

Endogenous IFN γ in chronic HCV genotype 4 patients treated with PEG-IFN α and ribavirin

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Abstract

Introduction: Hepatitis C virus (HCV) infections remain an increasingly prevalent and emergent health problem worldwide, causing a wide spectrum of liver diseases. Combination therapy with pegylated interferon (PEG-IFN) of peginterferon alfa-2a and oral ribavirin is currently recognized as the standard treatment of chronic HCV infection. Several complex immunological mechanisms are involved during the course of HCV treatment using interferons. The role of endogenous interferon gamma (IFN γ) in Egyptian patients infected with chronic HCV and treated with PEG-IFN/ribavirin is uncertain. The goal of this study was to evaluate the association of IFN γ and chronic HCV infection among patients treated with combination therapy of PEG-IFN/ribavirin.

Methodology: Samples from 20 patients infected with HCV genotype-4 (HCV-4) and 20 non-infected individuals as healthy controls were used in this retrospective study. IFN γ levels in peripheral blood monocytes were analyzed, along with liver enzyme alanine aminotransferase (ALT) levels, and single nucleotide polymorphism (SNP) of the myxovirus resistance-A (*MxA*) gene.

Results: The results showed that an increase of IFN γ and a decrease of ALT levels in chronic HCV-infected patients after 12 weeks of treatment with combination therapy.

Conclusion: Enhanced IFN γ secretion and decreased liver enzyme ALT production are indicative of HCV clearance and improvement of liver function. In addition, the SNP of the *MxA* gene is an important host genetic factor that independently influenced the response to IFN α in patients with chronic HCV infection, especially in those with a low viral load.

Key words: hepatitis C; interferon γ ; interferon α ; *MxA*; SNP

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Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease in humans. HCV is an enveloped single-stranded RNA virus with a genome of approximately 9.6 kb and consists of a single open reading frame encoding a polyprotein and non-translated regions (NTRs) located at the 5' and 3' termini [1]. HCV is the only species member of the genus *Hepacivirus* within the family *Flaviviridae* [2]. HCV is classified into eleven major genotypes (designated 1-11), many subtypes (designated a, b, c, etc.), and about 100 different strains based on the genomic sequence heterogeneity [3]. The lowest sequence variability between genotypes is found in the NTR leader sequence (5'UTR), where specific sequences and RNA secondary structures are required

for replication and translation. HCV genotypes have been further classified into more than 80 subtypes [4]. HCV is a blood-borne infection reported to have chronically infected approximately 170 million people [5]. More than 350,000 people die annually from hepatitis C-related liver diseases worldwide [2]. There are more cases of HCV and endemic chronic HCV infection in Egypt than in any other country worldwide [6]. The highest prevalence rate worldwide has been reported in Egypt (15%), where HCV genotype-4 represented 90% of all HCV cases [5,7].

The discovery of pathogen-associated molecular patterns (PAMPs) that are recognized by specific toll-like receptors (TLRs) has dramatically advanced the understanding of innate host responses to viral infection [8]. Interferons (IFNs) are divided into type I

and type II IFNs, where type I represent all types of IFNs; IFN γ is type II. Production of type I IFNs and other cytokines (including IL-12, IL-15, and IL-18) from hepatocytes activates natural killer (NK) cells and induces IFN γ production from these cells [8]. Type I IFNs activate the expression of hundreds of IFN-stimulated genes (ISGs) that also have antiviral activity [9]. The best characterized type I IFNs include the RNA-dependent protein kinase (PKR), 2', 5'-oligoadenylate synthetase, RNase L, adenosine deaminase, and the Mx protein GTPases [10,11]. The spontaneous clearance of HCV is associated with a strong HCV-specific CD4⁺ T cell response [12]. Successful cellular immune responses in recovered patients appear to be HCV-specific and sustained, with CD4⁺ T cells playing major roles [13]. Not only do cytotoxic T lymphocytes (CTLs) contribute to the elimination of HCV-infected cells; it is now clear that HCV-specific CD4⁺ T cells also play critical roles [14].

HCV infection is curable by therapy. The current standard-of-care (SOC) treatment is based on the administration of pegylated interferon (PEG-IFN) combined with oral ribavirin for 24 or 48 weeks [15]. The main endpoint of the treatment is a sustained virological response (SVR), defined as undetectable HCV RNA in peripheral blood 24 weeks after the end of the treatment, which generally corresponds to a permanent cure [16]. Quantitative measurement of the viral load at the beginning of therapy and after 12 weeks of treatment is needed to make decisions about the usefulness of further treatment [17-19]. Factors affecting the response to current therapy should be studied and evaluated – specifically, host genetic factors including interferon-stimulated genes (ISGs) in addition to immune elements such as IFN γ .

The main objective of the present study was to identify predictive therapeutic markers or early correlates of therapeutic response in order to select the more appropriate group of patients for the current therapy and enroll those non responders in more appropriate therapeutic schedules. The study aimed to evaluate the association between endogenous IFN γ in the response of HCV genotype-4 chronically infected patients to IFN α therapy using peginterferon alfa-2a combined with ribavirin.

Methodology

Patients

All patients in the present study provided written informed consent. Ethical approval for the research was obtained from the review board of the Faculty of

Medicine, Zagazig University. Forty patients diagnosed with chronic HCV genotype-4 (HCV-4) were included in the study. Twenty healthy blood donors were used as healthy controls. Patients were selected according to the following criteria:

1. Positive HCV RNA in serum samples, as confirmed by real time polymerase chain reaction (qPCR)-based assay more than six months ago.

2. Positive HCV antibodies in serum samples as confirmed by ELISA assays.

3. Elevated alanine aminotransferase (ALT) levels in patients with an F1 Metavir score (portal fibrosis without septa) of liver biopsy.

4. F2 (portal fibrosis with few septa) and F3 (numerous septa without cirrhosis) Metavir scores of liver biopsy without esophageal gastric varices.

The clinical protocol called for patients to be treated for up to 12 weeks with peginterferon alfa-2a (Pegasys, Hoffmann-La Roche, Basel, Switzerland) and daily oral ribavirin [20]. All patients' serum samples were subjected to HCV RNA quantitative RT-PCR-based assay and ALT assay before treatment and tests were repeated 12 weeks later. According to the results of the viral loads determined by the quantitative RT-PCR on the 12th week following treatment, the patients were classified into 3 groups:

Group I (responders) defined as patients who had a greater than twofold decrease of HCV RNA levels. This definition of responders is valid also for the current study, which included complete clearance at 48 weeks of treatment [20].

Group II (nonresponders) defined as patients who had persistent viremia after therapy.

Group III (healthy controls) defined as healthy donors with negative HCV RNA and negative HCV antibodies in their serum samples. Human peripheral blood samples (five mL) were obtained from healthy donors and from 40 patients with chronic HCV infection; the samples were obtained before starting and 12 weeks after starting the combined therapy of peginterferon alfa 2a and ribavirin.

Preparation of human peripheral blood mononuclear cells (PBMCs) and culture condition

Whole blood was diluted with an equal volume (10 mL) of phosphate-buffered saline (PBS), carefully layered over a ficoll-hypaque gradient (Amersham/Pharmacia, Piscataway, USA), and centrifuged at 100 xg for 20 min at room temperature. The buffy coat layer was transferred to a 50 mL RNase-free tube and diluted with PBS, centrifuged at 100 xg for 15 minutes at room temperature; the

supernatants were discarded, and PBMCs were retained [21].

Enzyme linked immunosorbent assay (ELISA)

Prepared PBMCs were resuspended into RPMI 1640 media as previously described [22]. Stimulation of PBMCs or lymphocyte subsets was performed using freshly isolated PBMCs which were seeded at 2×10^6 /mL (2 mL) into 96-well microtiter plates, and phytohemagglutinin (PHA) (Sigma Aldrich, Saint Louis, USA) at a concentration of 10 μ g/mL was added to each well. For stimulation, the final ratio of PBMCs and PHA was 10:1. For control treatment, PHA (1 μ g/mL) or the culture medium alone was added to the PBMCs suspension. To determine the cytokine expression by PBMCs, the samples were incubated for 24 hr at 37°C in the presence of 5% CO $_2$. Subsequently, PBMCs were collected, washed using cold PBS, and centrifuged. Cell culture supernatants were collected separately. IFN γ secretion from PBMC/mL was determined in 24 hour culture supernatants using standard protocols of sandwich ELISA (OptEIA), BD Biosciences - Pharmingen, Franklin Lakes USA). The lower limit of IFN γ detection was 7.8 pg/mL.

MxA gene restriction fragment length polymorphism

DNA extraction and PCR were performed to all cell pellets using AxyPrep blood genomic DNA Miniprep kit (Axgen Biosciences, Union City, USA). Genotyping for the biallelic G/T polymorphism in the promoter region of *MxA* gene at position -88 from the transcription start site was done using restriction fragment length polymorphism (RFLP) by the enzyme HhaI (New England Biolabs, Ipswich, USA) to digest the PCR fragment of 351 bp as previously described [20]. PCR was performed using Taq PCR master mix kit (Bioron, Ludwigshafen, Germany) in a final volume of 20 μ L containing 10–100 ng DNA, a forward primer, with a sequence of 5'-TGAAGACCCCAATT ACCAA -3' (anneal to nucleotides 269 to 287 of the *MxA* gene sequence) and a reverse primer, with a sequence of 5'-CTCTCGTTCGCTCT TTCAC-3' (anneal to nucleotides 619 to 600). These primers produced amplicon at a size of 351 bp. For the HhaI restriction digest, 8 μ L of the amplicon were digested for at least 4 hours in a volume of 20 μ L with 5 IU of HhaI, according to the manufacturer's specifications. Digested PCR products of 10 μ L were visualized on 2% agarose gel containing ethidium bromide. Four fragments of 261, 51, 23, and 16 bp were obtained by

cutting the 351 bp in the presence of the G allele, and, in the presence of the T allele, three fragments of 312, 23, and 16 bp were obtained.

Statistical analysis

The paired t-test was used to compare between stimulation conditions in PBMCs from healthy donors. Chi-square and t-tests were used to examine the associations between baseline characteristics and outcome groups in the longitudinal study. Statistical analysis was used to compare the data from responders and nonresponders of nonparametric unpaired groups using the Student's t-test and the Mann-Whitney U test. The significance of the differences between nonparametric unpaired patients and controls was calculated using the Mann-Whitney U test; the Wilcoxon test was used to compare paired groups at different time intervals. Correlation was done using Spearman's rank. For data management and statistical analysis, the PrismV6.0 statistical software was used (Graphpad, San Diego, USA). All data were shown as means \pm standard deviation unless indicated differently.

Results

Comparison of ALT, IFN- γ and MxA genotypes between chronic HCV infected patients and healthy control groups

Different parameters were compared in chronic HCV infected patients and healthy individuals. The median IFN γ levels in chronic HCV-infected patients were lower than that of the healthy controls (453 vs. 869 pg/mL). The difference between the two groups was statistically significant ($p < 0.05$), as shown in Table 1 and Figure 1A. On the other hand, the median of ALT level in chronic HCV-infected patients was higher than that of the healthy controls (125.5 U/mL vs. 45.0 U/mL) with the statistically significant difference between two groups ($p < 0.05$) as shown in Table 1 and Figure 1B). *MxA* promoter genotype was determined by restriction fragment length polymorphism using cutting enzyme HhaI for PCR products for both chronic HCV-infected patients and healthy individuals. TT homozygous was less frequently found in the patients' group than in the healthy one (20% vs. 40%) (Table 1). The reverse was true for GG homozygous (45% in virally infected patients and 30% in healthy ones), but GT heterozygous was nearly the same in both study groups (35% vs. 30%).

Table 1: Comparison of distribution of ALT, IFN γ and *MxA* genotypes between chronic HCV infected patients (before treatment) and healthy control groups

		Chronic HCV patients	Healthy controls	X^2	P-value
ALT (U/ml)	Median	125.5	45.0	1.87	P< 0.05 Significant
	Range	100–232	38–55		
IFN γ (pg/ml)	Median	453.0	869	2.92	P< 0.05 Significant
	Range	102–1220	371–1298		
<i>MxA</i> promoter genotypes	GG homozygous	18 (45%)	6 (30 %)	2.85	P>0.05 Insignificant
	GT heterozygous	14 (35%)	6 (30%)		
	TT homozygous	8 (20%)	8 (40%)		

Table 2: Effect of combined therapy on viral loads, ALT, and IFN γ on chronic HCV infected patients.

Study groups	Viral loads			ALT			IFN γ		
	Baseline IU/ml	After 12 week IU/ml	Wilcoxon test	Pre U/ml	Post U/ml	Wilcoxon test	Pre pg/ml	Post pg/ml	Wilcoxon test
Responders									
Median	440000	3300	P=0.0001	154	50	P=0.0001	585.5	3779	P=0.0001
Range	900000–5600000	0-38000		100–232	40–60		250–1100	2000–7510	
Nonresponders									
Median	585000	6200000	P=0.08	119	179	P=0.0001	313.5	310	P=0.08
Range	510000–73000000	4300000–7200000		100–155	98–345		102–1220	89–1300	
Mann-Whitney U test	P=0.048	P=0.0001		P=0.0001	P=0.0001		P=0.02	P=0.0001	

P< 0.05 significant

Table 3: Median-fold increase in IFN γ in response to 12-weeks combined therapy among responders and nonresponders groups.

Study groups	Responders	Nonresponders	Mann-Whitney test
Median-fold	6.64	1.0	P=0.005
Fold range	4.07- 14.88	0.87- 1.51	

P< 0.05 significant

Table 4: Correlation between baseline levels of IFN γ , viral load, ALT, and median-fold increase in IFN γ

	IFN γ median-fold increase	
	R	P value
Baseline IFN γ level	0.3	0.002
Baseline viral load	0.39	0.08
Baseline ALT level	0.27	0.04

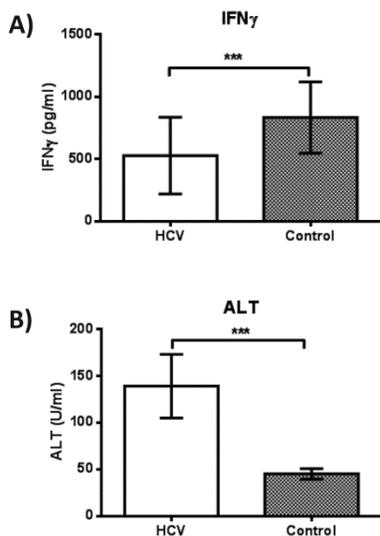
P< 0.05 significant

Table 5: Distribution of *MxA* promoter genotypes among responders, and nonresponders groups.

<i>MxA</i> promoter genotypes	Responders	Nonresponders	X^2	P-value
GG homozygous	5 (25%)	13 (65%)	11.50	0.003
GT heterozygous	7 (35%)	7 (35%)		
TT homozygous	8 (40%)	0 (0 %)		

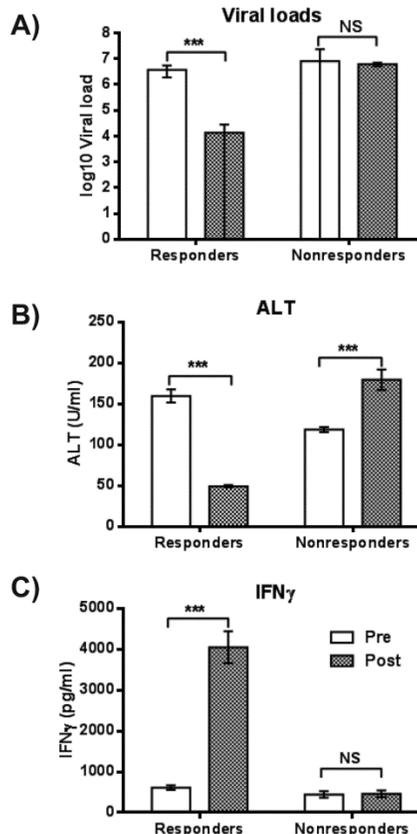
P< 0.05 significant

Figure 1: Distribution of IFN γ and ALT levels in chronic HCV infected patients and healthy individuals.



A) Significant decrease of IFN γ secretion in chronic HCV infected patients than in healthy control. B) Significant increase of liver enzyme ALT in chronic HCV infected patients than in healthy control. The data display the mean \pm standard error (SEM) of 40 infected patients and 20 healthy controls. The statistical significance was calculated with the Student *t* test for unpaired values and significance level indicated by asterisks (***) $P < 0.001$.

Figure 2: Effect of combined therapy (PegIFN/Ribavirin) on chronic HCV infected patients after 12 weeks of treatment.



Effect of combined therapy on viral loads (A), liver enzyme ALT (B), and IFN γ levels (C) of chronic HCV infected responders and nonresponders. The data display the mean \pm standard error (SEM) of 20 responders and 20 nonresponders. The statistical significance was calculated with the Student *t* test (Wilcoxon test) for paired values of before (Pre) and after (Post) therapy and significance level indicated by asterisks (***) $P < 0.001$. NS is non-significant.

Effect of combined therapy on viral loads of chronic HCV infected patients

To determine the effect of combined therapy after 12 weeks, chronic HCV-infected patients were classified into responders and nonresponders based on the quantitative RT-PCR result of the HCV genome. In the responder group, the median of the baseline viral load before therapy was significantly higher than the median of the viral load after 12 weeks of therapy (440000 IU/ml vs. 3300 IU/mL) (Table 2). The Wilcoxon test for paired matched samples showed a result of $p = 0.0001$ (Figure 2A). Among nonresponders, there was no statistical difference between the median of the baseline viral load and that after 12 weeks of treatment as (Wilcoxon test for

paired matched samples, $p = 0.08$) (Figure 2A). The Mann–Whitney U test was used to compare responders and nonresponders as unpaired groups; the results showed that the baseline viral load and the viral load after 12 weeks in the responders were statistically significantly lower than that in the nonresponders ($p = 0.048$ and $p = 0.0001$, respectively) (Table 2).

Effect of combined therapy on liver enzyme (ALT) of chronic HCV infected patients

Figure 2B shows that the median ALT level in the responders after 12 weeks of combined therapy was significantly lower than that before treatment (50 U/mL vs. 154 U/mL, Wilcoxon test, $p = 0.0001$), indicating that the therapy improved liver function.

Among the nonresponders, the median of ALT level before treatment was significantly higher than it was after 12 weeks of therapy (179 U/mL vs. 119U/mL, Wilcoxon test $p = 0.0001$) (Table 2, Figure 2B), indicating that the combined therapy did not affect liver function. The Mann–Whitney U test was used to compare the two groups; the baseline and 12-week ALT levels in the responders were significantly lower than that in the nonresponders ($p = 0.0001$ and $p = 0.0001$, respectively), as shown in Table 2.

Effect of combined therapy on IFN γ of chronic HCV infected patients

Figure 2C shows that the median IFN γ level after 12 weeks of therapy was significantly higher than the median at initial therapy (3778.0 pg/mL vs. 585.5 pg/mL, Wilcoxon test $p = 0.0001$). For nonresponders, there was no statistical difference between the median of the baseline viral load and that after 12 weeks of therapy (Wilcoxon test $p = 0.08$) (Table 2). The Mann–Whitney U test showed that, among responders, the baseline and after 12-week IFN γ levels were significantly higher compared to the nonresponders ($p = 0.02$ and $p = 0.0001$, respectively) (Figure 2C). Statistical analysis was used to determine if there was an association between the baseline IFN γ level and response to treatment. There was a significant association between baseline IFN γ level and the median fold increase of IFN γ level from one side and the response to treatment on the other side. The median-fold increase of IFN γ was significantly higher among responders compared to nonresponders (6.64 vs. 1.0, $p > 0.005$), as shown in Table 3. Spearman's rank was used to determine the correlation of the median fold increase of IFN γ level and each of baseline IFN γ level, baseline plasma viral load, and baseline ALT level. There were significant correlations between median fold increases of IFN γ levels and both baseline IFN γ level and baseline ALT level as shown in Table 4.

MxA gene polymorphism

Using PCR-RFLP of MxA promoter of both responders and nonresponders, agarose gel showed four fragments of 261, 51, 23, and 16 bp long in the G allele, and, in the T allele, only three fragments of 312, 23, and 16 bp long. The results showed that GG homozygous was less frequent in responders than in nonresponders (25% vs. 65%, $p < 0.005$). Conversely, TT homozygous was significantly more frequent in the group of responders than in the group of nonresponders (40% vs. 0%; $p < 0.005$) (Table 5).

Furthermore, the T allele was significantly more frequent in the group of responders than in the group of nonresponders. The reverse was true for the G allele; among responders, it was significantly more frequent than the in the nonresponders. Moreover, the MxA promoter polymorphism and the response to treatment and baseline viral load were independent determinants of the outcome of the combined therapy of PEG-IFN/ribavirin. However, there was a significant correlation between MxA promoter polymorphism and the baseline viral load.

Discussion

Antiviral treatment should be considered in all cases of chronic HCV-infected patients. Current antiviral treatment is a long-term regimen and is associated with a number of side effects [23]. The successful rate of treatment is about 50% in patients infected with HCV genotype 4 after 24 to 48 weeks of treatment [17]. Despite the significant improvement of the rate of treatment for patients with chronic HCV since the use of combined therapy of peginterferon and ribavirin, the treatment is still associated with considerable side effects, especially in nonresponders [23]. There is a need for improved alternative treatment regimens. The antiviral activity of IFN α superfamily genes is the rationale for their therapeutic use in viral hepatitis; however, the secreted cytokines are pleiotropic immune regulators [24].

The data shown in the present study provide further insights into the complex effects of IFN α therapy on IFN γ production in responders and nonresponders. The therapy induces significant secretion of IFN γ among responders (Figure 1A). Previous studies reported that type I IFN was essential for IFN γ production by CD8 $^+$ T cells [25,26], and other studies demonstrated the IFN α / β -derived IFN γ secretion by CD4 $^+$ T memory cells [27]. Enhanced IFN γ production has been predicted to favor HCV clearance [28]. In the current study, IFN γ secretion levels were significantly higher than baseline levels after IFN α therapy in the responders; in nonresponders, IFN γ secretion levels were not affected (Table 2 and Figure 2C). The present study examined the effect of IFN α which induced IFN γ in vivo. IFN γ has immunomodulatory effects and is a marker of successful cellular immune responses to HCV infection which is represented by a significant decrease of viral loads (Figure 2A) and liver enzyme ALT (Figure 2B) and eventually improve liver function. These findings suggest that IFN γ induced by IFN α therapy may modulate immune responses in

several ways. First, IFN γ enhances NK cell activity and induces the expression of inflammatory and potentially antiviral cytokines such as TNF α [29]. Second, IFN γ facilitates induction and effector functions of T cells via the upregulation of MHC class I and II proteins and promotes antigen processing via the induction of immunoproteasomes [30]. HCV clearance is correlated to the frequency and pool of HCV-specific IFN γ -producing Th1 and Tc1 immune cells [31].

In this study, there was no significant correlation between baseline viral load and IFN γ induced by IFN α ($r=0.39$, $p=0.08$). This finding supports that of Byrnes *et al.*, who noted that IFN α -driven IFN γ was not significantly affected by the baseline viral load [27,32]. Our observations showed that a significant correlation between baseline ALT levels and the mean fold increase in secreted IFN γ ($r = 0.27$, $p < 0.05$), similar to results reported in other studies [21,33].

With regard to *MxA* promoter genotype distribution, TT homozygous was less frequently found in patients than in healthy controls; the reverse was true for GG homozygous with no statistical significant differences as shown in Table 1, consistent with other studies [34]. Several viral as well as host factors contribute to the response of chronic HCV-infected patients to combined PEG-IFN/ribavirin therapy. Host genetic factors are associated with the efficiency of chronic HCV treatment [35]. The present study highlights the important roles of host genetic factors, particularly variation in the interferon-induced *MxA* gene in the modulation of the responses of HCV to IFN α therapy. GG homozygous occurred less frequently in responders than in nonresponders (25% vs. 65%, $p < 0.005$), which had been reported in a previous study [34]. The reverse was true for TT homozygous; TT homozygous occurred more frequently in the responders than in the nonresponders (40% vs. 0%; $p < 0.005$).

The results of PCR-RFLP of *MxA* promoter showed that the T allele occurred more frequently in the responders than in the nonresponders. The reverse was true for G allele, as shown in Table 1. These results showed that the single nucleotide polymorphism (SNP) of the *MxA* gene is one of the important host factors that independently influenced the response to IFN α in patients with chronic HCV infection, especially those with a low viral load. GG genotype expressed less *MxA* mRNA than the GT or TT genotype in IFN-treated PBMCs *in vitro* [36]. This can be explained by the involvement of that SNP in a genetic element which is highly homologous to the

IFN-stimulated response element consensus sequence; the G-to-T change could have contributed to the increase in this homology.

The present study showed that among all patients, HCV RNA level and the SNP of the *MxA* gene were independent and significant determinants of the outcome of IFN therapy. An additional biological marker used for monitoring IFN α therapy is the SNP at the interleukin-28B (IL28B) gene, which seems to be more relevant as an indicator of the responders to PEG-IFN/Ribavirin therapy in chronic HCV infected patients. [5,35,37-39].

HCV genotypes show major differences in progression risk and response to therapy; it is critical to compare subjects infected with similar HCV genotypes. The same is true for identifying the association of specific SNPs with diseases in different populations. Cases of HCV patients of the same ethnic group must be compared to their specific control populations. In general, the enhanced IFN γ production is predicted to favor HCV clearance. The increment of IFN γ from the baseline following treatment with IFN α is a predictor of the IFN γ levels following treatment. Finally, the SNP of the *MxA* gene is one of the important host genetic factors that independently affected the response to IFN α in patients with chronic HCV infection, especially those with a low viral load.

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