



Investigation of Transforming Growth Factor- β 1 Gene Polymorphisms Among Iranian Patients With Chronic Hepatitis C

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ABSTRACT

Background: Chronic hepatitis C infection is caused by the hepatitis C virus (HCV), and its clinical complications include liver cirrhosis, liver failure, and hepatocellular carcinoma. Transforming growth factor- β 1 (TGF- β 1) is an important cytokine in cell growth and differentiation, angiogenesis, extracellular matrix formation, immune response regulation, and cancer development and progression.

Objectives: The aim of this study was to investigate the relationship between single nucleotide polymorphisms (SNPs) in TGF- β 1 and chronic HCV infection among patients referred to the Taleghani Hospital, Tehran, Iran between 2008 and 2010.

Patients and Methods: In this case-control study, samples were collected using a convenience sampling method. We genotyped 164 HCV patients and 169 healthy controls for 3 SNPs in the TGF- β 1 gene (-509 promoter, codon 10, and codon 25). We determined the SNP genotypes by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. To confirm the PCR-RFLP genotyping results, 10% of the samples were re-genotyped using a direct sequencing method.

Results: There were no significant differences in the allelic frequency distribution of SNPs at -509 C/T, +869 C/T, or +915 G/C between HCV patients and the healthy controls. Genotyping results for all three polymorphic sites were similar with no statistically significant differences between the groups.

Conclusions: Most of the Iranian patients (over 85%), both healthy controls and HCV patients, had the GG genotype at the +915 G/C position, resulting in a high level of TGF- β 1 production. Therefore, we concluded that the SNPs investigated by us cannot be considered as prognostic factors for HCV infection in our population, despite being reported as prognostic markers in other populations. Moreover, there is a possibility that most of the population is susceptible to HCV infection.

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► Implication for health policy/practice/research/medical education:

Transforming growth factor beta-1 (TGF- β 1) is a multifunctional cytokine that acts as a regulator of proliferation and cellular differentiation. Investigation of the association of single nucleotide polymorphisms (SNPs) in TGF- β 1 gene sequence with increased or decreased individual's susceptibility to chronic diseases such as hepatitis C infection could be helpful to find appropriate and population specific genetic biomarkers.

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1. Background

Approximately 175 million people worldwide are infected with hepatitis C virus (HCV), and about 80% of these individuals develop chronic HCV infection. Some individuals with persistent HCV infections develop liver cirrhosis, which can lead to hepatocellular carcinoma in

some patients (1-4). Hepatitis C has a complex etiology; factors such as genetic predisposition and cytokine production levels contribute to the pathogenesis (5). Cytokines play a vital role in the body's ability to fight viral infections: they determine the main type of immune response triggered and directly inhibit viral proliferation (6). Host genetic variations such as single nucleotide polymorphisms (SNPs) in cytokine genes can affect the rate of HCV production or secretion (7). Transforming growth factor- β (TGF- β) is an important cytokine for cell growth and differentiation, angiogenesis, extracellular matrix formation, immune response regulation, and cancer development and progression (8-10). Changes in the secretion of immune suppressive factors such as TGF- β can cause dysregulation of host immune response in chronic HCV patients (5, 11).

The TGF- β superfamily consists of 3 main isoforms, namely, TGF- β 1, TGF- β 2, and TGF- β 3. TGF- β 1 is a dimeric protein that acts either as a hormone or locally as a regulator of proliferation, differentiation, extracellular matrix production, and cell death (12). Characteristics of the extracellular matrix may also contribute to the clinical symptoms of liver diseases such as HCV infection (13). Several studies have reported differences in cytokine production among patients; this difference could be attributable to variation in the levels of gene expression or in secretion levels of cytokines.

2. Objectives

In this study, we investigated three important SNPs in the TGF- β 1 gene. Two SNPs are found in the signal sequence of the early producing protein and one in the promoter region. These polymorphisms can affect the gene expression or secretion levels of the mature protein (14, 15). One SNP in codon 25 of TGF- β 1's signal sequence was previously associated with an individual's susceptibility to chronic HCV infection (3). The aim of the present study was to determine whether any of the three selected TGF- β 1 polymorphisms are associated with chronic HCV infection in an Iranian population.

3. Patients and Methods

3.1. Study Population

We conducted a case-control study by using samples collected using a convenience sampling method from individuals at the Taleghani Hospital of Tehran between March 2008 and October 2010. The study population consisted of 333 Iranian individuals, including 164 patients with HCV infection and 169 healthy controls. In the patient group, HCV infection was diagnosed using a third-generation enzyme-linked immunosorbent assay with anti-HCV antibodies (DRG International Inc., USA). We excluded patients co-infected with hepatitis B virus (HBV) or human immunodeficiency virus (HIV); these patients were identified using tests for hepatitis B surface antigen and/or anti-HIV antibodies (DRG International Inc., USA). To confirm HCV infection in patients who yielded

positive results in the anti-HCV antibody test, circulating viral RNA was identified using reverse transcription-polymerase chain reaction (RT-PCR). The healthy control group consisted of volunteer blood donors who had no history of hepatitis or hepatobiliary disorders and showed negative results for anti-HCV antibodies and HCV-RNA.

3.2. RNA Extraction and RT-PCR

Viral RNA was extracted from 200 μ L of plasma with the QIAmp viral RNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Complementary DNA was synthesized in a total volume of 32.5 μ L, consisting of 1 μ L of random hexamer primers, 5 μ L of ssRNA template (100 ng), 4 μ L of 5 \times buffer, 0.5 μ L of Ribolock RNase inhibitor, 2 μ L of dNTP mix, 200 U of RevertAidTM M-MuLV reverse transcriptase, and 19 μ L of RNase-free water (Fermentas, Latvia). Reverse transcription was carried out at 42 $^{\circ}$ C for 1 h.

3.3. TGF- β Gene Polymorphisms

Peripheral blood samples from all 333 subjects were collected and stored at 4 $^{\circ}$ C. Genomic DNA was extracted from whole blood by using a standard phenol-chloroform method. Genotyping of the 3 SNPs in the TGF- β gene, namely, the -509 C/T SNP in the promoter, the +869 T/C SNP in codon 10, and the +915 G/C SNP in codon 25, was performed using PCR-restriction fragment length polymorphism (RFLP). The PCR conditions for the SNPs in codons 10 and 25 were as follows: 95 $^{\circ}$ C for 5 min; 35 cycles of 95 $^{\circ}$ C for 30 s, 58.4 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 30 s; and a final extension at 72 $^{\circ}$ C for 10 min. The PCR conditions for the -509 SNP were as follows: 95 $^{\circ}$ C for 5 min; 30 cycles of 95 $^{\circ}$ C for 30 s, 62 $^{\circ}$ C for 45 s, and 72 $^{\circ}$ C for 30 s; and a final extension at 72 $^{\circ}$ C for 10 min. For genotyping the SNP at -509, we used previously described primers (16). The target sequence was amplified by PCR to yield a 153-bp product. Subsequently, the PCR product was digested using the Eco8II restriction enzyme (Fermentas, Latvia).

For genotyping the SNPs of codons 10 and 25, we designed a pair of primers that amplified both polymorphic sites. This PCR product was used as a template for two RFLP reactions with MspAII (Promega, USA) for codon 10 and BglI (Fermentas, Latvia) for codon 25. The primer sequences, digestion conditions, and restriction enzymes for the detection of each SNP are given in Table 1. Amplification of 100 ng of template DNA for each of the 2 PCR reactions was performed using a total volume of 25 μ L containing 10 pmol of forward and reverse primers, 1.25 U of Taq polymerase, 200 μ M of each dNTP (Fermentas, Latvia), 37 mM of MgCl₂, 1.25 μ L of dimethylsulphoxide (Sigma Aldrich, Germany), and distilled deionized water. The PCR products were run on a 1.5% agarose gel and stained with ethidium bromide for visualization under UV light. The RFLP products for codon 25 and -509 promoter SNPs were visualized on a 2% agarose gel; the RFLP products for codon 10 were separated and visual-

Table 3. Genotypic Frequencies of TGF- β Gene Among Patients With Chronic HCV Infection and Healthy Blood Donors

Polymorphism	Genotype	Chronic HCV Patients, No.(%) (n = 164)	Healthy Blood Controls, No.(%) (n = 169)	P value
TGF- β codon 10	T/T	49 (29)	50 (30.5)	0.956
	C/T	85 (50.3)	81 (49.4)	
	C/C	35 (20.7)	33 (20.1)	
TGF- β codon 25	G/G	145 (88.4)	151 (89.3)	0.780
	G/C	18 (11)	16 (9.5)	
	C/C	1 (0.6)	2 (1.2)	
TGF- β -509 (Promoter)				0.920

Table 4. Restriction Fragments for Three RFLP Reactions

TGF- β 1 Polymorphism	Restriction Enzyme	Genotype	Restricted Fragments, bp
Codon 10 (+869)	MspA1I	CC	12, 40, 67, 108, 230
		CT	12, 40, 67, 108, 230, 242
		TT	40, 67, 108, 242
Codon 25 (+915)	BglI	GG	212, 252, 60
		GC	212, 252, 312
		CC	212, 312
Promoter (-509)	Eco81I	TT	153
		CT	153, 117, 36
		CC	117, 36

controls. According to results of the chi-square test, there were no statistically significant differences in genotype variants of the studied polymorphic sites between the HCV and control groups ($P > 0.05$). The mean age of the HCV patients was greater than that of the healthy controls, and the percentage of men was higher in the HCV group than in the healthy control group; therefore, we used the logistic regression method to remove these confounding variables. The results of RFLP genotyping for the three polymorphic sites were confirmed using the direct sequencing method (Table 4); this showed that the results of the two methods were consistent. Figure 2 shows a sample of the polymorphic sequences for codons 10 and 25.

5. Discussion

A number of genes contribute to the difference in the cytokine levels observed in individuals; however, the mechanism of function of these genes has not been fully understood (7, 17, 18). Because cytokines regulate the immune response, polymorphisms in cytokine genes or variations in their expression may affect an individual's susceptibility to infectious diseases (19, 20). TGF- β 1 has a regulatory role in proliferation, differentiation, and activation of immune cells. Therefore, differences in its production or secretion in the individuals in a population might result in different responses to a given infectious

agent (21, 22). An appropriate immune response to HCV infection is essential for its clearance from the blood and bodily fluids. Therefore, defective or insufficient production or secretion of cytokines could affect an individual's ability for virus clearance and prevention of chronic disease. As such, genetic variations, such as SNPs, in a population may affect an individual's susceptibility to chronic infectious diseases.

The most commonly studied polymorphisms of TGF- β 1 include a T/C transition at the -509 position of the promoter region, a T/C transition in codon 10, and a G/C transversion in codon 25. The genotypic distribution of TGF- β 1 polymorphisms varies in different populations (23). Several studies have investigated the relationship between polymorphisms in the TGF- β 1 promoter or signal sequence and various diseases in the Iranian population. To our knowledge, however, there is limited information on the relationship between these polymorphisms and HCV infection in the Iranian population. Eurich *et al.* (2011) observed that the genotypes of TGF- β 1 polymorphisms in codons 10 and 25 are significantly different in HCV patients with different degrees of hepatic fibrosis (24).

Pereira *et al.* (2008) investigated the association of polymorphisms in TGF- β and three other cytokines with HCV infection in a Brazilian population. There was a statistically significant relationship between the polymor-

phism in codon 25 of TGF- β 1 and HCV infection; however, there was no such association with the polymorphisms in codon 10 (3). In contrast, Migita et al. observed that the site in codon 25 is not polymorphic among a Japanese population sample (25); this finding was confirmed by Suzuki et al. (23). A study in China by Xie et al. showed that all the 186 HBV patients in their study had the GG homozygous genotype in codon 25; they also found no significant difference in codon 10 or promoter region (-509) polymorphisms between the case and control groups (17). These studies suggest that the site in codon 25 is not polymorphic among East Asian populations; this could be attributable to an ethnic difference between East Asians and other populations, such as Brazilians and Iranians. However, our results regarding codon 25 differ from those of previous studies. The polymorphism in codon 25 was detected in both the HCV and control groups; however, there was no significant difference in the frequency of the genotypes between the two groups. For the polymorphism in codon 10, our findings were consistent with those of a previous study conducted on a Brazilian population (3); in both studies, there was no statistically significant difference in the genotype between the chronic HCV patients and healthy controls.

Awad et al. (14) and Dunning et al. (15) observed that individuals with the G allele in TGF- β 1 codon 25 exhibited a high cytokine-production phenotype. In our study, most of the Iranian patients in both the HCV and control groups exhibited high TGF- β 1 production. Furthermore, according to Pereira et al. high TGF- β 1 production is associated with increased HCV susceptibility; therefore, we hypothesize that patients with the GG genotype in codon 25 are more susceptible to HCV than other populations. Because of the predominance of a high-production phenotype among the studied Iranian population and the effect of high TGF- β levels on increased susceptibility to viral infections similar to HCV, we assume that most of the patients in our population are more susceptible than those in other populations, such as Brazilians. However, the results of our study indicate that TGF- β 1 polymorphisms cannot be used as prognostic factors for chronic HCV infection in the Iranian population.

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