

## RESEARCH ARTICLE

# Interleukin-6-174 Promoter Polymorphism and Susceptibility to Hepatitis B Virus Infection as a Risk Factor for Hepatocellular Carcinoma in Iran

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### Abstract

**Background:** Hepatitis B virus (HBV) is a major risk factor for hepatocellular carcinoma (HCC). Cytokines play an important role in the regulation of immune responses and defense against viral infections. Human interleukin 6 (IL6) is a multifunctional cytokine that participates in these processes. **Objective:** The aim of this study was to assess the IL6-174 gene polymorphism in patients with chronic hepatitis B virus (HBV) infection as compared with healthy controls in an Iranian population. **Materials and Methods:** Totals of 297 HBV patients and 368 control individuals were evaluated. Genomic DNA was extracted from peripheral blood and the SSP-PCR (sequence specific primer-polymerase chain reaction) method was applied for genotyping. **Results:** The frequencies of genotypes C/C, G/G and C/G in HBV cases were 4.7%, 34.3%, 60.9% and in controls were 12.8%, 39.7% and 47.6%, respectively. The frequencies of G and C allele in patients and controls were 78.1%, 21.9% and 67.4%, 32.6% respectively. There was a significant difference in the frequencies of G/G genotype (CI=1.8-7.1, OR=3.47, P=0.00001) and G allele (CI=1.34-2.23, OR=1.72, P=0.0001) between HBV patients and the control group. **Conclusions:** These findings suggest that the IL6-174 C/G genotype and the G allele are strongly associated with susceptibility to HBV infection. Demographic information showed that most of the subjects were male (74.4%). According to high frequency of G/G genotype in male participants (63.1%) men probably are more susceptible to hepatitis than women.

**Keywords:** Hepatitis B virus (HBV) - hepatocellular carcinoma (HCC) - interleukin-6 - Iran - polymerase chain reaction

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### Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer. Cirrhosis is the most important risk factor for HCC while the main agents that lead to the development of HCC are hepatitis C virus (HCV) and hepatitis B virus (HBV) (Colombo et al., 1989). It is estimated about 350 million people are infected to chronic hepatitis B virus (HBV) throughout the world and is a major cause of liver cirrhosis and HCC (Wang et al., 2004; Gao et al., 2012).

Some investigations in Iran show majority of HCC patients are positive for at least one of the HBV markers (Shamszad and Farzadegan, 1982; Merat et al., 2000) and a study in South of Iran demonstrated that the outstanding cause for HCC was hepatitis B (Hajiani et al., 2005). Other evaluations mentioned that prevention of infection with HBV decreases the risk of subsequent HCC (Pourhoseingholi et al., 2010; Wang et al., 2015).

It is believed that HBV does not have a direct cytopathic effect on hepatocytes but some researchers have confirmed that in HBV-positive patients, the viral antigens that are presented in infected hepatocytes and targeted by

the cellular immune response and consequently, cause the liver damage. Studies have indicated that the immune response genes are the genetic component in chronic HBV infection. Human leucocyte antigen (HLA) class II genes and cytokine genes are associated with susceptibility to chronic HBV infection (Kummee et al., 2007).

Cytokines have a key role in the regulation of the immune response and defensive effect against viral infections. Polymorphisms in the cytokines genes determine the capacity of cytokine production among various individuals (Rapicetta et al., 2002; Lu et al., 2015). Human interleukin 6 (IL6) is a multifunctional cytokine that interfere in the regulation of immune response (Kakumu et al., 1993; Galun et al., 2000). Studies represented, serum levels of IL6 is increased significantly in HBV patients (Malaguarnera et al., 1997; Song et al., 2000). It has been shown that human interleukin 6 (IL6) facilitate hepatitis B virus infection *in vivo* and *in vitro* (Galun et al., 2000).

There are three single nucleotide polymorphisms (SNP) at positions -597 G/A, -572 G/A and -174 G/C within the IL6 gene promoter region that prior studies indicated a cooperative influence of these SNPs on

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regulation of IL6 transcription (Fishman et al., 1998; Terry et al., 2000).

It is indicated that a polymorphism in the promoter region of IL-6 (position -174), would affect on the level of IL6 expression (Fishman et al., 1998). Based on previous reports, the polymorphism of this position is associated with some disease like diabetes (Fernandez-Real et al., 2000; Hamid et al., 2005) Alzheimer (Capurso et al., 2004) and multiple sclerosis (MS) (Shahbazi et al., 2010). Previous studies also confirmed the association of this polymorphism and HCV infection (Nattermann et al., 2007; Cussigh et al., 2011).

In case of the IL6-174 G/C promoter polymorphisms in susceptibility to hepatitis B virus (HBV) infection, there are deferent findings. Indeed, this relationship is not fully understood and was denied in some studies (Park et al., 2003; Migita et al., 2005). Fabris C. et al in 2009 for the first time indicated a relationship between this position and the course of chronic HBV infection (Fabris et al., 2009).

The aim of this study was to evaluate whether IL6-174 G/C promoter polymorphisms are related to HBV infection resistance in an Iranian population in the north of Iran.

## Materials and Methods

### Study population

A total number of 665 Iranian people were classified in two different groups; HBV infected cases (n=297) with seropositive Hepatitis B surface antigen (HBsAg) and healthy controls (n=368). The HBV infected cases were collected from people whose HBV titer was tested by the Medical Cellular and Molecular Research Center (MCMRC) of Talghani Hospital (Gorgan, Iran). Healthy people who referred to Gorgan blood donation centers in Golestan province with HBS-Ag negative without history of autoimmune or inflammatory disorders were selected from the Northeast of Iran.

### Demographic information

Average age of patients were 32±8.6 years and healthy individuals 36±8.43 years and sex ratio male to female in patients and controls was female: 25.6%, male: 74.4% and female: 44.48%, male: 55.52% respectively. For the above samples people with precluded environmental factors with the same sex and ethnicities were selected.

### Serological markers

HBsAg and antibodies to HBsAg (anti-HBs antigen) measurement were assessed by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Hepanostika, BioMérieux, Boxtel, Netherlands).

### HBV DNA extraction

Peripheral blood samples were gathered in EDTA tubes and genomic DNA was elicited using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

### HBV load measurement

HBV viral load was measured by real-time polymerase chain reaction (RT-PCR) using the Artus Light Cycler

HBV DNA Kit (Qiagen; Hilden, Germany) and Applied Biosystem 7300 Real Time PCR System (ABI).

### Genomic DNA extraction

Genomic DNA was extracted from 5 ml peripheral whole blood by modified standard protocol (Shahbazi et al., 2002). Briefly, red blood cells were lysed three times by lysis buffer. Then, SDS (10%), EDTA and 10 µl proteinase K were added to the remained white cells. After incubation for 1 hour at 65 °C, a phenol/chloroform/ isoamylalcohol mix was added and centrifugation was performed after strongly mixing. The supernatant was separated and isopropanol and sodium acetate were added for precipitating the DNA. Visualized DNA was extracted after centrifugation and was dissolved in distilled water. Then, the concentration was determined by spectrophotometer at 260 nm (Techne, UK) and DNA samples were maintained in -20°C.

### Interleukin-6 genotyping

The SSP-PCR (sequence-specific primer-polymerase chain reaction) method was applied for genotyping (Mansoori et al., 2015); Each PCR reaction contained 100-150 ng of DNA, 9.5 ml master mix containing 20 mM dNTP, 1X Ready load PCR buffer, 12% Sucrose (Merck, Germany), one unit Taq DNA polymerase (GenetBio, Korea), 6 mM human growth hormone (HGH) primer as an internal control, and 30 mM of each specific IL6 primer. The sequences of used primers are presented in Table 1.

The PCR reaction was carried out in a Thermal Cycler (Techne, UK), with the following program: 1min at 95°C followed by 10 cycles of 15 s at 95°C, 50 s at 58°C, 40 s at 72°C, followed by 20 cycles of 20 s at 95°C, 50 s at 54°C and 50 s at 72°C, with 5min at 72°C as final extension.

PCR product was submitted to electrophoresis using 1.5% agarose gel (Merck, Germany) and stained by ethidium bromide. Using a gel documentation system (UVITEC, UK), DNA bands were visualized under the UV radiation.

### Statistical analysis

Genotyped were tested for deviation from Hardy-Weinberg equilibrium using web program (<http://ihg.gsf.de/cgi-bin/hw/hwa2.pl>). Statistical analysis was done by the Statistical Program for Social Sciences (SPSS version 17.0) and the means of parametric variables were calculated. Data were presented as Mean±SD for parametric values and as percentages for non-parametric

**Table 1. Primer Sequences Used for the IL6 SSP Genotyping Method**

Primer position	Primer sequence
IL-6 (-174)C	5'-CCC CTA GTT GTG TCT TGC C-3'
IL-6 (-174)G	5'-CCC CTA GTT GTG TCT TGC G-3'
IL-6 (-174) Generic	5'-GCC TCA GAG ACA TCA CCA GTC C-3'
*HGH (sense)	5-GCCTTCCCAACCATTCCTTA-3
HGH (antisense)	5-TCACGGATTCTGTGTGTTTC-3

\*HGH=human growth hormone

**Table 2. Frequency of the IL-6 Allele and Genotypes among Patients (N=297) and Controls (N=368).**

Alleles and genotype	Control n (%)	Hepatitis n (%)	OR*	95% CI*	P-value*
C/C	47 (12.8 %)	14 (4.7 %)	1	-	-
C/G	146 (39.7%)	102 (34.3 %)	2.35	1.2-4.8	0.0082
G/G	175 (47.6%)	181 (60.9 %)	3.47	1.8-7.1	0.00001
C/G + G/G	321(87.3%)	283(95.2%)	-	-	-
C/G + C/C	193(52.5%)	116 (39%)	-	-	-
C/G + G/G vs. C/C	-	-	2.96	1.56-5.94	0.0004
C/G + C/C vs. G/G	-	-	0.58	0.42-0.80	0.0006
C	240 (32.6%)	130 (21.9%)	1	-	-
G	496 (67.4%)	464 (78.1%)	1.72	1.34-2.23	0.00001

P values were determined by fisher's exact test for allele frequencies, and by logistic regression test followed by Bonferroni's correction for genotype frequencies

**Table 3. L6 Sex Cross Tabulation in Study Participants**

IL6 Genotype	sex		Total
	Men n (%)	Women n (%)	
CC	13 (21.3%)	48 (78.7%)	61(100.0%)
GG	209 (63.1%)	122 (36.9%)	331(100.0%)
CG	142 (60.7%)	92(39.3%)	234(100.0%)
Total	364 (58.1%)	262(41.9%)	626(100.0%)

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.

## Results

In the present study we evaluated position IL6-174 SNP in 297 HBV patients and 368 control subjects using the SSP-PCR method. The study has been carried out at North-Eastern Iran. Each subject participated voluntarily, however they could cancel their participation at any time.

### IL-6 Gene Promoter Polymorphisms Comparison between Patients and controls

Results showed that there was a significant difference in allele frequencies between the two groups. C allele were more frequent among healthy people compared with hepatitis B patients (OR=1.72, 95% CI=1.34-2.23, P=0.0000)

In addition, the G/G genotype was more frequent among patients (60.9 %) in comparison with control subjects (47.6%). The frequency of the C/G genotype was not significantly different between patients (34.3 %) and controls (39.7 %). Complementary data are presented in Table2.

## Discussion

HCC distribution in the world is mostly due to spread of hepatitis B and hepatitis C virus. In Eastern Asia and sub-Saharan Africa, high frequency of HBV infection is the major risk factor for acquiring HCC (Parkin et al., 2005; Fallot et al., 2012).

To date, the association of polymorphisms of several genes and HBV infection has been assessed by several studies (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).

In the present study we aimed to verify the possible interference of IL6 -174 G/C polymorphism in HBV infection. Subsequently, we evaluated 297 HBV patients and 368 control individuals via SSP-PCR and gel electrophoresis methods.

This simple PCR method is a cost effective method that apply three primer types for determining one of two alleles in each DNA sample (Table 1). HGH (human growth hormone) internal control has been used for eliminating liar responses in each PCR reaction.

The human IL6 gene is located on chromosome 7p21 and is composed of five exons and four introns. IL6 is a cytokine that plays a key role in the maturation and differentiation of the immune system (Galun et al., 2000; Rapicetta et al., 2002).

As discussed above, Carlo Fabris and et al. demonstrated a relationship between IL6 -174 polymorphism and HBV infection (Fabris et al., 2009). This relationship was not detected by Migita K and Park BL in Far East Asian patients (Park et al., 2003; Migita et al., 2005).

In this evaluation, a highly statistically significant difference in the frequency of the G/G polymorphism was observed between HBV patients and controls. Our study exhibited that the G allele and G/G genotype at the IL6 -174 position had higher distribution among Iranian HBV patients (78.1% and 60.9 % respectively) in comparison to controls (67.4% and 47.6 % respectively) (Table 2). The majority of the patients (60.9 %) revealed the potential for producing G/G genotype compared with 47.6 % of the control group (p=0.000).

In conclusion, these findings suggest that the IL6 G allele was strongly associated with susceptibility to HBV infection.

Demographic information showed most of the subjects was male. This finding supports previous reports (Kumar et al., 2014). In general analysis, G/G genotype frequency in men is more prevalent while C/C genotype frequency in women is more common. According to G/G genotype frequency in patients can be said perhaps the men more than the women are susceptible to hepatitis (Table 3).

Taken together, the results could be explained by divergences in the genetic background of the Iranian population. Although we did not study the IL6 serum levels in our population and it is still unclear, but it can be evaluated in the future. It is predicted that there is an association between genotype and expression level of IL6 gene.

It has been previously confirmed that substitution from the G to C allele at this position suppresses the transcription of IL6 (Shahbazi et al., 2002) which could explain how this SNP decreases the risk of development of disease.

In conclusion, the IL6 gene can be an important factor that affects the outcome of HBV infection and subsequent HCC and people with allele C at position -174 in the IL-6 gene may be protected against HBV infection;

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