African Journal of Biotechnology Vol. 10(62), pp. 13376-13382, 12 October, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1522
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Correlation between a single nucleotide polymorphism (G/T at nt –88) in the *Mx1* gene promoter and the response to interferon therapy for hepatitis C virus in Egyptian patients

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Accepted 7 September, 2011

Interferon used in the treatment of hepatitis C virus (HCV) patients stimulates the expression of a number of host genes encoding enzymes with antiviral activities, including myxovirus resistance gene-1 (*Mx1*). *Mx1* gene was found to have a single nucleotide polymorphism (SNP) at position -88 in the promotor region that affect the expression of Mx 1 protein and was suggested to be associated with the response of HCV. In this study, we assessed the relation between the SNP in the *Mx1* gene and the responsiveness of Egyptian HCV patients to pegylated interferon and ribavirin treatment along with other host-related and virus-related predictors of treatment outcome. We genotyped the biallelic G/T SNP in the promoter region of *Mx1* gene at position -88 from the transcription start site by restriction fragment length polymorphism (RFLP) in 42 interferon treatment-naïve Egyptian patients that were treated with pegylated interferon and ribavirin. We found that *Mx1* nt-88 SNP is not significantly correlated to achieving sustained virological response (SVR) after pegylated interferon alpha and ribavirin combined treatment. We conclude that *Mx1* gene polymorphism at codon nt-88 cannot be considered as biological marker to potentially identify responders and non-responders of HCV patients to achieve a sustained virological response to treatment with interferon (IFN) in combination with ribavirin.

Key words: Hepatitis C virus (HCV), interferon (IFN), myxovirus resistance protein (Mx1 protein), myxovirus resistance gene-1 (*Mx1 gene)*, single nucleotide polymorphism (SNP).

INTRODUCTION

Hepatitis C virus (HCV) is a major etiologic agent of chronic hepatitis that may lead to the development of liver cirrhosis and hepatocellular carcinoma. HCV infection is one of the most common blood-borne chronic infections with an estimated 170 million infected people worldwide (Yu et al., 2008). HCV infection has a high rate of persistence and usually evolves to chronicity in 70 to 80% of cases (Jamall et al., 2008). In an infected individual, the HCV genome population circulates as quasispecies. The dominant sequence, as well as the consensus sequence, changes sequentially during the course of infection. HCV genetic heterogeneity has important implications in diagnosis, pathogenesis, treatment, and vaccine development (Simmonds et al., 2005). There is no vaccine against HCV so far and the only approved therapy is pegylated interferon plus

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ribavarin (Pearlman and Sjorgen, 2010). IFN does not inhibit a specific viral enzymatic function, but rather induces modifications of specific immune responses and the establishment of a nonspecific antiviral state in infected cells by the activation of numerous cellular genes (Poynard et al., 2000). Ribavirin does not significantly reduce HCV viral load when used alone but increases rates of SVR when combined with Peg-IFN. However, despite recent successes after the introduction of combination therapy with IFN and ribavirin, about 60% of patients still fail to respond. Response prediction to treatment has health and economic impacts, and is a multi-factorial problem including both host and viral factors for example, age, sex, ethnicity and pre-treatment viral load (Asahina et al., 2005; Elhefnawi et al., 2010). Therefore, resistance to antiviral therapy remains a serious problem in the management of chronic hepatitis C (Jain and Zoellner, 2010).

In a previous study, it was shown that patients who did not have SVR to IFN therapy constitute a heterogeneous group, some experienced persistent viremia and alanine aminotransferase (ALT) abnormalities (nonresponse) whereas others had an initial response followed by reactivation while on IFN therapy (virological breakthrough) or by relapse after its discontinuation (transient response) (Su et al., 2008). The outcome of antiviral treatment seems to depend on many factors both host-related and virus-related factors. The virus-related parameters appear to play an important role. These include the HCV genotype and the level of both the viral replication and the genetic complexity of the quasispecies population before the start of treatment (Elhefnawi et al., 2010). On the other hand, host-related factors such as age, sex, race and innate immune response also act as strong predictors of treatment response (Reddy et al., 2009). A crucial component of the innate immune response is the interferon system. Type I interferons induces numerous proteins with antiviral effects such as myxovirus resistance I (Mx1), 2-5 oligoadynylate synthetase 1 (OAS-1) and double stranded RNA (dsRNA)-dependent protein kinase (PKR) (Knapp et al., 2003). In humans, there are two Mx genes, *Mx1* and *Mx2*, encoding the Mx1 and Mx2 proteins, respectively. *Mx1* protein is found in the cytoplasm of cells and has a GTPase activity. *Mx1* also has an antiviral effect, which is dependent on the GTPase activity (Samuel, 2001). The high levels of *Mx1* protein were found in most of the IFN-treated hepatitis C patients in contrast to minimal levels before treatment even in patients with high viremia (Chieux et al., 1998). Also, the levels of the *Mx1* protein were greater in virological responders than in non-responders (NR) (Fernandez et al., 1999). The SNPs in the promoter region of the *Mx1* gene was found to be most likely associated with the levels of IFN-induced expression of the *Mx1* protein, and thus further with the response of the hepatitis C patients to the IFN therapy (Knapp et al., 2003; Hijikata et al., 2000; Suzuki et al., 2004). In this study, we evaluated the correlation between *Mx1* promoter nt-88 single nucleotide polymorphism and the response to treatment with pegylated-IFN-alfa2b and ribavirin among 42 HCV infected Egyptian patients.

MATERIALS AND METHODS

Study subjects

From March 2008 to August 2009, 42 interferon treatment-naïve patients with chronic hepatitis C infection were recruited for this study. 20 of them were males and 22 were females. All patients were positive for anti-HCV antibodies. Their ages ranged from 26 to 59 years old. All patients were treated with the current standard course of HCV therapy which is a weekly injection of pegylated interferon (Pegasys or PegIntron) plus daily oral weight-based ribavirin for 24 to 48 weeks.

Initial assessment

For initial assessment, 10 mls of whole blood were drawn from each patient onto three separate vacutainer tubes, 3 ml per each tube: two Lavender top ethylene diamine tetraacetic acid (EDTA) tubes and one red top (non-additive, with clot activator) tube. Plasma and serum were separated from one lavender and red tubes, respectively, and centrifuged at 5000 rpm for 5 min using a heraeus biofuge centrifuge for separation from cellular blood components.

HCV antibodies and viremia

Patients' sera were used to determine baseline ALT reference range (17-49 U/L) and aspartate transaminase (AST reference range: 8-38 U/L) levels while patients' plasma were used in the extraction of viral ribonucleic acid (RNA) which was then used for detection and quantification of baseline viral HCV RNA by real-time polymerase chain reaction (RT-PCR) technique (Abbott 2000, USA).

HCV genotyping technique

HCV genotyping was conducted using the Inno LiPA HCV Kit V2.0 (Innogenetics, Gent, Belgium).

Assessment of patient responsiveness to therapy (HCV-RNA viral load determination)

All patients were evaluated for their response to interferon therapy by measuring the level of plasma viral RNA six months after the end of treatment using the same method of RT-PCR HCV quantification used in the initial assessment.

Screening test for novel polymorphisms

Whole blood from the second lavender top tube was used for

extraction of host (patient) genomic deoxyribonucleic acid (DNA) from the buffy coat according to the manufacturer protocol (EZ1 DNA Blood Kit, Qiagen). The host DNA was used for amplification of the *Mx1* gene by traditional polymerase chain reaction (PCR). The biallelic G/T polymorphism in the promoter region of *Mx1* at position -88 from the transcription start site was genotyped by RFLP using the enzyme *HhaI* (New England Biolabs) to digest the PCR fragment of 351 bp. Amplification was carried out in a volume of 20 µl, containing 100 ng DNA , 2.5 mM $MgCl_2$ (Promega,USA), 500 nM of each primer (Eurofins,UK); Gene bank accession number X55639 (Knapp et al., 2003), Primer (Forward): 5'-GAAGA CCCCCAATTACCAA-3', Primer (Reverse): 5'-CTCTCGTTCGC CTCTTTCAC-3', 500 mM dNTP's (Promega,USA), 1x PCR buffer (Promega,USA), and 1U GoTaq DNA polymerase enzyme (Promega,USA). The cycling conditions in an applied biosystems 9700 machine were: denaturation at 94°C for 5 min, subsequently 35 cycles of denaturation at 94°C for 30 s; annealing at 58°C for 30 s; and extension at 72°C for 1 min. This was followed by a final extension step at 72°C for 7 min. For the *HhaI* restriction digest 8 µl of the PCR product were digested for at least 4 h in a volume of 20 µl with 5U of *HhaI* according to the manufacturer's specifications. 10 µl of digested PCR product were run out on 2% agarose gels and analysed. In the presence of the G allele, the 351 bp long product was cut into fragments of 261, 51, 23 and 16 bp and in the presence of the T allele into fragments of 312, 23 and 16 bp.

Statistical evaluation of host and virus related determinants with treatment response

A patient is considered a responder if SVR is achieved. SVR refers to undetectable plasma-viral RNA by PCR for at least 24 weeks after completing full-dose combination therapy, (FCT = 48- week-long injection of interferon alpha 2b plus daily oral dose of ribavirin). If SVR is not achieved, the patient is considered a non-responder. The baseline characteristics (age, gender and baseline ALT) of the 42 HCV patients were included in this study. In addition, their virus genotypes and their *Mx1* -88 genotypes were correlated with the response to pegylated alpha-interferon and ribavirin combination therapy. Results were expressed as number and percentage. Moreover, multinomial logistic regression was used to evaluate the effect of genotypes as predictors for viral responses in HCV patients. Statistical analysis was performed with the aid of the SPSS computer program (version 12 windows). P value of less than 0.05 was considered statistically significant.

RESULTS

Response to treatment

In this study, we used RT-PCR assay for quantification of HCV RNA in the plasma of 42 interferon treatment-naïve Egyptian patients that were treated with pegylated interferon and ribavirin. The complementary deoxyribonucleic acid (cDNA) was amplified using fluorescent Taqman probes for Real-Time quantification. HCV quantification was done before and six months after the end of treatment to assess responsiveness. RT- PCR HCV showed that n=21 (50%) of the patients were responders, achieving sustained virological response, while n=21 (50%) were nonresponders to treatment.

HCV genotyping

The extracted viral RNA was also reversed transcribed into cDNA amplified using conventional PCR for hybridization to oligonucleotide probes on HCV genotyping strips. HCV genotyping results revealed that n=7 (16%) patients were infected with GT 1, n=13 (30%) with GT 2, n=1 (2.3%) with GT 3, n=19 (45.5%) with GT4 and n=2 (4.7%) were untypable. Untypable or uninterpretable results can be attributed to cases with mixed infections of more than one HCV genotype.

Frequencies of *Mx1 gene* SNP at –88 G/T

We extracted host genomic DNA and used it for the amplification of the *Mx1* gene fragment in a conventional PCR. All the *Mx1* PCR products of the 42 patient DNA samples were 351 base pairs in length before digestion with Hha1 as shown in Figure 1. The *Mx1* PCR amplicons were genotyped using restriction fragment length polymorphism. The RFLP system used to detect the genotype and allelic polymorphism at nt-88 of the *Mx1* gene was based on the recognition site of the *Hha1* enzyme. In the presence of the G allele, the 351 bp long product was cut into fragments of 261, 51, 23 and 16 bp, and in the presence of the T allele into fragments of 312, 23 and 16 bp. The 351-nt PCR products from all samples were digested with *HhaI* (recognition site: GCG↓C) and electrophoresed in 2% agarose gels to see whether one or both of the 216-nt and 312-nt bands were generated as shown in Figure 2. 29 patient samples which generated only one 261 bp band have homozygous G-G genotype. 11 patient samples which generated only one 312 bp band had homozygous T-T genotypes and two patient samples which generated both 261 and 312 bp bands had heterozygous G-T genotypes.

Statistical significance of host and virus related determinants with treatment response

The correlation between patients' baseline characteristics, as well as their virus genotypes and their *Mx1* -88 genotypes was assessed with the response to pegylated alpha-interferon and ribavirin combination therapy and these data are represented in Table 1. We found that more than 60% of the responders had baseline ALT levels less than 66U/L. The effect of genotypes as predictors for viral responses in HCV patients was evaluated (Tables 2 and 3). In Table 2, 95% confidence

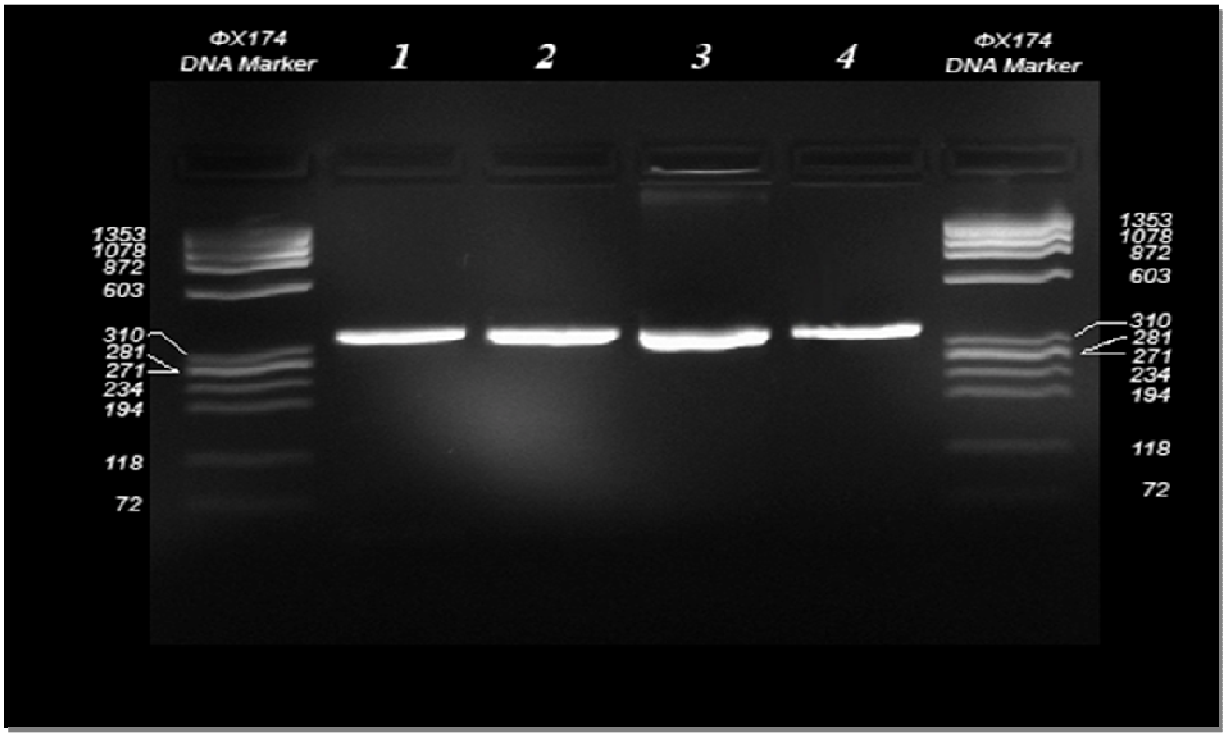

Figure1. Mx1 gene PCR: 351 bp amplicons of samples 1 to 4.

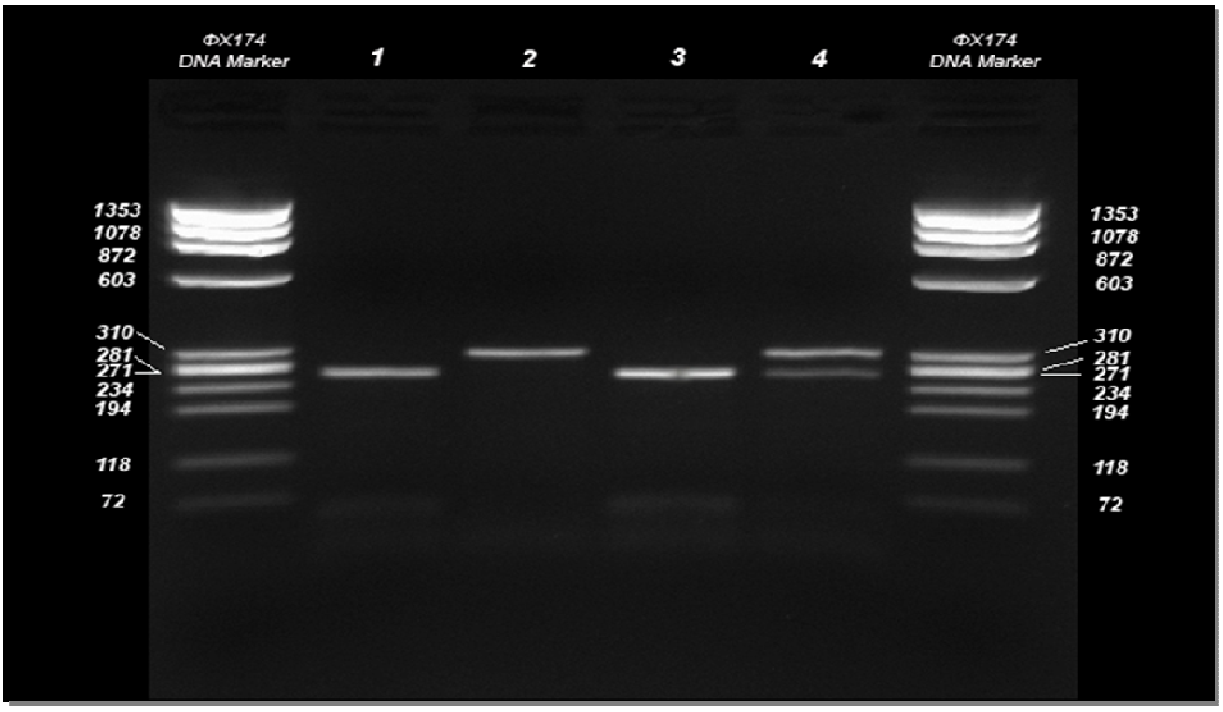

Figure 2. Mx1 PCR products after restriction digestion with *Hha1* enzyme of samples 1-4. Lane1, homozygous G-G genotype (261 bp band); lane 2, homozygous T-T genotypes (312 bp band); lane 3, homozygous G-G genotype (261 bp band); lane 4, eterozygous G-T genotypes (Both 261 and 312 bp bands).

Table 1. Correlation between predicting factors and treatment responsiveness.

Predicting factor	Responder (N= 21)	% Among responder	Non responder (N= 21)	% Among non responder
Age				
<49 years (N=23)	11	52.38	12	57.15
>49 years (N=19)	10	47.62	9	42.85
Gender				
Male (N=20)	9	42.85	11	52.38
Female (N=22)	12	57.15	10	47.62
ALT				
<66 U/L (N=22)	13	61.9	9	42.85
>66 U/L (N=20)	8	38.1	12	57.15
Virus genotype				
1 (N=7)	4	19	3	14.28
2 (N=13)	7	33.33	6	28.57
3 (N=1)	1	4.75	0	0
4 (N=19)	8	38	11	52.38
Uninterpretable(N=2)	1	4.75	1	4.75
Mx1 -88 genotype				
GG (N=29)	16	76.19	13	61.9
GT (N=11)	3	14.28	8	38.1
TT (N=2)	2	9.52	0	0

ALT, alanine aminotransferase. Mx1 -88 represents myxovirus resistance gene-1 at nt –88.

Table 2. Statistical analysis of Mx1 -88 genotypes GG, GT and TT.

Mx1-88 Genotype	SVR (n= 21)	NR (n= 21)	95% confidence interval	P value
GG (n= 29)	16 (55.2%)	13 (44.8%)	0.59-2.56	0.58
GT (n= 11)	3 (27.3%)	8 (72.7%)	0. 10-1.41	0.15
TT (n= 2)	2 (100%)	0 (0%)	---	---

SVR, sustained virologic response; NR, non responders. P value of less than 0.05 is considered statistically significant.

intervals and P-values were determined for the *Mx1* -88 genotypes GG, GT and TT separately. In Table 3, genotypes GT and TT were assessed together. *Mx1* -88 genotypes GG and GT had P-values of 0.58 and 0.15, respectively. *Mx1* -88 genotypes GT+TT together had a P-value of 0.41. These results show that none of the P-values were significant. So, there is no association between the presence of neither GT nor TT polymorphisms and increasing the rate of SVR. Moreover, GT+TT genotypes were not correlated with the rates of SVR.

DISCUSSION

In this study, 42 interferon treatment-naïve Egyptian patients with chronic hepatitis C infection were recruited. All patients were treated with the current standard course of HCV therapy which is pegylated IFN (Pegasys or PegIntron) plus ribavirin for 24 to 48 weeks. In this study, RT-PCR HCV quantitation revealed that 50% of patients responded to treatment which is considered moderate and this result is concurrent with globally reported response rates which lie between 48 and 88% (Malta et al., 2010). In addition, HCV genotyping test results reveal that HCV genotype 4 is the most prevalent genotype in Egypt as previous reports stated (Shaker et al., 2009). Current standard-of-care therapy consists of pegylated IFN (Pegasys or PegIntron) plus weight-based ribavirin (Pearlman and Sjorgen, 2010). A previous study showed that treatment regimen is strongly affected by HCV genotype (Ferenci et al., 2008). In this study, we

Table 3. Statistical analysis of Mx1 -88 genotypes GG and GT+TT.

Mx1 -88 genotype	SVR (n= 21)	NR (n= 21)	95% confidence interval	P value
GG (n= 29)	16 (55.2%)	13 (44.8%)	0.59-2.56	0.58
GT +TT (n= 13)	5 (38.5%)	8 (61.5%)	0.20-1.91	0.41

SVR, sustained virologic response; NR, non responders. P value of less than 0.05 is considered statistically significant.

evaluated the correlation of treatment responsiveness with HCV genotype (Table 1). The results show that n=4 (19%) of the 21 responders had HCV GT1, n=7 (33.33%) of the responders had HCV GT2, n=1 (4.75%) of the responders had HCV GT3, n=8 (38%) of the responders had HCV GT4 and n=1 (4.7%) of the responders had untypable HCV GT. Moreover, we used the RFLP system to detect the genotype and allelic polymorphism at nt-88 of the *Mx1* gene.

The results reveal that out of 42 patients, n=29 (69%) patients had homozygous G-G since they generated only one 261 bp band while n=11 (26%) patients had homozygous T-T since they generated only one 312 bp band and n=2 (4.7%) patients had *Mx1* -88 genotype: heterozygous G-T since they generated two bands, 261 bp and 312 bp. Also in this investigation, baseline ALT level was shown to be a strong predictive factor, since n=13 (61.9%) of the responders had ALT baseline levels less than 66 U/L while only n=8 (38.1%) of the responders had ALT baseline levels higher than 66 U/L (Table 1). In our study, correlation of treatment responsiveness with *Mx1* -88 genotype showed that n=16 (76.19%) of the 21 responders had GG while n=3 (14.28%) of the responders had GT and n=2 (9.52%) had TT genotypes (Table 1). Our study reveals that *Mx1* -88 genotypes GG and GT had P-values of 0.58 and 0.15, respectively (Table 2). *Mx1* -88 genotypes GT+TT together had a P-value of 0.41 (Table 3). None of the P-values were significant. No association was found between the presence of GT or/and TT polymorphisms and the increase of the rate of sustained virological response. Our results suggest that *Mx1* nt-88 single nucleotide polymorphism is not significantly correlated to achieving SVR after IFN-alpha and ribavirin combined treatment while other factors may have stronger predictive value such as HCV genotype and baseline ALT level.

Conclusion

We observed that *Mx1* nt-88 SNP is not significantly correlated to achieving SVR after IFN-alpha and ribavirin combined treatment while other factors may have stronger predictive value such as HCV genotype and baseline ALT level. Further studies are needed to be carried to confirm our results.

ACKNOWLEDGMENT

The authors are thankful to all the staff members of the Department of Tropical Medicine, Faculty of Medicine, Cairo University, Egypt for their valuable help in the samples collection.

Abbreviations

SNP, Single nucleotide polymorphism; **HCV**, hepatitis C virus; **FCT**, full-dose combination therapy; **RFLP**, restriction fragment length polymorphism; **SVR,** sustained virological response; **ALT**, alanine aminotransferase; **Mx1**, myxovirus resistance I; **OAS-1**, oligoadynylate synthetase 1; **dsRNA**, double stranded ribonucleic acid ; **PKR**, protein kinase; **NR**, non-responders; **EDTA**, ethylene diamine tetraacetic acid; **AST**, aspartate transaminase; **RNA,** ribonucleic acid; RT-PCR, real-time *polymerase chain reaction;* PCR, *polymerase chain reaction;* **cDNA,** complementary deoxyribonucleic acid; **IFN**, Interferon.

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