

RESEARCH ARTICLE

Tumor Necrosis Factor Alpha -308 G/A Single Nucleotide Polymorphism and Susceptibility to Hepatocellular Carcinoma Via Hepatitis B Infection

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Abstract

Background: Hepatitis B virus (HBV) is a key factor for hepatocellular carcinoma (HCC). About 350 million people are affected by chronic infection which is related to the rapid development of liver diseases as well as hepatitis, cirrhosis and hepatocellular carcinoma. Expression of tumor necrosis factor alpha (TNF- α) in the liver demonstrates a major genetic polymorphism which is involved in resistance or susceptibility to chronic HBV infection. **Materials and Methods:** In this study, two populations were studied by the sequence specific primer-polymerase chain reaction (SSP-PCR) method: HBV cases (n=409), who were HBS-Ag+, and healthy controls (n=483). **Results:** The results shown that the frequency of TNF- α -308 G/G genotype in healthy controls (47.2%) was significantly higher than in HBV infected patients (28%) (CI = 1.29–2.61, OR = 1.83, P = 0.0004). Also TNF- α -308 A/A and A/G genotype frequencies in the healthy controls were 4.6% and 48.2% and in patient group were 19.5% and 52.5% (CI = 2.23–7.12, p: 0.0001, OR: 3.94) respectively. **Conclusions:** We found that among Iranian people TNF- α -308A allele not only has the highest genotype frequency but also it has the highest frequency in the world population. In addition, TNF- α -308 G/G polymorphism was associated with HBV resistance, whereas TNF- α -308A (A/A or A/G) polymorphism appeared to associated with chronic HBV infection. These data suggested that among the Iranian population, the -308 G/G polymorphism of TNF- α gene promoter region has the potential to influence the susceptibility to HBV infection and it may be responsible for viral antigen clearance.

Keywords: Gene susceptibility - hepatitis B virus - predisposing factor - tumor necrosis factor alpha

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Introduction

Hepatitis B virus (HBV) is a hepatotropic virus which replicates within hepatocytes and causes liver cirrhosis and hepatocellular carcinoma (HCC) progression. According to the World Health Organization, it estimates that 2 billion people have been infected with hepatitis, and more than 350 million people in the world are infected with Chronic HBV. (Wang et al., 2004; Gao et al., 2012). The carrier rate varies from low (0.1-2%) in USA and Western Europe, to intermediate (2-8%) in Mediterranean countries and Japan and high (8-20%) in sub-Saharan Africa and parts of Asia. In highly endemic areas, the majority of individuals infected in either prenatally or in early childhood, whereas in low prevalence areas the infection is acquired primarily in adulthood. (Di Bisceglie, 2009).

According to some studies in Iran, most HCC patients are positive for at least one of the HBV markers (Shamszad and Farzadegan, 1982; Merat et al., 2000). A study in Iran represented that the original cause of HCC was hepatitis B (Hajjani et al., 2005). Other assessments mentioned that

the risk of HCC decreases via prevention of infection with HBV (Pourhoseingholi et al., 2010; Wang et al., 2015).

HBV has an unusual circular DNA which is not fully Double strand. The full length strand has 3020-3320 nucleotides and the short length-strand has 1700-2800 nucleotides. (Beck and Nassal, 2007). The virus particle consists of an outer lipid envelope and an icosahedral nucleocapsid core composed of protein. (Kay and Zoulim, 2007; Pungpapong et al., 2007). It is possible that both host and viral factors are involved in the severity of chronic HBV infection. For this reason the main focus of HBV studies is to determine the infection situation in order to understand whether an infection will persist or terminate. (Seeger and Mason, 2000). It is generally believed that HBV does not have a direct cytopathic effect on hepatocytes. However some investigators shown that in patients with HBV-positive, the cellular immune response against the viral antigens presented in infected hepatocytes will damage the liver. It is already shown that there is a relation between susceptibility to chronic HBV infection, human leukocyte antigen (HLA) class II genes,

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and cytokine genes. (Zuckerman, 1996; Kummee et al., 2007). Therefore, it expected that the genetic components like Tumor necrosis factor alpha (TNF- α) in chronic HBV infection have immune response genes.

Tumor necrosis factor alpha (TNF- α) is a powerful pro-inflammatory cytokine that plays a vital role in the modulation of immune functions. It has been shown that TNF- α mediate host responses to infections and also it has a role in development of the autoimmune diseases. (Zuckerman, 1996). Since TNF- α gene encoding is highly inducible, genetic variations in the promoter region may influence TNF- α production. On a basis of this hypothesis, a number of single nucleotide polymorphisms (SNPs) in the TNF- α promoter region have been investigated for their effect on gene transcription as well as for their possible correlation with a particular disease. It is already known that SNPs at position 376 (TNF376), 308 (TNF308) and 238 (TNF238) are G to A substitution. (Locksley et al., 2001). In 2005, Xu et al. consider the TNF- α 308 G/A frequency alleles. They confirm that TNF- α 308 G/A and the allele A in chronic patients with severe disease has higher frequency relative to controls, asymptomatic patients and patients with mild disease. They find that the level of TNF- α serum is significantly high in G/A genotype patients with severe disease in compare with those with G/G genotype. (Wirz et al., 2004). A few years later, Ming-Hua Zheng et al. (2010) proposed that the frequency of TNF- α is influenced by ethnicity, too. They showed that decreased risk is associated with -308 variant genotypes (G/A and A/A) in Mongoloid populations. However, no significant associations were found in Caucasoids. Nevertheless, no significant associations were found in the group of healthy cases. (Xu et al., 2005). Furthermore, in the same year, Wang B et al showed that besides ethnicity, the gender can also be a possible effective factor on the liver cirrhosis patients. In the case of females in compare with persistent infection, liver cirrhosis patients had significantly high frequency of A allele (Zheng et al., 2010).

In the present study TNF- α promoter polymorphism at position -308G>A was considered as a candidate to investigate in 892 Iranian people. Meanwhile, the distributions of genotypes were compared between HBV infected cases and healthy controls by statistical analysis. Finally, our results guide us toward a hypothesis that there is the possible correlation of TNF- α promoter polymorphisms with HBV infection resistance.

Materials and Methods

Subjects

A total number of 892 northeastern Iranian people were classified in two different groups; HBV infected cases (n=409) with HBS-Ag-positive, and healthy controls (n=483) (Table 1). HBV infected cases were collected among the people whose HBV titer was tested by the Medicine Cellular and Molecular Research Center (MCMRC) located in Gorgan city. Healthy individuals were selected from blood donation centers of Golestan province through HBS-Ag negative with no medical history of autoimmune or inflammatory disorders.

Furthermore, in all cases precluded environmental factors with the same sex and ethnicities were also considered. The study was performed by approval of the Local Ethical Committee, and informed consent was obtained directly from research participants. None of the approached subjects refused to participate.

DNA Extraction and Genotyping

Red blood cells were lysed three times with a buffer containing ammonium chloride, potassium dihydrogen phosphate, and disodium hydrogen phosphates. Then, SDS (10%), EDTA, and proteinase K were added to the pellet and incubated for 1 h at 65°C. After incubation, a phenol/chloroform/isoamyl alcohol mix was added to samples. After that the mixtures were centrifuged. To precipitate DNA, isopropanol and sodium acetate were added to the supernatant and DNA was extracted after centrifugation. DNA samples were aliquot in graded distilled water, and DNA concentrations were determined by a UV spectrophotometer at 260 nm (Technique, UK). All samples were diluted and stored at -80°C for future analysis.

Polymerase Chain Reaction

The SSP-PCR (Sequence Specific Primer-Polymerase Chain Reaction) method was used for genotyping (Mansoori et al., 2015); 100ng of genomic DNA was amplified in a 15 μ l reaction mix with 9.5 μ l master mix containing 20 μ M dNTP, 1X ready-load PCR buffer, 12% sucrose (Merck, Germany), one unit Taq polymerase (QIAGEN, Germany), and 30 μ M of each specific primer (MWG, Germany).

The primer for TNF- α promoter -308 was designed as specific G and specific A (Table 2). Polymerase chain reaction amplification was performed in a thermal cycler (Technique, UK). The cycling conditions for promoter 308 were set as follows: 1 minute at 95°C; 10 cycles of 15 seconds at 95°C, 50 seconds at 64°C, and 40 seconds at 72°C; 20 cycles of 20 seconds at 95°C, 50 seconds at 58°C, and 50 seconds at 72°C; and 5 minutes at 72°C as final extension. The PCR product size was 117-bp. At the end 10 μ l of the products were loaded into 1.5% agarose gel containing ethidium bromide for electrophoresis. The polymorphism was divided into A/A homozygote, G/G homozygote and A/G heterozygote types.

Statistical analysis

Data were analysed by SPSS v-16 program, and the means of parametric variables were calculated. Data were presented as Mean \pm SD for parametric variables and as percentages for non-parametric values. Alleles and genotype frequencies were calculated and compared by non-parametric tests followed by Fisher's exact analysis using STATA v-8 (CA, US). P-values were determined, and those less than 0.05 were considered to be significant.

Table 1. Demography of Patients and Healthy Control Individuals

	Case	Control
Average Age	32 \pm 8.6	36 \pm 8.43
Sex	f: 25.6%, m: 74.4%	f: 44.48%, m: 55.52%

Age (year); f: female; m: male

Table 2. Primer Sequences Used for the TNF Sequence-Specific Primer Genotyping Method

Primer Position	Primer Sequence	Tm
TNF-308 A	5`-ATAGGTTTTGAGGGGCATGA-3`	60°C
TNF-308 G	5`-ATAGGTTTTGAGGGGCATGG-3`	
Generic antisense	5`-TCTCGGTTTCTTCTCCATCG-3`	
HGH (sense)	5`-GCCTCCCAACCATTCCCTTA-3`	64°C
HGH (antisense)	5`-TCACGGATTTCTGTTGTGTTTC-3`	62°C

HGH=human growth hormone; Tm=melting temperature; TNF= Tumour Necrosis factor.

Table 3. Allele and Genotype Frequencies of -308 Position in HBV Infected Cases and Controls

Genotype	Case (%)	Control (%)	P	OR	95%CI
A/A	80(19.5%)	22(4.6%)	1	-	-
A/G	215(52.5%)	233(48.2%)	0.0001	3.942	2.228-7.119
G/G	114(28%)	228(47.2%)	0.0004	1.836	1.293-2.615
Allele "A"	375(45.84%)	277(28.67%)	1	-	-
Allele "G"	443(54.16%)	689(71.33%)	0.0001	2.1006	1.672-2.637

OR= odds ratio. CI= 95% Confidence Interval of the odds ratio. P= p-value

Results

In order to realize that the TNF- α promoter polymorphism (-308G>A) is related to hepatitis B virus infection or not, a total number of 892 northeastern Iranian people were selected as two groups; HBV infected and healthy controls. After taking the individuals blood, their DNA was extracted and then their genotype was identified, too. Afterward, the frequencies of genotypes were analyzed statistically. The results showed that the frequency of TNF- α -308G/G genotype in healthy controls (47.2%) was significantly higher than the HBV infected patients (28%) (CI = 1.29-2.61, OR = 1.83, P = 0.0004). While the frequency of TNF- α -308 A/A and A/G genotype in healthy controls was 4.6% and 48.2% and in HBV infected patients was 19.5% and 52.5% (CI = 2.23-7.12, p: 0.0001, OR: 3.94) respectively. The frequency of allele A and G in healthy controls (28.67% and 71.33%) was quite different (CI = 1.672-2.637, p: 0.0001, OR: 2.1006) from the patient group (45.84% and 54.16%) (Table 3).

Discussion

The TNF- α gene is located in the class III region of the major histocompatibility complex (MHC) between HLA-B and DR. The expression of this gene is tightly controlled at the transcriptional and post-transcriptional level. (Wang et al., 2010). Up to now, the association of polymorphisms of various genes and HBV infection has been evaluated (Ben-Ari et al., 2003; Kim et al., 2003; Li et al., 2012; Tayebi and Mohamadkhani, 2012; Jeng et al., 2014; Chanthra et al., 2015; Limothai et al., 2015; Nun-Anan et al., 2015).

TNF-alpha gene polymorphism in HBV patients cause effective inhibition of HBV and progression of End Stage Liver Disease (ESLD). Therefore, it can be a valuable factor to predict ESLD development in patients with chronic HBV infection.(Kim et al., 2003).

In the present study the frequency of TNF α -308 alleles were measured in 409 HBV patients and 483 healthy controls of Iranian northeastern population. Following, the polymorphisms were identified at TNF- α -308 position for all cases. We found that the frequency of

TNF- α -308 A/A polymorphism was significantly higher (19.5%) in HBV-infected individuals than those of healthy controls (4.6%). Besides, TNF- α -308 G/G polymorphism was significantly more common in control group (47.2%) than the HBV carriers (28%). We also found that TNF- α -308 A allele frequency was significantly more common (45.84%) in HBV-infected individuals (both patients and carriers) than those of healthy controls (28.67%). Statistically significant differences were still kept after Bonferroni correction of the p-values for only TNF- α -308 G allele frequency in patients or carriers.

In 2008, Basturk B et al reported that patients carrying the -308G/G genotype had a significantly increased risk of HBV persistence in compared with those with G/A or A/A genotype which is not in agreement with our results.(Basturk et al., 2008). They reported that the polymorphisms -308 G in the TNF- α gene promoter region might be a risk factor for HBV persistence. The TNF- α -308 G/A has been shown to be associated with elevated TNF- α transcriptional activity. (Kim et al., 2003). Whereas the previous studies on the TNF- α -308 gene promoter polymorphism in chronic HBV infection have reported conflicting results. (Wang et al., 2010).

We presume that the ethnicity might play an important role in HBV infection outcome which leading to inconsistent results.

Taking all previous studies together with our finding we can suggest that TNF- α -308 promoter polymorphisms do not play a direct role in the susceptibility and pathogenesis of HBV infection. In addition, our data shown that A allele is significantly related to HBV infection.

As shown in Table 1, gender can influence in the HBV infection. Demographic information showed most of the subjects was male. This finding supports previous reports (Kumar et al., 2014). Somi et al in 2006 revealed that TNF- α promoter polymorphism -308A is common in Iranian population, but has no association with development of chronic HBV infection. Our result showed that TNF- α -308 G allele and G/G genotype were associated with HBV resistance whereas TNF- α -308A (A/A or A/G) polymorphism were associated with chronic HBV infection in the studied population. Apparently this association is not depend on transcriptional activity of

TNF- α -308 Polymorphism. Hence, the effect of SNP on the production of TNF- α is not established. Assessment of concurrent local and the amounts of TNF-plasma might elucidate this phenomenon. Finally we have provided some degree of evidence about the interaction between TNF- α -308 A as a part of genetic predisposing factor to HBV. The data reported here suggesting that TNF- α -308 G/G polymorphism is associated with HBV resistance whereas TNF- α -308A (A/A or A/G) polymorphism is associated with chronic HBV infections. More studies are needed to explain how TNF- α is involved in the pathogenesis of HBV infection.

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