

RESEARCH ARTICLE

Lack of TNF- α Gene Polymorphism (rs1799724) Association with Sustained Virological Response in Iranian Patients with Chronic HCV Infection

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Abstract

Infection with the hepatitis C virus is a major public health concern which can lead to carcinoma and liver failure. It has been shown that single nucleotide polymorphisms can affect the level of gene activity of tumor necrosis factor (TNF) which has an important role, especially in viral infections which can lead to apoptosis of infected hepatocellular cells. We investigated the impact of three possible genotypes for rs1800629 or A/G single nucleotide polymorphism located downstream of TNF α gene promoter in groups of control (n=76) and chronic hepatitis C patients (n=89) focusing on the response to treatment among sensitive and resistant groups. Genomic DNA was extracted from 500 μ l prepheral whole blood and PCR and RFLP were used to amplify the region of interest and genotyping. With statistical analyzes a p-value <0.05 was considered meaningful. There was no significant difference in distribution of possible three genotypes among healthy individuals and patients (P=0.906, OR=1.194, CI=0.063-22.790). However, the frequency of G allele was higher in patients whereas A allele was more common among healthy individuals (p<0.0001). Further studies with more samples seem to be necessary.

Keywords: Hepatitis C - sustained virological response - tumor necrosis factor-alpha - polymorphism, rs1800629 - Iran

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Introduction

The worldwide prevalence of hepatitis C virus (HCV) infection is approximately 3%. Hopelessly, Only 15% of those who contract HCV are able to spontaneously clear the virus. Thus 85% of infected people are at the risk for developing cirrhosis and hepatocellular carcinoma in further years after infection. Furthermore, HCV infection is the most common reason for liver transplantation in the United States. Combination of pegylated interferon and ribavirin is the Standard- of-care treatment for fine hepatitis C; however, this treatment is expensive, hardly tolerated and only successful in 40% to 50% of infected individuals with HCV. Moreover, patients receiving the therapy usually experience serious adverse effects, differing from psychological depression up to bone marrow suppression. Due to the high cost of treatment, the low response rate for HCV 1, and adverse side effects, identifying factors which could predict response to therapy would be valuable. (Hoofnagle, 2002; Sy and Jamal, 2006; Ray Kim, 2010; Klevens et al., 2012)

Several related factors to the virus, host, and viral kinetics are associated with the efficiency of antiviral treatment (Manns et al., 2001; Henderson, 2003; Klevens

et al., 2012) including younger age, female gender, nonblack race and normal immune system, which increase the ability to suppress the virus. The most prevalent HCV genotype globally is genotype 1 and also it could be connected with more severe liver disease (Chloe, 2008; Kau et al., 2008; Thio, 2008; Aman et al., 2012; Mirjam et al., 2013; Zeisel et al., 2013) Based on the study conducted by Khodabandehloo (Khodabandehloo, 2014) the frequency of HCV genotypes was calculated for subtype 1a 39% (34-44%, 95% CI); following by subtype 3a, 32% (26-39%, 95% CI); and 1b subtype, 13% (10-15%, 95% CI); and also genotype 4, 5.18% (3.27-7.5%, 95% CI); and genotype 2, 3.6% (1.6-8.3%, 95% CI), respectively in Iran.

Among several genetic factors associated with the clearance the virus and response to the combined therapy, cytokines play a significant role to support the human immune system.

Tumor necrosis factor (TNF) refer to a group of cytokines that can lead to apoptosis. TNF α , the best-known member, is a monocyte-derived cytotoxin that has been involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation.

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Furthermore, it has been implicated in a variety of diseases, such as insulin, autoimmune diseases resistance and cancer (Kau et al., 2008; Guo et al., 2012; Mohd Hanafiah et al., 2013). Recently, multiple independent researchers have shown genome-wide association and reported several single-nucleotide polymorphisms (SNPs) associated with response to the treatment. rs1800629 or A/G single nucleotide polymorphism is located downstream of TNF α gene promoter and play an important role in level of gene activity specially in viral infections which can lead to apoptosis of infected hepatocellular cells (Andrea et al., 2012; Gane et al., 2013; Heba et al., 2013). This study investigated evaluating the distribution of this polymorphism among Iranian population and also the association with the response to the Hepatitis C treatment.

Materials and Methods

Sampling

This research was conducted according to the Declaration of Helsinki, and relevant local regulations. The Sampling protocol was approved by the ethical committee of Pasteur Institute of Iran (Code of ethical approval: 91-0201-7997). In this cross-sectional study, we investigated in convenience sampling from September of 2012 to January of 2014, 89 chronic Hepatitis C patients (including 68 patients with positive SVR and 21 non-SVR individuals) from both genders (83 males, 6 females), older than 18 years old (average of 41.84) and positive for viral RNA. Patients who cleared the infection spontaneously were excluded from the study. The control group included 76 non-relative healthy individuals who were testified by anti HCV Ab ELISA kit (DiaPro, Italy) test, from both genders (47 females, 29 males) older than 18 years old (average of 35.14). Moreover, the clinical data of patients such as BMI, viral load, positive or negative SVR, the liver stage and its enzymes level were collected under the supervision liver disease specialist. Finally, all patients declared their agreement to join our research.

Genomic DNA extraction

In order to isolate genomic DNA from 500 μ L from leukocytes, 5 mL of peripheral blood were collected into EDTA tubes. Genomic DNA extraction kit (DynaBio, Iran) was used according to the manufacturer's instructions and the whole DNA preparations were stored at -20°C for the further usage.

The rs1800469 genotypes detection

Specific primers for rs1800469 forward 5' CCCGGCTCCATTTCCAGGTG 3' and reverse 5' GGTCACCAGAGAAAGAGGAC3', used to amplify the target region. The PCR procedure consisted of one cycle of initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 40 seconds, annealing at 60°C for 40 seconds and extension at 72°C for 60 seconds and final extension at 72°C for 5 minutes. The 117 bp PCR product was identified on the 1% agarose gel (Figure 1A).

The rs1800629 SNP (A/G) genotyping was acquired by RFLP (restriction fragment length polymorphisms)

using NcoI endonuclease (Thermo scientific, Lithuania). The total digestion reaction tube (30 μ L) included 10 μ L of qualified PCR product, [1X] buffer Tango and 1 μ L of NcoI incubated at 37°C for two hours. In order to make the fragments of interest visual, we used 20% polyacrylamid gel in vertical electrophoresis gel and AgNO₃ used to stain the bands of interests. NcoI digestion of genotype AA yields fragments of undigested 117 base pairs (bp), whereas DNA containing GG genotype polymorphism results in fragments of 97 and 20 base pairs. The heterozygote genotype yields for 117, 97 and 20 bp as well (Figure 1B).

Statistical analysis

The final data based on genotyping A/G polymorphism in TNF α gene promoter for all three possible genotypes (AA/AG/GG) from both groups (patients and healthy individuals) and also the clinical data of the patients including both groups (positive SVR and NVR) were analyzed using SPSS 19 software. Furthermore, X² test and t-student test were used to generate variables data and p-value <0.05 was considered meaningful.

Results

The 117 bp PCR product of interest amplified gene with two specific primer was identified on polyacrylamid gel (Figure 1A). For A/G polymorphism in TNF α gene promoter, which has been shown to influence on the response to treatment was analysed by RFLP method using NcoI digestion. The digestion of genotype AA yields fragments of 117 base pairs, whereas DNA containing the allele GG polymorphism results in fragments of 97 and 20 base pairs (Figure 1B). The statistic analysis of demographic information of totally 165 samples including 89 cases (68 positive SVR and 21 negative SVR) and also 76 healthy individuals were shown in Table 1. In comparison between case and control groups, the race and the gender (p-value <0.001) were significantly meaningful. However, there was no correlation in term of

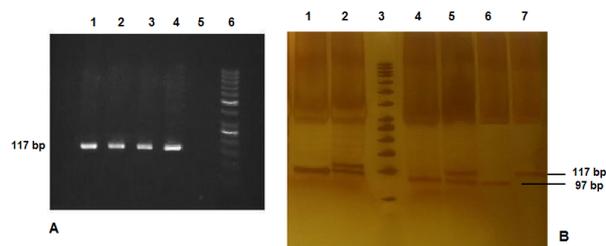


Figure 1. (A): PCR product of TNF- α on agarose gel 2% electrophoresis: Lanes 1 and 2: Healthy individual samples, lane 3: HCV infected samples with SVR, lane 4: HCV infected sample with NVR, lane 5: negative sample, lane 6: DNA Ladder marker 50 bp (Thermo scientific, Lithuania). (B): Acrylamide gel electrophoresis of digested PCR products of different groups: lane 1, 4 and 6: mutant homozygote GG, lane 2 and 5: AG, lane 7: undigested, normal homozygote AA, lane 3: DNA Ladder marker 50 bp (Thermo scientific, Lithuania)

Table 1. Demographic and Clinical Information of Patients (Sensitive and Resistant) and Control Groups

Specifics	Patients		p- value** (case series)	Total patients N(%)	Total healthy N(%)	p-value*** (case & control)
	Positive SVR N(%)	Negative SVR N(%)				
Number of samples	68(76)	21(24)	-	89(54)	76(46)	-
Average age (Years)	40.35	46.67	0.349	41.84	35.14	0.349
Gender (male/famale)	65(95.6)/3(4.4)	18(85.7)/3(14.4)	0.135	83(93)/6(11.3)	29(38.2)/47(88.7)	<0.001*
BMI	24.32	25.06	0.437	24.56 \pm 3.36	25.85 \pm 4.08	0.012
Ethnicity						
Iranian Fars	66(97.1)	18(85.7)	0.073	84(94.4)	52(38.4)	<0.001*
Other Iranian	2(2.9)	3(14.3)		5(5.6)	24(31.6)	
Marital status						
Single	27(39.7)	7(33.3)	0.6	34(38.2)	39(51.3)	0.091
Married	41(60.3)	14(66.7)		55(61.8)	37(48.7)	
Risk factors						
Tatoos	14(20.6)	6(28.6)	0.61			
Intervascular injection	65(95.6)	13(61.9)	<0.015*			
Sexual contact	6(8.8)	5(23.8)	0.681			
Blood transmission	3(4.4)	1(4.8)	0.24			
ALT (max-min)	24.46(12-175)	59.45(11-140)	<0.001*			
AST (max-min)	34.80(10-329)	56.18 (21-130)	<0.001*			
Liver status						
Chronic	(98.5)67	14(66.7)	<0.001*			
Cirrhosis	(1.5)1	7(33.3)				
Viral load (log)	10.79 \pm 2.75	15.32 \pm 0.94	<0.01			
HCV Genotype						
1a	41(60.3)	20(95.2)	0.014			
3a	27(39.7)	1(4.8)				
Co-infection						
HCV	57(83.8)	16(76.2)	0.626			
HCV/HIV	11(16.2)	5(23.8)				

*-statistically significant, **-p-value between sensitive and resistant groups, *** - p-value between case and control groups.

Table 2. Genotype Frequencies of Case (Sensitive and Resistant) and Control Groups

Host Genotype	Positive SVR (n%)	Negative SVR (n%)	p-value	OR**	95% CI***
GG*	55 (80.90)	15 (71.4)	-		
AG	12 (17.6)	6 (28.6)	0.295	1.833	0.590 – 5.699
AA	1 (1.5)	0 (0)	1	0	0
G*	122 (89.7)	36 (85.7)			
A	14 (10.3)	6 (14.3)	0.476	1.452	0.521 – 4.052
Host Genotype	Control (n%)	Case (n%)	p-value	OR**	95% CI***
GG*	35 (46.1)	70 (78.7)	-		
AG	40 (52.6)	18 (20.2)	0.002	0.231	0.091 – 0.581
AA	1 (1.1)	1 (1.3)	0.906	1.194	0.063 – 22.790
G*	110 (72.4)	158 (88.8)	<0.0001		
A	42 (27.6)	20 (11.2)		0.332	0.185 – 0.595

*Reference group **odds ratio ***confidence interval

age (p-value=0.349) and marital status (p-value=0.091) among patients and controls.

In addition, there was a significant association between the liver status (p-value<0.001) between positive SVR and NVR patients. It is to say that the majority of patients with Positive SVR showed chronic Hepatitis C(98.5%) whereas most of NVR patients developed cirrosis(33.3%). Moreover, the viral load was higher in NVR patients in comparison with SVR group (p-value<0.01). The distributin of HCV genotype showed that 1a genotype is highly frequented in NVR group (95.2%) whereas it is 60.3% frequented among SVR patients. 3a genotype is only 4.8% common in patients with NVR (p-value=0.014).

Genotype and allele distributions of Rs1800629 polymorphism are summarized in Table 2. Distributions of the genotypes were as GG:46.1%, AG: 52.6% and AA:

1.1%among control group and GG:78.7%, AG:20.2% and AA: 1.3 among patients. The frequency of alleles between healthy individuals were G:72.4%, A:27.6% and G:88.8%, A:11.2% among case group (P<0.0001, OR=0.332, CI=0.185-0.595). So, there is no significant difference between distribution of possible three genotypes among healthy individuals and patients(P =0.906, OR=1.194, CI=0.063-22.790)

However, the frequency of G allele was higher in patients whereas A allele was more frequented among healthy individuals (p<0.0001). As the result G allele can increase the risk of Hepatitis C among Iranian population.

Genotyping between two groups of patients also was calculated as GG:80.9%, AG: 17.6% and AA: 1.5% among positive SVR and GG:71.4%, AG: 28.6% and AA: 0% among NVR patients. The frequency of alleles were

G:89.7%, A:10.3% among SVR and G:85.7%, A:14.3% among NVR group ($P<0.476$, $OR=1.452$, $CI=0.521-4.052$). Therefore, there is no association between rs1800629 SNP (A/G) TNF α polymorphisms and response to the current standard treatment of Hepatitis C among studied Iranian population.

Discussion

The standard care for chronic Hepatitis C is based on combination of pegylated interferon (PEG-IFN) with ribavirin (RBV) which leads to intolerant side effects and high costs for patients (Chloe, 2008; Aman et al., 2012; Mirjam et al., 2013). Recently, it has been demonstrated that TNF α of cytokine family, can actively support immune system through apoptosis of infected hepatocellular cells. So, characterising rs1800629 or A/G single nucleotide polymorphism with the purpose of response to the standard treatment of Hepatitis C could be highly valuable to decrease side effects and also costs of curement and.

In another study which was done in Egypt, 440 patients and 220 healthy individuals were studied and genotyping results showed: AA+AG, GG were 23.4% and 76.6% among patients whereas 15% and 85% among controls. The frequency of AA+AG among SVRs was 19.5% and GG was 80.5% whereas 27.3% and 72.7% among NVRs. Moreover, A allele frequency was 10.2% frequented among SVRs and 15.2 among NVRs ($p\text{-value}<0.03$). They concluded that A allele may result in NVR (Heba et al., 2013).

Talaat. R studied a total 90 Egyptian individuals including 45 HCV infected and 45 healthy people. distribution of G/G, G/A, and A/A genotypes were 87%, 7%, and 6% among patients with liver cirrhosis and were 94%, 4%, and 2% in HCC patients. Moreover, the frequency of GG, AG and AA was: 56%, 29% and 15% among healthy individuals. The frequency of the G allele was significantly higher in HCV infected patients than in healthy controls ($p\text{-value}<0.05$), whereas the frequency of the A allele was higher in healthy controls ($p\text{-value}<0.001$). finally, they indicated that inheritance of the TNF promoter genotype at position -308 is not associated with clinical features of HCV infection (Roba et al., 2011)

Dogra G et al. studied 70 Hepatitis C patients and 70 healthy Indian individuals. In this study the distribution of rs1800629 polymorphism for GG, AG and AA was 87.1, 4.28% and 8.57% among infected people and 87.1%, 11.4% and 1.4% among controls. In this study they showed that there is an association between genotypes and risk of the disease ($p\text{-value}=0.05$). furthermore, they found that prevalence of GG genotype among patients with lower stage of liver disease was 96.5% whereas it was 71.4% among who suffered from severe form of Hepatitis C. however the correlation was not significant ($P\text{-value}=0.07$) (Gaurav et al., 2011)

In a study conducted in Egypt 280 patients with HCV including 152 cirrhosis and 128 with HCC and 160 healthy controls, AA+AG were calculated as 28.9% in patients whereas in healthy individuals it was 15% frequented. Moreover, GG genotype was 85% among controls and

71.1 among patients. The frequency of A allele was 7.5% between healthy people whereas 15.8 among case group suggesting that A allele can increase the risk of cirrhosis (Mohamed et al., 2012). By the contrast, in our study, frequency of A allele among controls was 27.6% and 11.2% among patients with chronic Hepatitis C whereas G allele was 72.4% frequented among healthy individuals and 88.8% among infected people, suggesting the G allele can increase the risk of Hepatitis C ($p\text{-value}<0.0001$).

In a research that was carried out by Cheng et al in 2003, 141 Taiwanese infected patients were studied. HCV 1b genotype was reported as the main subtype among Hepatitis C infected people. The distribution of -308 region of TNF α showed: GG:76.6%, AG:21.3% and AA:2.1% among patients. The frequency of G allele was obtained 82.2% and A allele was 12.8%. moreover, 40% of who had G allele achieved SVR whereas 0.8% of who had A allele. Therefore they concluded that A allele in this position (-308) can increase the risk of NVR ($p\text{-value}=0.029$) (Chia et al., 2006)

In current study, 33.3% of NVR patients achieved cirrhosis and A allele was 14.3% frequented among NVRs and 10.3% among SVRs whereas G allele was 85.7% among NVRs and 89.7% among SVRs, therefore there is no correlation between frequency of alleles and risk of developing cirrhosis ($p\text{-value}=0.476$). In the present study, GG genotype was frequented 87.7% among chronic Hepatitis C patients and 46.1% of healthy individuals. AA was 1.3% among patients and 1.1% among controls ($p\text{-value}=0.906$). the prevalence of G and A allele was 88.8% and 11.2% among cases and 72.4% and 27.6% among controls ($p\text{-value}<0.0001$). As a result the only association was found between presence of allele and risk of Hepatitis C among Iranian people.

Conclusion: the results showed AG+GG genotype can affect on susceptibility to HCV compared to AA in studied population ($P=0.906$, $OR=1.194$, $CI=0.063-22.790$), frequency of G allele was higher in patients whereas A allele was more frequented among healthy individuals ($p<0.0001$). As the result G allele can increase the risk of Hepatitis C among Iranian population. However there was no significant association between genotypes and sustained virological response among patients ($p=0.476$). In conclusion, the polymorphism had a similar distribution between infected groups with sensitive or resistant response to treatment. However, further studies with more samples are necessary.

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