 promoter region utilized the primers and two TaqMan® minor groove binder (MGB) probes, a modification that increases the melting temperature (T_m) producing robust allelic discrimination. One of the probes was labeled with the reporter dye VIC and the other was labeled with the reporter dye FAM. Briefly, an amount of 20 ng DNA was mixed with Allelic Discrimination Assay Mix (900 nM each forward and reverse primer, 200 nM each reporter dye FAM or VIC-labeled probe) and TaqMan Universal PCR Master Mix (Applied Biosystems, CA). PCRs were carried out in a total volume of 25 μL/well in 48-well PCR plates using StepOne™ Real-Time PCR System (Applied Biosystems, CA). The PCR amplifications were subjected to the following cycling parameters: 95°C for 10 minutes to activate the AmpliTaq Gold Enzyme, followed

by 40 cycles of 92°C for 15 seconds for denaturation and 60°C for 1.5 minutes for annealing and extension. Genotypes were assessed by the TaqMan allele-specific assay method using the ABI Prism® 7500 Sequence Detection System software, according to the manufacturer's protocols (Applied Biosystems).

Statistical methods. Data was analyzed using Statistical Package for Social Science software computer program version 16 (SPSS, Inc., Chicago, IL, USA). Quantitative data was presented in mean and standard deviation, while qualitative data was presented in number and percentage. Analysis of variance (ANOVA) and least significant difference (LSD) were used for comparing quantitative means of the three groups. Kruskal–Wallis test and Mann Whitney U test were used to compare means of the skewness data. Chi-square "χ^2" or Fischer's exact tests, as indicated, were used to compare the qualitative data. *P* value less than 0.05 was considered statistically significant.

Results

The HIV, HBsAg, HBcAb, HBeAg, and schistosoma IgG of all the included cases were negative. ANA, ASMA, AMA, and LKM were also negative. All cases were HCV genotype 4.

100 HCV patients were successfully genotyped for IL-10 single polymorphism at position -1082. The frequencies of dimorphic polymorphisms in HCV persistent infection and viral clearance groups and in those with normal and abnormal ALT levels are shown in Table 1. None of the IL-10 single polymorphisms at position –1082, were differentially distributed between the chronic (persistent infection) HCV patient and resolved (HCV clearance) patient groups (P = 0.414, OR = 0.72; 95% CI: 0.32–1.59).

Taq-Man real-time PCR was used for allelic discrimination assay (Fig. 1a and b). The frequency distribution of IL-10 (-1082) A/G alleles among chronic HCV and resolved HCV patients were 63% and 60% for G allele, but were 37% and 40% for A allele, respectively. The IL-10 (-1082) A/G allele frequencies were also not significantly different between the two groups (P value = 0.663, OR = 0.88; 95% CI: 0.50–1.56).

Table 1. Differential distribution of IL-10 (-1082) genotypes among persistently infected (HCV RNA$^+$) and resolved (HCV RNA$^-$) patient groups; and in HCV patients with normal and abnormal ALT levels.

HCV PATIENT GROUP (*n*)	GG *n* (%)	GA *n* (%)	AA *n* (%)	*P* VALUE
HCV RNA$^+$ (50)	22 (44%)	19 (38%)	9 (18%)	0.414
HCV RNA$^-$ (50)	18 (36%)	24 (48%)	8 (16%)	
Abnormal ALT (21)	7 (33.3%)	10 (47.6%)	4 (19%)	0.782
Normal ALT (79)	33 (41.8%)	33 (41.8%)	13 (16.5%)	

Moreover, genotypic and allelic differential distribution among chronic HCV patients, sustained responders, and spontaneously resolved patients were not significantly different (Table 2). The odds ratio for IL-10 (-1082) AA homozygous versus -1082 GG homozygous in chronic HCV, sustained responders, and spontaneously resolved groups were 0.64 (0.19–2.19), 0.528 (0.200–1.391), and 2.86 (0.31–26.75), respectively. While the OR for each of IL-10 (-1082) G/A alleles among chronic HCV, sustained responders, and spontaneously resolved groups were 0.73 (0.40–1.36), 1.468 (0.59–3.67), and 2.000 (0.78–5.13), respectively.

Male gender was significantly more predominant in the chronic group (P = 0.041). The odds ratio for ALT measurement abnormality among chronic HCV, sustained responders, and spontaneously resolved groups were 1.308 (0.458–3.739), 1.895 (0.371–9.686), and 1.448 (0.262–8.003), respectively.

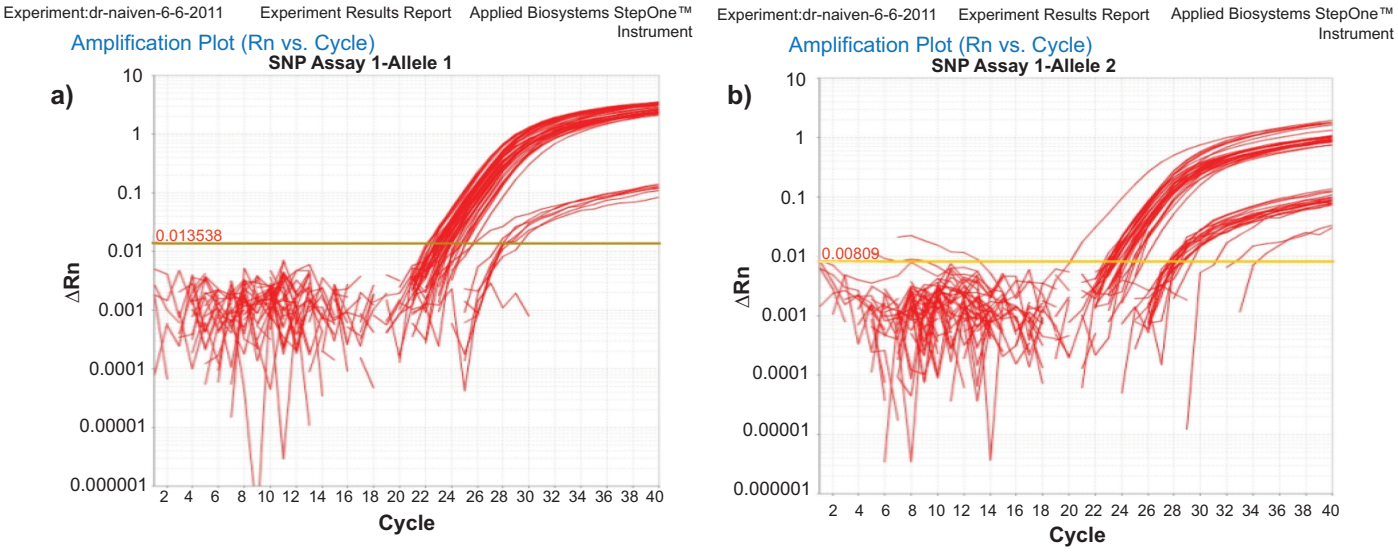

Figure 1. (**a, b**) Amplification curves showing each allele (allele 1(G) and allele 2 (A)) separately in real-time PCR.

Table 2. Genotypic and allelic frequencies among chronic HCV, sustained responders, and spontaneously resolved patients.

GENOTYPES & ALLELES	CHRONIC HCV GROUP (50)	SUSTAINED RESPONDERS (36)	SPONTANEOUSLY RESOLVED (14)	*P* VALUE
GG	22 (44%)	11 (30.6%)	7 (50%)	0.556
GA	19 (38%)	18 (50%)	6 (42.9%)	
AA	9 (18%)	7 (19.4%)	1 (7.1%)	
G	63 (63%)	40 (55.6%)	20 (71.4%)	0.311
A	37 (37%)	32 (44.4%)	8 (28.6%)	

Upon evaluating IL-10 (-1082) GG genotype versus non-GG genotype (Fig. 2), we found that the IL-10 genotype frequencies showed no significant differences between males and females, neither within the three studied groups ($P = 0.358$) nor within the chronic HCV group when studied alone ($P = 0.667$). Additionally, we found no significant gender differences when we evaluated females versus males with elevated ALT levels in the chronic HCV patient group (P value = 1).

Discussion

Host immune factors are important in the outcome of HCV infection.[14] While multiple studies have reported the association of IL-10 and TNF-α gene promoter polymorphisms with the natural course of HCV infection,[15–18] these results remain controversial as many of these studies have used relatively small subject numbers in regionally disparate areas. IL-10, whose secretion is regulated by monocytes and lymphocytes,[19,20] plays an important role in the pathogenesis of HCV infection.[7,21] In our study, no significant difference in the genotype profile of IL-10 (-1082) was detected between the chronic HCV (persistent infection) and resolved HCV (HCV clearance) patient groups (P values 0.414 and 0.556). Similarly, Kusumoto and collegues found no significant association between polymorphism in the IL-10 (-1082) and HCV clearance or even the severity of hepatitis.[22] In 2012, Pasha et al, found that there was no association between IL-10 gene polymorphism and susceptibility to HCV infection and response to treatment.[23] Moreover, two additional studies, examining 606[24] and 259[25] subjects could not confirm any association. In accordance with our results, a meta analysis identifying studies that examined the association of IL-10 polymorphism and HCV infection

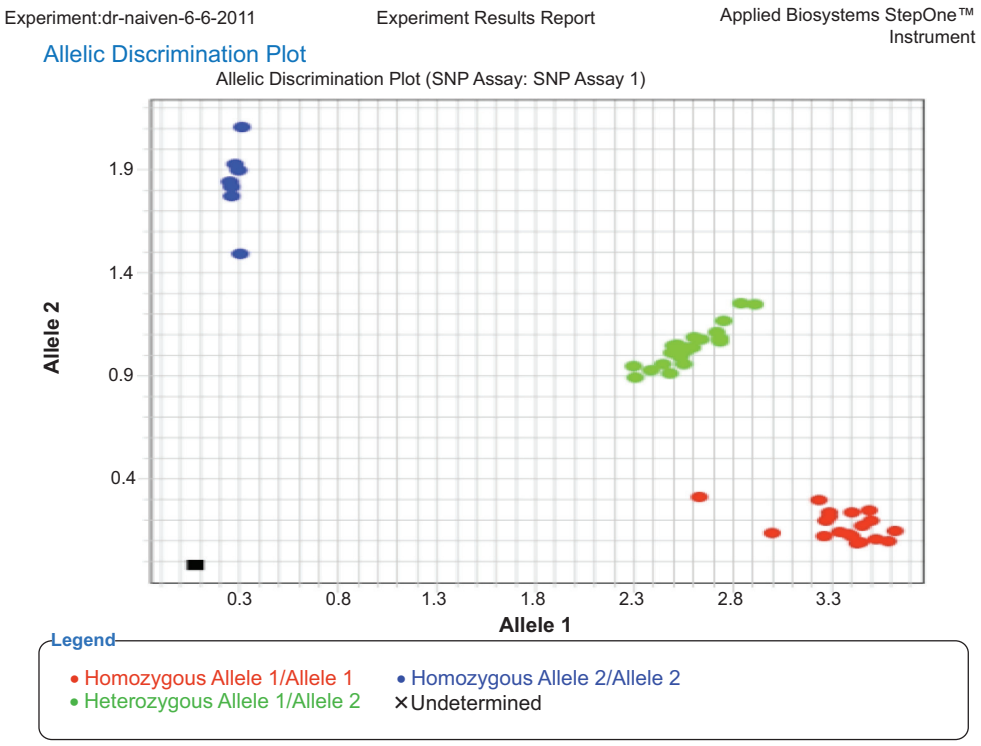

Figure 2. Allelic discrimination plot for G/A alleles revealing genotype plotting on the graph, blue dots represented the A/A genotype near the *X*-axis, G/G genotype was plotted near the Y-axis as red dots, while G/A genotype was plotted as green dots in the middle between the two axes.

revealed no association between polymorphism in the IL-10 (-1082) and HCV infection outcome.[26] On the contrary, the study by Knapp and colleagues revealed an association of the G/G genotype at position 1082 with persistent infection; a study of 659 subjects examining the relationship between IL-10 polymorphisms and viral clearance.[27] The association of the IL-10 (-1082) GG genotype with persistent infection was confirmed in the study by Vidigal and colleagues, though this association was based on a comparison with healthy controls rather than patients with self-limiting infection.[28]

An association between male gender and chronic hepatitis C outcome was found in this study (*P* value 0.041) that is consistent with the known natural history of hepatitis C.[29] Males have a 10-times more rapid progression in HCV severity than females, regardless of age.[30] Although gender affects the hepatitis C outcome, we found no significant difference in genotype frequencies and gender. Additionally, we found no significant gender differences when we evaluated females with elevated ALT versus males with elevated ALT levels in the chronic HCV patient group (*P* value = 1). Conversely, Natalia Paladino and colleagues demonstrated an increase in the GG frequency as well as a decrease of the GA frequency in female HCV patients, especially, in those patients with elevated levels of ALT.[31] Moreover, we observed that IL-10 polymorphism did not contribute to ALT abnormalities and it was not differently distributed between the persistently normal and abnormal ALT groups (*P* value 0.782). Our results are in line with similar findings reported by Kusumoto and colleagues.[22]

A vigorous $CD4^+$ and $CD8^+$ T-cell response with a predominant Th1 cytokine profile seems to be responsible for recovery from an HCV infection.[32] Conversely, patients who develop a chronic infection show a predominant Th2 response that downregulates the Th1 response and therefore favors persistent HCV infection.[33] IL-10 is a cytokine that downregulates the proinflammatory response and has a modulatory effect on hepatic fibrogenesis and is a potent anti-inflammatory Th2 cytokine that downregulates the expression of major histocompatibility complex (MHC) class I and class II molecules, as well as the production of Th1 cytokines.[34–36] Moreover, chronically HCV-infected patients who received a short treatment with recombinant IL-10 showed decreased hepatic inflammation and reduced liver fibrosis.[37] On the other hand, a 12-month IL-10 therapy in patients with advanced fibrosis led to increased levels of serum HCV RNA and a reduction in fibrosis score,[38] suggesting that high levels of IL-10 not only decrease fibrogenesis but also lead to an increased HCV viral burden. This could be achieved by decreasing the number of HCV-specific $CD4^+$ and $CD8^+$ gamma interferon-secreting T cells and polarizing the immune response towards a Th2-dominant profile. IL-10 levels differ widely between individuals, possibly because of polymorphisms in the promoter region of the IL-10 gene.[39,40]

In our study, the IL-10 (-1082) A/G allele frequencies were also not significantly different between persistently infected and resolved HCV patient groups (*P* values 0.663 and 0.311) respectively. Notwithstanding, Gao and colleagues found that -1082 G allele was associated with a reduced risk of HCV RNA replication while -1082 A allele was associated with an increased risk.[41] Conflicting results between different studies can be a reflection of one or several factors, such as differences in sample size, genetic heterogeneity of various populations and different gene–gene or gene–environment interactions. Underlying differences in ethnicity of patients investigated for the various studies are also likely to affect the observed differences in findings between studies, which makes comparisons very difficult.

However, it had been published that the antibody-induced blockage of the IL-10 receptor generates a favorable balance of $CD4^+$ T-cell response to HCV.[42] As this anti-IL-10 receptor reverses the inhibitory effect of IL-10 on HCV-specific T-cell proliferation.

In conclusion, the relationship between inherited variations in IL-10 expression at -1082 was not associated with alterations in HCV clearance or ALT levels in this study.

Considering that HCV infection is a complex disease, the heterogeneity of disease may cause research results to be inconsistent, and the statistical power of small sample associated analysis is too low to detect minor genes. Therefore, there is a need for larger studies to suggest any role of cytokine gene polymorphisms in HCV outcome and in order to orient molecular epidemiological research of IL-10 polymorphisms sites and HCV infection susceptibility in the future.

Author Contributions

SFH and HEG conceived and designed the experiments. HEG, EHT, and MAY analyzed the data. EHT and NAH wrote the first draft of the manuscript. EHT contributed to the writing of the manuscript. SFH, HEG, EHT, MAY, and NAH agree with manuscript results and conclusions. SFH, HEG, EHT, MAY, and NAH jointly developed the structure and arguments for the paper. All authors reviewed and approved of the final manuscript.

DISCLOSURES AND ETHICS

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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