The Impact of IFN- γ Gene Polymorphisms on Spontaneous Clearance of HCV Infection in Fars Province, Southern of Iran

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Background: Certain polymorphisms in cytokine genes such as IFN- γ may influence the outcome of hepatitis C virus (HCV) infection. Here the frequency of the genotype, allele, and haplotype of IFN- γ gene at some loci is investigated in HCV-infected patients. **Methods:** Totally 255 patients with chronic HCV infection and 44 spontaneously cleared individuals were included. The chronic or clearance states were confirmed using enzyme-linked immunosorbent assay (ELISA) and two different qualitative reverse transcriptase polymerase chain reaction (RT-PCR) techniques. IFN- γ gene polymorphisms were performed by PCR using sequence-specific primers and PCR-RFLP on extracted genomic DNA. **Results:** The frequency of GG genotype ($P = 0.0001$,

OR: 5.69 and CI: 2.21–14.62) and allele ($P = 0.0003$, OR: 2.73 and CI: 1.54–4.83) of IFN- γ gene at +2109 locus was significantly higher in cases that spontaneously cleared the infection. Haplotype analysis showed the association of AG haplotype ($P = 0.0046$, OR = 6.14 and CI = 1.56–25) with spontaneous clearance of the infection. **Conclusion:** Our finding indicated that individuals with GG genotype at +2109 loci of IFN- γ gene and also AG haplotype (A allele at +874 loci and G allele at +2109 loci) may clear HCV infection more frequently than those with AA and AG genotype at +2109 loci and AA, TA, and TG haplotype. *J. Clin. Lab. Anal.* **30**:301–307, 2016. © 2015 Wiley Periodicals, Inc.

Key words: HCV clearance; hepatitis C virus; interferon- γ gene profile; haplotype analysis

BACKGROUND

Hepatitis C virus (HCV) has remained among the most important causes of chronic liver disease worldwide (1). More than 170 million people are infected with HCV and the infection becomes chronic in more than 70% (130 million) of them (2). According to the recent data, the prevalence of HCV is less than 1% in Iranian population (3). Unfortunately, following acute infection, spontaneous clearance is uncommon for reasons that have remained vague, and in most studies, the majority of patients (50–85%) progress to chronicity with subsequent risks for liver diseases. Although a number of factors, including age, sex, jaundice, viral genotype, transmission route, and coinfections with human immunodeficiency virus or hepatitis B virus have been assigned as variable impacts on the outcome of HCV infection (4–7), the emerging field of immunogenetic has confirmed the significant role of heritability and ethnicity in host immune response to infection (8, 9).

There is a significant relationship between ethnicity and response to pegylated interferon plus ribavirin therapy, as approximately only 20–28% of black patients achieve sustained virological response compared with 40–52% of white patients with genotype 1 infection (11, 12) and also 57% versus 82% for genotype 2/3 (12). Similarly, it has also been reported that racial differences are related to spontaneous viral clearance following acute infection (13, 14). These racial differences are proposed to introduce some genetic influences on HCV infection outcome. Recently, genetic variation in the interleukin-28B gene (IL-28B) has been shown to strongly predict HCV viral

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Received 16 March 2014; Accepted 8 April 2015

DOI 10.1002/jcla.21855

Published online in Wiley Online Library (wileyonlinelibrary.com).

clearance (8, 15). It has also been reported that spontaneous clearance of HCV infection is associated with some variations in HLA class I and II (16, 17), IL-10 (18), and IL-4 gene sequences (19). Furthermore, Raghuraman et al. reported the association of appearance of neutralizing antibodies and reversal of T-cell exhaustion with spontaneous clearance of HCV infection (20).

IFN- γ is a secretory protein mainly produced by T and NK cells. Its gene is placed on chromosome 12q24.1 and consists of four exons and three introns, which encode a short polypeptide of 166 amino acids. A pioneer study by scientists showing that IFN- γ level is associated with control of HCV replication implicated the crucial role of this cytokine in diseases progression inhibition (21). Also polymorphisms in the cytokine gene have been implicated in several autoimmune and chronic inflammatory conditions (22). Some polymorphisms have been reported in regulatory and coding regions of IFN- γ gene, including -183 G/T, +764 C/G, +874 A/T, +2109 A/G, +3810 G/A, and +5644 G/A loci. In the present study, three functional polymorphism sites of the IFN- γ gene that had been previously reported were selected. One polymorphism was located at 874 bp downstream from the translation start site that coincides with the nuclear factor- κ B (NF- κ B) binding site (23). Others were located at 2,109 bp downstream and 183 bp upstream from the translation start sites that were reported to be involved in transcriptional regulation of IFN- γ gene (24, 25).

Some investigators have shown the association between the outcome of some diseases including tuberculosis, hepatitis B, and brucellosis with these polymorphisms (26–28). To the best of our knowledge, information on these polymorphisms on the outcome HCV infection is very limited. Accordingly, in this study we aimed to determine the genotypes and allele frequency of IFN- γ at +874 A/T, +2109 A/G, and -183 G/T loci among patients infected with HCV, Fars, Southern Iran, and whether their genotype, allele, and/or haplotype frequency are significantly different in spontaneous clearance individuals versus patients with chronic infection.

PATIENTS AND METHODS

Patients

Subjects ($N = 299$) were recruited consecutively from the Gastroenterohepatology Research Center at Nemazee Hospital in Shiraz, Iran, from September 2010 to May 2013. Of the total subjects, 44 patients spontaneously resolved HCV infection cases and the other 255 patients had the confirmed history of chronic HCV infection.

The stepwise diagnosis of chronic HCV infection was documented based on a persistent elevation of serum aminotransferase level in the presence of HCV antibody,

detected using enzyme immunoassay, and the presence of HCV RNA by reverse transcriptase polymerase chain reaction (RT-PCR). The diagnosis of spontaneous clearance in subjects was based on the presence of HCV Ab, normal level of serum aminotransferase, and the negative results of qualitative RT-PCR for two tandem samples with 6 months interval. All the coinfection cases of HIV/HCV or HBV/HCV were also excluded from study.

The study was approved by the Ethics Committee of Shiraz University of Medical Sciences and written informed consent was obtained from each participant before sampling.

ELISA Tests

To document the spontaneous clearance of HCV infection, the serologic status was tested for presence of anti-HCV Ab using fourth generation anti-HCV enzyme-linked immunosorbent assay kits (ELISA) diagnostic systems (RPC Diagnostic System, Ltd., Russia) on serum samples according to the manufacturer's instructions.

Virologic Evaluation

Viral RNA extraction

Viral RNA genome was extracted by a commercially available kit. Briefly, viral RNA was extracted from 200 μ l serum samples by the help of a column-based viral RNA extraction method, Invisorb Spin virus RNA mini kit (Invitex, Inc., Germany), with respect to company recommended protocol and eluted solution in final volume of 50 μ l. The eluted solution was introduced into cDNA synthesis as soon as possible or stored in -80°C until use.

Qualitative RT-PCR

As virologic assay, for confirmation of the absence or presence of HCV viral genome inside the serum samples, an in-house nested PCR method and a commercial real-time PCR test were employed in parallel. For cDNA synthesis, "RocketScript RT Pre-Mix" kit (Bioneer, Inc., S. Korea) that contained lyophilized mixture of reverse transcription reaction was employed. For this purpose, totally 10 μ l of extracted RNA virus was introduced to each reaction tube and also random hexamer was added as primers followed by 1 hr incubation at 45°C .

An in-house nested PCR method that was developed in our laboratory was applied in all sera as a virus presence screening tool. Briefly in the first and second rounds of PCR, all the reaction mixtures were prepared similar to standard PCR condition with the exception of primer concentration that was used in concentration of 0.2 μM in final volume of 25 μ l and also

3 μ l input DNA for each round. Primer pairs sequence were so designed to cover amplification of all genotype especially 1a, 1b, and 3a outlined as follows: HC-Out1: 5'-CCCCTGTGAGGAACTACTGTC-3', HC-Out2: 5'-GCACGGTCTACGAGACCTC-3', HC-in1: 5'-ACGCAGAAAGCGTCTAGCCATG-3', HC-in2: 5'-TCGCAAGCACCTATCAGGCAG-3'. The first round was run for 25-cycle repeat of 55°C annealing temperature for 50 sec, while the second round was a 35-cycle repeat of amplification with 65°C annealing temperature for 45 sec. Final PCR products were run on 2% agarose gel and a 230 bp band was indicative of positive result.

Qualitative real-time PCR

In addition, for more accurate virologic evaluation, a commercial real-time PCR detection method, Amplisens HCV-FRT (Russia), was employed to confirm the presence/absence of viral genome inside the serum. Briefly, 10 μ l of the extracted virus genomes was introduced into one-step real-time reactions for 40 cycles of amplification by iQ5 real-time instrument (Bio-Rad Company, Hercules, CA, USA). The program was designed with respect to the recommended company protocol and final amplicons were detected using FAM and HEX dyes related filters. After all, based on control negative tubes and Ct threshold value evaluation, real-time negative results were established as an indicator of virus cleared patients in this confirmatory step. The detection limit of this kit was acclaimed to be near 10 IU/ml viral copy number.

Genomic DNA Extraction and Analysis of Cytokine Polymorphisms

A blood sample of 5 ml was drawn from each participant in EDTA anticoagulant. DNA was extracted from the peripheral blood leukocytes using the salting out procedure. Cytokine gene polymorphisms were studied by PCR using a thermal cycler (5530 Master Cycler, Eppendorf, Germany). Polymorphism at position +874 of IFN- γ gene was identified using allele-specific nucleotide PCR (ASO-PCR) as described by Pravica et al. with some modifications (23). Polymorphism at position +2109 and -183 of IFN- γ gene was identified by PCR-RFLP-based method using Aci I and Alu I restriction enzymes digestion, respectively, as described elsewhere (24, 25).

Statistical Method

The data were analyzed by χ^2 test using EPI-Info 2000 and SPSS version 15 software. Arlequin software package was used for haplotype analysis and also for determining the consistency of the genotype frequencies with

Hardy-Weinberg equilibrium. *P* values less than 0.05 were considered significant.

RESULTS

Patients and ELISA

A total of 299 patients infected with HCV were enrolled. The mean ages of chronic and clearance groups were 38.14 and 35.2 years, respectively; so, the difference was not significant ($P = 0.37$). Two hundred twenty-one of the subjects with chronic infection were male and 39 were female. In the case of subjects who had cleared the infection, 39 were male and five were female. Statistical analysis did not show any difference between the two groups ($P = 0.9$).

The genotyping data from available medical records (only from major part of chronic cases) also emphasized the prevalence of genotype 3a (49%), 1 (47%), and 2 (<0.5%) in our population as reported in our similar study (31).

All of the participants, including those who had chronic HCV infection or spontaneously cleared infection, were concluded to be positive for HCV Ab based on kit's controls absorbance.

Viral Genome Detection

For more certification and screening the spontaneous clearance of HCV infection, an in-house qualitative RT-PCR beside a commercial assay was employed on these group's sera two times with a 6-month interval. Individuals who had detectable HCV genome in qualitative RT-PCR were included in the chronic infection group and those with negative results were assigned in the spontaneous clearance category.

IFN- γ Gene Profile in Chronic and Spontaneously Clearance Groups

Of the 299 subjects, the polymorphisms of IFN- γ at +874, +2109, and -183 loci were determined using PCR method in 210, 255, and 260 of subjects, respectively. After ASO-PCR performing, analysis of gel electrophoresis showed 262 bp band correspond to IFN- γ A or T allele. Hence, homozygosity and heterozygosity for T or A allele also was revealed by this method, as depicted in fig. 1A. Analysis of PCR-RFLP (Fig. 1B) indicated the coincidence presence of 106 and 256 bp bands for homozygous A and G allele, coincidence presence of 106, 256 and 365 bp bands for heterozygous G allele and 365 bp band for homozygous G allele. The distribution of cytokine genotype and allele of +874, +2109 loci in spontaneously cleared and chronic cases is shown in Tables 1 and 2. No

TABLE 1. IFN- γ Genotypes Frequency Between Chronic and Spontaneous Clearance HCV Infection Groups

IFN- γ genotype	Chronic	Clearance	<i>P</i> -value
+874 AA	34(20.5%)	10(24.3%)	0.28
TT	50(29.5%)	16(39%)	
TA	85(50.2)	15(36.5)	
Total	169(100%)	41(100%)	
+2109 AA	167(78.4%)	28(66.6%)	0.0001
GG	14(6.5)	12(28.5%)	
AG	32(15%)	2(4.7%)	
Total	213(100%)	42(100%)	

TABLE 2. IFN- γ Allele Frequency Between Chronic and Spontaneous Clearance HCV Infection Groups

IFN- γ allele	Chronic	Clearance	<i>P</i> -value
+874 A	153(45.21%)	35(42.6%)	0.76
T	187(54.7%)	47(57.3%)	
Total	338	82	
+2109 A	366(85.9%)	58(69.1%)	0.0001
G	60(14.1%)	26(30.9%)	
Total	426	84	

statistically significant difference in the genotype distribution was observed between the two clinical groups for IFN- γ +874 polymorphisms. However, the frequency of IFN- γ 2109 GG genotype was higher (28.5% vs. 6.5%) in spontaneous clearance subjects compared to those with chronic HCV infection ($P = 0.0001$) as presented in Table 2. The IFN- γ 2109 G allele was associated with a higher probability of spontaneous clearance (OR: 2.73; CI: 1.54–4.83). Thus, subjects carrying the IFN- γ 2109 A/A genotype had a higher risk of chronic infection than those with the IFN- γ 2109 G/G genotype. In the case of –183 loci, interestingly no polymorphism was seen and all the subjects had G/T genotype.

Haplotype Analysis

It has been reported that these polymorphisms were under linkage disequilibrium. Haplotypes were calculated using Arequin 3.1 software package (29). A total of four haplotypes (a, b, c, and d) were constructed. The distribution of different haplotype frequencies in both cases and control groups is shown in Table 3. The presence of AA and TA haplotype was not associated with HCV infection outcome but the presence of AG haplotype was more frequently observed in spontaneous clearance subjects than those with chronic infection (OR = 6.14 and CI: 1.56–25). In the case of TG haplotype, although the frequency of this haplotype was 1.7-fold in the sponta-

TABLE 3. Distribution of the Estimated Haplotype Frequencies for IFN- γ Gene in Chronic and Spontaneously Clearance Groups

Haplotype	Chronic	Clearance	<i>P</i> -value
AA	110(44.7%)	27(35.5%)	0.0046
TA	100(40.6%)	25(32.7%)	
TG	32(13%)	17(22.3%)	
AG	4(1.6%)	7(9.7%)	
Total	246(100%)	76(100%)	

neous clearance group compared with the chronic group, the P -value was not significantly different ($P = 0.07$, OR: 0.52, CI: 0.26–1.05) between the two groups.

DISCUSSION

A number of factors, including age, sex, viral genotype, coinfections with HIV/HBV, and ethnicity may affect the outcome of HCV infection: clearance, chronic infection, or responsiveness to therapy (4, 6–9). Race factor and genetic background has special place to determine the fate of HCV infection as recently diagnosed polymorphisms around IL28B cytokine is associated with spontaneous clearance and responsiveness to treatment (8, 9). For this reason we designed a study to investigate immunogenetic polymorphism among a population from Southern Iran. It has been reported that polymorphisms in cytokine gene including IFN- γ have been implicated in several autoimmune and chronic inflammatory conditions, such (30, 31) infection (29, 30).

In this study, we showed that the frequency of GG genotype ($P = 0.0001$, OR: 5.69 and CI: 2.21–14.62) and allele ($P = 0.0001$, OR = 0.2.73, CI: 0.1.54–4.83) of IFN- γ +2109 polymorphisms in individuals who cleared their infection was higher than those with chronic infection. To the best of our knowledge, data regarding this polymorphism and outcome of HCV infection are limited. We previously showed that this polymorphism is associated with response to therapy in patients infected with HCV genotype 1 (32). In the case of other diseases, although Chevillard et al. (33) have reported association of G allele at 2109 locus with fibrosis following hepatic schistosomiasis, Etokebe et al. (26) and Karahrudie et al. showed no association with pulmonary tuberculosis (34). Although this change may increase the transcription of IFN- γ , the exact effect of this polymorphism on IFN- γ production is not clear yet. Therefore, we cannot make extra conclusion about the effect of this polymorphism on HCV infection outcome, regarding the upregulation/downregulation of IFN- γ gene transcription.

Investigators have also reported the association of other immunogenetic polymorphisms, such as IL-10 (–1082 A/G), IL-4 (+33 C/T), and HLA class I and II with

spontaneous clearance in hepatitis C infection (16, 17). More importantly, scientists have recently shown that genetic variation in the IL-28B gene strongly predicts viral clearance (8, 15). In this study, we found that individuals with GG genotype and/or G allele at IFN- γ gene at 2109 loci are more competent in clearing HCV infection than those with AA genotype and/or allele.

Another polymorphism at +874 A/T loci has been associated with HBV and HCV infection outcomes (26, 35), but not with HPV infection and colorectal cancer (36, 37). In Iran, some studies have reported the association of this SNP with pulmonary tuberculosis (38), brucellosis (27), breast cancer (39), HCV infection in kidney transplant recipients (40) and liver transplantation rejection (41), while some others reported no association of this polymorphisms with HBV infection (42) and recurrent pregnancy loss (43).

Dumoulin et al. and Radaeva et al. reported that high levels of intrahepatic IFN- γ mRNA correlated with lack of response to therapy in chronic HCV patients (44, 45). In another studies by Dai et al. (35), Oxenkrug et al. (46), and Bouzgarrou et al. (47), it was shown that patients with the T allele, higher producer genotype of +874 IFN- γ had a significantly higher rate of liver cirrhosis or HCC and are more prone to IFN- α therapy related depression. Based on our knowledge, there is limited published data that investigate the association of this polymorphism with HCV infection clearance and in this study we did not find the impact of +874 A/T polymorphism on HCV infection outcome.

Haplotype analysis is more helpful for identification of predisposing genes of complex diseases (26). In the present study, haplotypes was calculated to clarify the effect of different haplotype on HCV infection outcome (28). The distribution of different haplotype frequencies in both groups is shown in Table 3. The result of an association analysis demonstrated that the subjects carrying the haplotype AG, which contained the A allele at position +874 and the G allele at position +2109, were associated significantly with spontaneous clearance of the HCV infection. The probability of HCV infection clearance in participants with haplotype AG was higher ($P < 0.005$) than those without the haplotype AG, suggesting that the presence of haplotype AG is a factor independently affecting HCV infection outcome in Iranian southern population. Furthermore, although TG haplotype was not significantly different between the two groups ($P = 0.07$), this haplotype was more frequent (1.7-fold) in subjects who spontaneously cleared the infection. Mangia et al. have reported that IL-10 ATA haplotype was more frequent in patients with spontaneous HCV RNA clearance (36.0%) than those with persistent infection (17). In a study by Lue et al., association of IFN- γ gene haplotype in the Chinese population with chronic HBV infection

Fig. 1. Results of polymorphism detection on gel electrophoresis. (A) Agarose gel electrophoresis of ASO-PCR products for IFN- γ (+874 T $\rightarrow$ A) SNP; detected 262 bp bands correspond to IFN- γ A or T allele and the 105 bp bands are indicator of internal controls. M: 100 bp DNA size marker; lanes 1 and 2 show homozygosity for T allele; lanes 3 and 4 show heterozygosity for A and T alleles; lanes 5 and 6 show homozygosity for A allele. (B) Results of AclI digestion and subsequent RFLP-PCR patterns. M: DNA size marker; 1: homozygous for the A and G allele (coincidence presence of 106 and 256 bp band); 2: heterozygous for the G allele (coincidence presence of 106, 256, and 365 bp band); and 3: homozygous for the G allele (a 365 bp band).

was demonstrated (26). According to genotype, allele, and haplotype analysis, the presence of G allele at 2109 loci of IFN- γ gene may contribute to HCV infection clearance.

Henri et al. (23) reported a polymorphism at -183 loci of IFN- γ gene that may influence AP-1 binding domain and subsequent promoter activity, which would influence the transcription of the IFN- γ . It has been reported that this polymorphism is associated with chronic HBV infection in Chinese population (48). Interestingly, all of our subjects had G/T genotype at -183 of IFN and no polymorphisms were seen in this study group. Although it is too early to conclude about the prevalence of this polymorphism in our area and reliable conclusion needs investigation on larger population, at least, according our finding, we can say that this polymorphism is very rare in our area, Fars province, Southern Iran.

Although sex and age have been reported as the factors that influence the outcome of HCV infection, in this study statistical analysis did not show any association between sex ($P = 0.6$), age ($P = 0.47$) and outcome of HCV infection (4, 49).

In conclusion, in this study we showed that the frequency of AG haplotype is significantly higher in clearance subjects compared to those with chronic infection. We also showed that GG genotype and G allele at +2109 loci are more frequently observed in clearance subjects compared to those with chronic infection; therefore, we suggest that the presence of G allele at IFN- γ +2109 loci is a genetic factor that may influence the outcome of HCV

infection in Iranian patients infected with HCV. Moreover, IFN- γ gene polymorphisms at +874 might not have any effect on the outcome of infection in HCV infected patients and -183 loci may have no/or very rare polymorphic in our general population, Fars, Southern Iran. It can be noted that one of the limitations of this study was a relative small sample size of those who spontaneously cleared the infection because these cases are very rare and finding such cases takes long.

ACKNOWLEDGMENTS

The authors thank Dr. Nasrin Shokrpour for improving their article writing. They also express their thanks to Mastaneh Zeraatian, Maryam Nejabat, and Zahra Mansourabadi for their kind assistances during sample preparation and laboratory methods performance. The study was financially supported by Shiraz University of Medical Sciences (grant number: 92-01-5695).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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