Original Article

Relation of interleukin-1β gene to treatment response in chronic patients infected with HCV genotype 4

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Abstract

Introduction: Hepatitis C virus (HCV) infection results in chronic hepatitis in more than 70% of infected patients, while 20-30% of patients recover spontaneously. This strengthens the role of the host genetic factors in either spontaneous or drug-induced viral clearance. The aim of this study was to investigate the relationship between interleukin-1β +3953 gene polymorphism and the response to interferon therapy in chronic HCV patients infected with genotype 4.

Methodology: The interleukin-1β (+3953 C/T) (rs1143634) gene was amplified in 115 chronic HCV patients. Interleukin-1β single nucleotide polymorphism (SNP) plus several clinical and pathological factors were statistically analyzed in correlation with response to therapy.

Results: Genotypes C/T and T/T had a significant association with non-response to treatment compared to genotype C/C, which had a strong association with response to treatment (95% confidence; 6.4884-48.5818, p = 0.0001). Furthermore, analysis of allele frequency in this cohort revealed that the T allele is associated with non-response, higher fibrosis, and higher hepatic activity, while the C allele had a significant association with sustained virologic response lower fibrosis, and lower hepatic activity (p value = 0.0001).

Conclusion: This is the first study to examine the correlation between interleukin-1β (+3953 C/T) (rs1143634) gene polymorphism and the response of interferon therapy in genotype 4 HCV-infected patients. The results encourage further assessment of this SNP as a marker to predict response to therapy and disease progression, which can have major implications in saving money, time, and in avoiding unnecessary adverse effects.

Key words: HCV; Interlukin-1β; interferon therapy; sustained virological response; single nucleotide polymorphism

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Introduction

Hepatitis C virus (HCV) infection has a global prevalence of ~3%, affecting 130–170 million people worldwide. In most cases (60-85%), HCV infection progresses to chronic liver disease, which can lead to liver cirrhosis and hepatocellular carcinoma (HCC) [1]**.** The risk for developing HCC increases with the severity of inflammation and the progression of fibrosis [2]. The current standard for HCV G1 is the triple therapy with direct-acting antiviral (DAA) combined with pegylated interferon and ribavirin. The main goal of treatment is to achieve a state of sustained virologic response (SVR), with sustained disappearance of HCV RNA in the serum within approximately 24 weeks of treatment, while end of treatment response (ETR) is usually achieved after 48 to 72 weeks for patients infected with genotypes 1 and 4, while patients with genotypes 2 and 3 are treated only for 24 weeks. Treatment outcomes in patients with hepatitis C are influenced by several variables, including viral factors such as viral genotype and baseline viral load [3,4]. Host factors that influence the achievement of SVR include age, race, gender, obesity, steatosis, disease severity, and genetic factors [5]. Increasing evidence indicates that host genetic factors such as the protein kinase RNA (PKR) gene and suppression of cytokine signalling 3 (SOCS3) and most importantly IL28 influence the natural history of HCV infection and the response to combined therapy [6]. Interleukins are highly active molecules constituting a rapidly growing group of proteins termed cytokines [6,7]. Cytokines are multi-functional molecules with important biological activity against pathogens [7]. Interleukin-1β (IL-1β) production is an important initiating factor in the cascade of events resulting in inflammation. Elevated serum and liver

levels of IL-1β have been shown to inhibit IFN-α/β-activated signals and antiviral activity [8]. Three single nucleotide polymorphisms (SNPs) were reported at positions -31 (rs 1143627), -511 (rs16944), and +3953 of the interleukin-1β gene [9]. The common variant present in the coding region at exon 5 of IL-1β is the synonymous +3953 C/T (rs 1143634**)** [7]. The current study involved 115 chronic hepatitis C (CHC) Egyptian patients infected with genotype 4. All subjects received pegylated interferon alpha 2a (INF α 2a) and ribvarin (RBV) for 48 weeks, followed for 24 weeks if needed. The association of the C/C, C/T and T/T alleles of interleukin-1β +3953 SNP with treatment-induced HCV clearance was examined.

Methodology

Patients

The current study investigated 115 patients infected with chronic HCV genotype 4. All patients were Egyptian (97 males and 18 females with ages ranging from 21 to 64 years). Only those patients covered by the national health program for treating viral hepatitis (National Committee of Hepatitis Virus-Ministry of Health and Population) were included in the study. This criterion includes thebsence of other causes of liver disease and the absence of hepatitis B, HIV, or schistosomiases infections. Exclusion criteria also included thyroid dysfunction, uncontrolled diabetes mellitus, history of long-term drug or alcohol intake, and autoimmune hepatitis. The assessment of the stages of liver activity and fibrosis was based on the histo-pathological examination of the liver biopsy according to the Knodell score. The patients were divided based on different fibrosis stages (F0-F4) and different liver activity stages (A0-A3). Patients received a weekly S.C injection of pegylated IFN alfa 2a and daily oral ribavirin at a dose of 1000-1200 mg, depending on body weight. Patients who did not follow therapy in the proper schedule with the required dose were excluded. The study also included seventy healthy controls (negative for HCV Ab and RNA).

HCV RNA tests

The tests included nested RT-PCR, quantitative determination of serum HCV RNA, and genotyping of the HCV RNA genome. Methods used for these assays were: (a) nested RT-PCR [10] including amplification of the highly conserved 5'-UTR sequences with nested primers in two rounds of amplification, including RT-PCR beads (Amersham Biosciences, Pittsburg, USA) in the first round, 0.2 mmol/L from each dNTP, two units of Taq DNA polymerase in the second round; (b) quantitative test of HCV RNA using C Amplicore, HCV monitor test Roche with broad dynamic range of 615 to 7,700,000 IU/mL (Amplicore, Reading, UK); and

(c) HCV genotyping [11] including nested PCR amplification of HCV core gene using genotype specific primers in two rounds of amplification with 0.2 mmol/L from each dNTP and two units of Taq DNA polymerase.

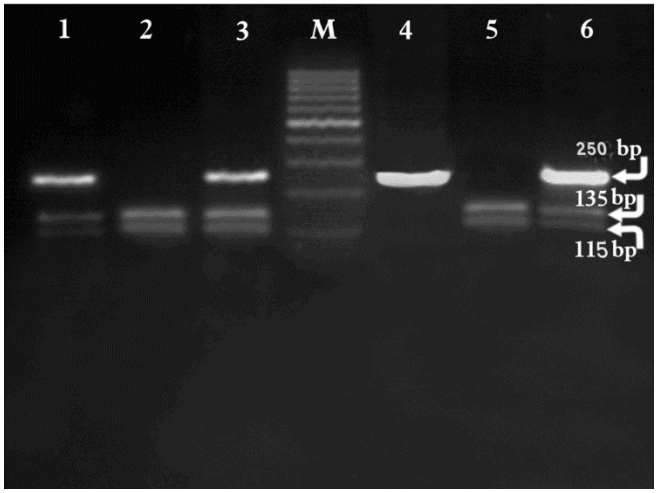
DNA extraction

DNA was extracted from whole blood using the salting out technique [12]. Genomic DNA was extracted from the peripheral blood mononuclear cells (PBMC) after digestion with proteinase K and subsequent salting out of cellular proteins using sodium chloride, followed by ethanol precipitation and final storage at -20°C until required.

Genotyping of IL-1β+3953 SNP

The region spanning the tested SNP was amplified by polymerase chain reaction (PCR) using sequence-specific primers as follows: forward primer (F) 5′-GTTGTCATCAGACTTTGACC-3′, and reverse primer (R) 5′-TTCAGTTCATATGGACCAGA-3′. The reaction mix (50 µL) contained 2 units of Taq polymerase (Finnzyme, Vantaa, Finland), 5 µL of 10X PCR buffer (supplied with the enzyme), 0.2 mM dNTPs (Promega, Madison, USA), 1.5 mM $MgCl_2$, 5 µM from each primer, 3 µL of DNA and DDW (double distilled water) to 50 µL. A negative control (with no DNA in the PCR reaction) was included in every reaction. Thermal cycling protocol comprised an initial denaturation at 96°C for 5 minutes, followed by 38 cycles. The first three cycles, were each comprised of 96°C for 90 seconds, 53°C for 90 seconds, and 72°C for 90 seconds, followed by 35 cycles each including 96°C for 1 minute, 53°C for 1 minute, 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. The amplification product was resolved on 2% agarose gel electrophoresis. A fragment of 250 bp indicated successful amplification. The amplified products were subjected to digestion with 2 units of TaqI restriction endonuclease (Amersham Pharmacia-Biotech, St Albans, UK) at 65°C for 3 hours. Restricted fragments were resolved on 2% agarose gel electrophoresis parallel with a DNA size marker (Amersham Pharmacia Biotech, Piscataway, USA). As shown in Figure 1 failure to cut the 250 bp fragment with a TaqI enzyme indicated a T/T genotype, while digestion cut results into 135 and 115 bp bands indicates a C/T heterozygote . If both alleles

Figure 1: Purified genomic DNA from each patient was subjected to PCR amplification using specific primers bracketing the +3953 C/T site of IL-1β gene followed by Taq1 digestion. The products were resolved on 2% agarose gel. Lanes 1, 3and 6 represent heterozygous C/T; lanes 2 and 5 show C/C genotype; lane 4 shows T/T genotype, while M indicates molecular weight marker.



were digested with TaqI enzyme (i.e., two bands of 135 and 115 bp), a homozygous for the C/C genotype of interleukin-1β +3953 SNP was assumed.

Statistical analysis

Statistical analysis was performed using the SAS statistical program for data analysis. Data were expressed as mean ± SD of the percentage (%) of each genotype among the subject population, whether sustained viral response (SVR) or non-responder (NR), where HCV RNA was kept detectable in the serum during or after the period of treatment. Data were also expressed as percent of SVR among each IL-1β+3953 genotype. Comparison between mean values of different variables in SVR and NR subgroups was performed using an unpaired student *t* test. Comparison between categorical data was done using the chi square test. Multinomial logistic regression with forward stepwise variable selection was used to identify predictors associated with SVR rates. Variables were described as odds ratio (OR) with a 95% interval (CI). The data were considered significant if p values were ≤ 0.05; highly significant if $p < 0.01$, and very highly significant if $p < 0.001$ [13,14].

Results

Demographic data

The data presented in Table 1 clearly demonstrates statistically significant differences between SVR and NR patients in the majority of parameters recorded, including higher values of mean age, alanine aminotransferase (ALT), alpha-fetoprotein (AFP), body weight, viral load, higher fibrosis, and histology activity index (HAI) indices among non-responder (NR) versus sustained viral response (SVR) patients. Low fibrosis and low liver activity indices were statistically elevated in SVR patients compared with NR patients. All chronic hepatitis C (CHC) patients in this study (n = 115) had genotype 4 of HCV RNA, determined using the method described previously [11].

Frequencies of interleukin-1β (+3953 C/T) genotypes in control Egyptian subjects versus CHC patients

The frequencies of interleukin-1β (+3953 C/T) genotypes in controls versus patients are shown in Figure 2. The interleukin-1β C/C allele constituted 51% of the healthy population and 45% of the CHC patients. There was a slight equilibrium between allele frequencies in the two groups for both C/T and T/T genotypes.

Frequencies of interleukin-1β (+3953 C/T) genotypes

Current data showed that 88.5% of patients carrying IL-1β C/C were associated with SVR ($p = 0.001$) versus 32% and 23% with C/T and TT genotypes of interleukin-1β, respectively. On the other hand, C/T and TT genotypes of interleukin-1β were associated with non-responder (NR) patients; 68% and 77% respectively, shown in Table 2 and Figure 3. This revealed greater association of IL-1β genotype CC with a response ($p = 0.0001$). Patients carrying the T allele of interleukin-1β represented 100% of patients suffering viral breakthrough, 70.5% of NR and 40% of relapsers (patients with an initial response to treatment of up to 50% who relapsed after treatment was discontinued). This showed that T allele showed poor response to IFN treatment ($p = 0.011$) whether was of pure (dominant) T allele of patients of T/T or is of carrier T allele of patients with C/T.

Prevalence of interleukin-1β (+3953 C/T) SNPs in different liver stages of CHC patient

The prevalence of interleukin-1β genotypes during different pathological changes in the liver of CHC patients was found to be highly significant during F1 and F2 stages of fibrosis ($p < 0.001$) as well as in A1

Table 1: The Demographic data of Egyptian CHC patients (SVR vs. NR).

Patients (n=115)	Non responders (n= 50)	Responders (n= 65)	P value
Age of all patients in years from 18-64 With mean ± SD 40.94± 10.83			
Min.-Max.	21-64	18-58	
Mean ± SD	44.62 ± 9.90	37.25 ± 11.76	0.001***
Gender			
Female	10 (20%)	8 (12.3%)	0.260 NS
Male	40 (80%)	57 (87.7%)	
Weight (Kg) 80.39±15.17	85.90 ± 12.21	76.15± 15.93	0.001***
ALT U/L 64.50±54.00	91.24 ± 61.92	43.92 ± 35.65	0.001***
AFP ng/ml 17.73±19.17	30.19 ± 22.19	8.15 ± 7.95	0.001***
Viral load (IU/ml) 397492.8± 622824	739566 ± 682573	138625± 21284	0.001***
Viral load (IU/ml)			
< 800000	35 (70%)	63 (96.92%)	0.001
> 800000	15 (30%)	2 (3.08%)	0.001
Low Fibrosis (F0-F1) (n= 47)	7(14.89%)	40 (85.1%)	0.001***
Fibrosis stages (F2-F4) (n= 68)	43 (63.24%)	25 (36.76%)	0. 029*
A0-A1 (n= 75)	21 (28%)	54 (72%)	0.001***
A2-A3 (n= 40)	29 (72.5%)	11 (27.5%)	0.001***

Data are expressed as mean ± SD or number (%). NS= not significant (p> 0.05).
(*) meant the data were considered significant if p values was ≤ 0.05.
***p< 0.001= very highly significant.

Table 2: The percentage of various genotypes of IL-1β (+3953 C/T) in SVR and NR patients.

	NR (n= 50)	SVR (n= 65)	Total(n=115)	P value
CC (n= 52)	6 (11.54%)	46 (88.46%)	52 (45.22%)	0.001***
CT (n= 50)	34 (68.00%)	16 (32.00%)	50 (43.48%)	0.011*
TT (n= 13)	10 (76.92%)	3 (23.08%)	13 (11.30%)	0.052 NS

Data are expressed as number (%).
NS= not significant (p> 0.05).
(*) meant the data were considered significant if p values was ≤ 0.05.
***p< 0.001= very highly significant.

Table 3: Frequencies of Interleukin-1β (+3953 C/T) SNPs in different liver fibrosis and activity stages

	CC (n= 52)		CT (n= 50)		TT (n= 13)		P value
	No.	*%*	*No.*	*%*	*No.*	*%*	
F0 (n= 12)	6	50.00	5	41.67	1	8.33	0.174 NS
F1 (n= 35)	23	65.71	7	20.00	5	14.29	0.001***
F2 (n= 36)	17	47.22	15	41.67	4	11.11	0.017*
F3 (n= 26)	6	23.08	18	69.23	2	7.69	0.001***
F4 (n= 6)	0	0	5	83.33	1	16.67	0.102 NS
A0 (n= 10)	5	50.00	4	40.00	1	10.00	0.273 NS
A1 (n= 65)	39	60.00	17	26.15	9	13.85	0.001***
A2 (n= 33)	6	18.18	25	75.76	2	6.06	0.001***
A3 (n= 7)	2	28.57	4	57.14	1	14.29	0.368 NS

NS= not significant (p> 0.05).
(*) meant the data were considered significant if p values was ≤ 0.05.
***p< 0.001= very highly significant.

Figure 2: Typing of IL-1β SNP was determined in 70 control subjects and 115 chronic hepatitis C (CHC) patients. The frequency of each IL-1β genotype in both patient groups was presented as columns (black bar: patients; white bar: controls).

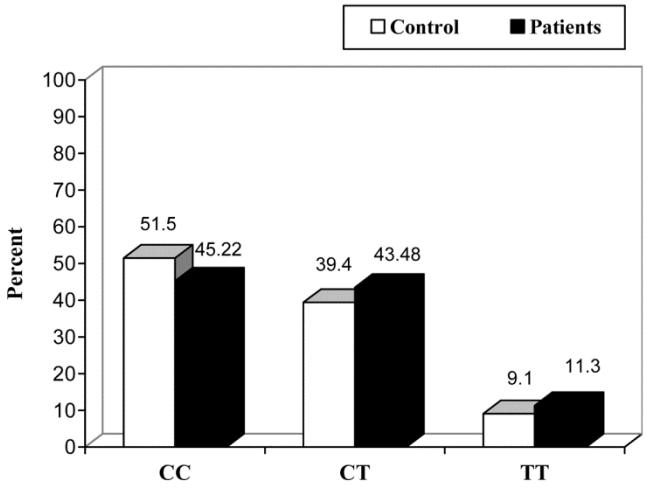
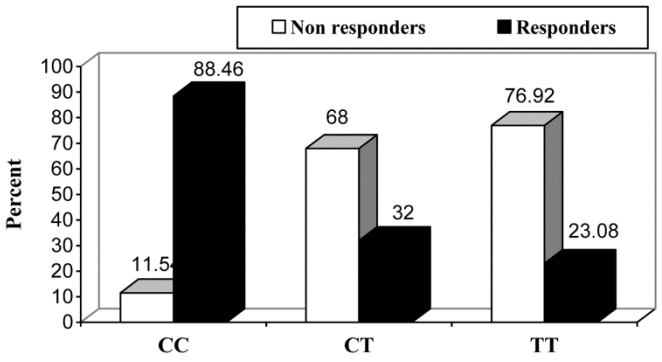


Figure 3: Genotyping of IL-1β was determined in 65 SVR patients and in 50 NR patients. Rates of SVR (black bar) and NR (white bar) were independently plotted as a function of each IL-1β genotype (i.e., C/C, C/T and T/T).



and A2 hepatic activity ($p < 0.001$) . The IL-1β C/C allele constituted the largest genotype frequency (65.7%) in early fibrosis and activity (60%), but turned down to the lowest value during advanced stages of fibrosis F4 (0%) and of hepatitis activity (28.6%), as shown in Table 3.

Multivariate analysis of different host factors including interleukin-1β variants in SVR and NR patients

The data presented in Table 4 summarizes the role of several factors in achieving SVR to combined therapy. Patients with advanced stages of fibrosis (F3-F4) were associated less with SVR than patients with low fibrosis (F0-F1) (p value = 0.0001). Otherwise, patients with higher liver activity (A2-A3) had lower chances of SVR compared to those with low activity (A0-A1) (p value = 0.0001). Patients with higher levels of AFP (>5 ng/mL) had less chances of SVR compared to those who had AFP levels below 5 ng/mL (p value = 0.0005). The IL-1β C/C allele had significant association with SVR compared with C/T and T/T variants (95% confidence; 6.4884-48.5818, p = 0.0001). In general, patients with a T allele had significant association with NR, while C-carriers had significant association with SVR (p value = 0.0001).

Discussion

In recent years, increased attention had been drawn to the role of polymorphisms in genes encoding immunoregulatory proteins, proinflammatory cytokines, and fibrogenic factors, which perhaps are associated with disease progression, and spontaneous or drug-induced viral clearance [7]. Interleukin-1β (IL-1β) is a pivotal initiating factor in the cascade of events resulting in inflammation and is specifically inhibited by naturally occurring antagonists, including the IL-1 receptor antagonist (IL-1Ra) [8]. The present study was designed to examine the possible relationship of (+3953 C/T) SNP with treatment-induced clearance of HCV in chronic patients infected with genotype 4 receiving the standard of care (SOC) for HCV infection, consisting of the combination of pegylated interferon (PEG-IFN) plus ribavirin [17].

A quick look at the present patient cohort revealed that age, gender, and body weight might be prognosis factors in determining the rates of SVR to combined therapy. The fact that older patients displayed lower rates of SVR is likely related to impaired cellular, humoral, and innate immunity, supporting earlier findings [15,16].

It has been reported that age, gender, cirrhosis, insulin resistance, diabetes, African-American ethnicity, and weight are all factors associated with poor response to pegylated-IFN treatment [17]. However, the present results demonstrated higher values in males than in females for both non-response and response, indicating that the high male HCV infection rates might be due to the increased exposure to the high risk factors of HCV. Conversely, it was suggested that the overall rate of SVR was similar in both sexes [18]. The mean values of body weight were higher in non-responders than in SVR patients, thus suggesting that being overweight might affect the

Table 4: Multivariate analysis of different clinical factors and alleles of IL-1β (+3953 C/T) gene polymorphism in responders and non-responders HCV patients

	Odds ratio	95% confidence intervals	P value
Age	0.5277	0.2170-1.2850	0.1591
Weight	1.0000	0.9000-1.0000	0.0460
ALT (≤45 IU)	5.8000	1.7000-19.400	0.0040
Viral load (≤ 130000)	34.300	9.5000-124.40	< 0.001
F0-F1 vs. F2-F4	10.170	3.9700 – 26.110	0.0001
A0-A1 vs A2-A4	14.750	6.2600 – 34.780	0.0001
AFP (≤ 5 ng/ml vs > 5 ng/ml)	18.990	7.4500 – 48.420	0.0005
CC vs CT+TT of IL-1β(+3953)	17.7544	6.4884 - 48.5818	0.0001

p< 0.001 = very highly significant.

outcome of therapy. Earlier reports showed that rates of SVR were decreased to about half in patients weighing > 90 kg [3,19]**.** Other studies have suggested an association between interleukin-1β +3953 gene polymorphism and obesity [20,21]**.** Furthermore, weight reduction in obese subjects had been shown to improve liver histological results, which also may improve the efficacy of IFN-alfa therapy in these patients [22]. The base line viral load was shown to play an active role in SVR rates, since patients with < 2 $x10^6$ copies/mL are three times more likely to achieve SVR than patients with > $2x10^6$ copies/mL [23], supporting the current results showing that initial viral loads were ~ 138 $x10^3$ in SVR versus $739x10^3$ in NR patients. Also, the regression coefficient analysis in Table 4 showed that ALT, viral load, and weight were probably shown to affect prognosis. A high ALT increased the risk of no response 5.8 times the rate of those with a low ALT value. An increased viral load increases the risk of no response 34 times more than low viral load. The mean serum levels of AFP in the SVR patients were significantly lower than those of NR patients (8 versus 30 ng/mL); this was supported by earlier reports [24,25] confirming that elevated AFP levels could be valuable markers for poor response. The present data demonstrated a significant association between interleukin-1β (+3953 C/T) gene polymorphism and IFN-induced HCV clearance in an Egyptian cohort infected with genotype 4, where C/C genotype frequency was eight times greater in SVR than in NR patients (88% vs. 11%). On the other hand, the frequency of C/T and T/T genotypes represented about two-thirds (68% and 77%, respectively) in NR patients. C allele frequency was higher in SVR than in NR (70% versus 30%), and this ratio was reversed for the T allele. An earlier report showed that serum and liver levels of IL-1β are elevated in chronic HCV patients [26]; this suggests that high levels of serum IL-1β may contribute to the reduced therapeutic responses to IFN-α in patients with chronic HCV infection, which is likely mediated via suppression of STAT1 activity in a proteosome-dependent manner [27]. Since genetic polymorphisms do not always reflect an altered expression of the corresponding protein unless they act as functional mutations, this study was designed to examine the statistical association of genetic polymorphisms (as a structural variant) with response to treatment. It was proposed that IL-1β could help viral clearance by regulating immune response and/or enhancing IFN-stimulated target gene expression, which could play a role in antiviral activity [28]. It has been reported that while the levels of serum IL-1β in all HCV patients are higher in comparison with healthy adults, its levels in patients with liver cirrhosis (LC) or hepatocellular carcinoma (HCC) were higher than that in patients without LC; such high levels of IL-1β result in a lengthening of the inflammatory process in the liver and in a high replication of the HCV [29,30]. However, other studies have reported that the interleukin-1β (+3954) T allele has been associated with increased IL-1β production in diverse pathological conditions, and also with an increased risk for the development of such pathologies [31,32]**.** In accordance with those findings, our data showed that the percentage of interleukin-1β SNPs in different stages of the liver of chronic C patients, where the percentage of C/C genotype was the highest in lower fibrosis stages i.e. F0 and F1 (50% and 65.71%, respectively) versus lower percentages for the T/T genotype at F0 and F1 (8.3% and 14.29%, respectively) and also higher percentages were shown for CC at lower grades of liver activity A0-A1 (50% and 60%, respectively) versus lower percentages for TT at A0-A1 (10% and 13.85%, respectively) and this was

highly significant for both factors F1 and A1 (p =0.001). Moreover, the frequency of the C/C genotype decreased gradually until it reached 0% at the advanced fibrosis stage (F4) and 18.18% at A2 (Table 4). In this study, multivariate statistical analysis showed that regardless of AFP levels, body mass index (BMI), and initial viral loads, the frequency of the C/C genotype was significantly higher in SVR, low fibrosis and reduced hepatic activity cases, while the frequency of the C/T and T/T genotypes were significantly higher in NR, high fibrosis and increased hepatic activity cases.

In summary, it is obvious that none of the host factors studied to date can be used as the sole predictive factor for response to therapy in actual clinical settings. As other polymorphisms were found in the genes of HCV-infected patients, they were shown to be associated with viral persistence or clearance; the HLA-DRB1*07 allele was found to be significantly associated with virus persistence, and HLA-DQB1*0301 was found to be associated with virus clearance [33]. Also, IL12B 3'-UTR 1188-C-allele carriers appeared to be capable of responding more efficiently to antiviral combination therapy as a consequence of a reduced relapse rate [34]. IL28 [6,35,36] held the promise that it could be utilized to categorize chronic patients into potential responders versus non-responders; however it is important to study together with IL28 SNPs of other host genes to develop the best mathematical model for the prediction of response to therapy.

Conclusion

This is the first report on the genetic association of interleukin-1β (+3953 C/T) gene polymorphism with IFN combined therapy induced HCV clearance in an Egyptian population infected with genotype 4. The observed significant association with SVR makes this locus a potential prognostic marker for response to IFN-based therapy, suggesting that patients with CT should be treated with IFN, as C-carriers had significant association with SVR (p = 0.0001), while patients with T/T should seek other treatment modality since the T allele had a significant association with NR to combined therapy, These findings should initiate the search for additional host factors for prediction of SVR with the aim of designing mathematical models based on these host factors, thus reducing unnecessary expenses and undesired adverse effects of ineffective treatment.

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