

Lack of Association of Interleukin 12 p40 Subunit Polymorphism (IL-12B +1188) and Risk of Chronic Hepatitis B Infection in Iranian Patients

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Article information	Abstract
<p>Article history: Received: 9 April 2011 Accepted: 17 September 2011 Available online: 27 June 2011</p> <p>Keywords: Hepatitis B IL-12 Single nucleotide Polymorphism</p> <p>*Corresponding author at: Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran E-mail: srmohebbi@gmail.com</p>	<p>Background: Hepatitis B virus is a major cause of incidence of chronic hepatitis in the world. Although the cause of chronic infection in individuals is not well known, cytokines that are of the main factors to regulate the immune reaction play an important role in determining the clinical course of infection of this virus, and host genetic background and single nucleotide polymorphisms have a decisive effect on the level and performance of cytokines. Thus, the aim of this study is to investigate the relationship between the single nucleotide polymorphisms of 3' noncoding region of IL-12 p40 subunit gene on position +1188, and susceptibility to chronic hepatitis B.</p> <p>Materials and Methods: This case-control study was performed on a population including 140 patients with chronic hepatitis B and 150 healthy people. PCR-RFLP method was used to determine genotype of individuals. In order to confirm the genotyping results, sequence of 10% of samples was reviewed using direct sequencing.</p> <p>Results: The frequency of AA, AC and CC genotypes of this gene at position +1188 was respectively calculated 56.4, 36.4 and 7.1% in group of patients and 59.3, 33.3 and 7.3% in the control group. No significant difference was found between both case and control groups ($p = 0.487$).</p> <p>Conclusion: The frequency of different genotypes of this gene in the studied population is similar to the results of most studies conducted in different countries. The results of this study shows no association between IL-12B +1188 polymorphism and the risk of chronic hepatitis B in Iranian population.</p> <p>© 2012 Zahedan University of Medical Sciences, ZJRMS. All rights reserved.</p>

Introduction

Clinical consequences of hepatitis B infections are widely varying from spontaneous recovery to chronic form, liver cirrhosis and hepatocellular carcinoma. In addition to the viral virulence factors, different function of immune system in various people is the most important factor in determining clinical course of infection. Also, host genetic background and polymorphisms in various regions of genes involved in immune system and cytokines play an important role in determining the immunization process and clinical course of viral infections [1, 2]. Cytokines are proteins with critical role as in regulation of stages of differentiation; maturation and activation of immune cells and their gene polymorphisms can influence the output of immune system activities. IL-12 is one of the most important pre-inflammatory cytokines involved in regulation of immune system which consists of two subunits (35 and 40 kDa) (P35 and P40). Gene of each subunit is located on a different chromosome (p35 on chromosome 3 and p40 on chromosome 5). This cytokine has the ability to adjust the balance between function of Th₁ and Th₂ and plays a key role in induction of interferon-gamma secretion, regulation

of Th₁-dependent immunity, stimulation of natural killer cells and generally in regulation of immune response to intracellular pathogens including viruses [3, 4]. In hepatitis B infection, induction of Th₁ cells activity stimulates cytotoxic T lymphocytes to the lysis of virus infected cells. Regional secretion of gamma interferon by cytotoxic T lymphocytes induces the secretion of IL-12, which in turn leads to the induction of Th₁ immunity and more secretion of interferon-gamma. Therefore, in patients with hepatitis B who are able to increase production of their IL-12 appropriately, interferon-gamma will be also produced at a proper level and will efficiently apply its effect on the inhibition of virus transcription in infected cells. Thus, increased production of IL-12 plays a key role in viral clearance in infected people [5].

Due to the broad and various functions of IL-12 in immune system, various studies have been conducted to understand the relationship between single nucleotide polymorphisms (SNP) of genes encoding its subunits and the risk of immune-related diseases. Several polymorphisms can be seen in the regions of promoter, introns and 3' untranslated region (3' UTR)

of IL-12B gene (encoding p40subunit), including +1188 single nucleotide polymorphism of 3' non-coding region of this gene, whose relationship with clinical course of various immune system-related diseases have been revealed by several researchers so far [6, 7]. Assuming that this polymorphism may affect the production of IL-12 and regulation of immune response to viral infections, the aim of this study is to determine the relationship of +1188 A/C single nucleotide polymorphisms (located in 3' non-coding region) of IL-12B gene with susceptibility to chronic hepatitis B in Iranian population

Materials and Methods

This case-control study was conducted by sampling from 140 patients with chronic hepatitis B who referred to Taleghani Hospital in Tehran during 2008-2010 whose infection was confirmed through ELISA test for HBs antigen and HBc anti-antibody and 150 healthy volunteers. All patients included in the study underwent the objectives of the research project and the consent form of Ethics Committee of research center for gastroenterology and liver diseases of Shahid Beheshti University of medical sciences was obtained from them. Complete genomic DNA of individuals was extracted from 4 ml of peripheral blood using phenol method [8] and PCR-RFLP technique was used to determine the genotype of individuals. PCR amplification stage was performed using a couple of primers with sequences listed in table 1 which were designed by Gene Runner software ver. 3.05 (Hasting software Inc.) and Primer BLAST Section of the Website of National Center of Biotechnology information of the United States (<http://www.ncbi.nlm.nih.gov>). A part of IL-12B gene including +1188 polymorphism was amplified through polymerase chain reaction under the following conditions:

100 ng genomic DNA was added to the reaction mixture containing Taq buffer, (10mM Tris-chloride (pH=9), 50mM potassium chloride, 0/1% Triton X-100), 2 units of Taq DNA polymerase (Super Taq, England); 1/5mM magnesium chloride, 0/2mM of each dNTP and 10pM of each primer in the final volume of 25µl. PCR was performed by automatic thermal cyler (Eppendorf, Germany) as follows: First, the initial denaturation was performed at 95°C for 5 minutes followed by 35 cycles of temperatures of 95°C for 30 seconds, 58/5°C for 30 seconds and 72°C for 45 seconds and then 72 degrees Celsius for 10 minutes for the final amplification of DNA fragment. PCR product was detected on Agarose gel (Roche, Germany) using ethidium bromide staining on ultraviolet light. After that, PCR product entered into enzymatic digestion with TaqI enzyme (Fermentas, Lithuania) as follows: 10 µl of the PCR product was directly added to a mixture containing Taq I buffer (10 mM Tris-chloride (pH= 8), 5 mM magnesium chloride, 100 mM sodium chloride, and

0/1 mg/ml of bovine serum albumin) and 2 units of Taq I enzyme in a final volume of 20 µl and incubated for 16 hours in 65 degrees Celsius. Digestion product was also revealed with 2% Agarose gel electrophoresis. To confirm the genotyping results, 10 percent of samples were sequenced through direct sequencing using ABI genetic analyzer 3130xl system. Statistical analysis of data was performed using SPSS-13 software and variables were compared by 2 test. $p < 0/05$ was considered significant. The mean difference was also performed based on independent t-test.

Results

Specifications of patients group and healthy control subjects are presented in table 2 in terms of age, gender and body mass index. As observed, there is no significant difference between case and control groups in terms of gender, age and the average body mass index (BMI).

Table 1. Specifications of the primers used in PCR

Primer	Annealing Temperature	Primer sequence (5' to 3')	G+C content
Forward	55.4	GGCATGAAATCCTGAAACC	50
Reverse	53.2	TACATCCTGGCAGACAAACG	50

Table 2. Specifications of the study population

		Case	Control	p-Value
Gender(n%)	Male	77 (55)	78 (52)	0.876
	Female	63 (45)	72 (48)	
Age (year)		42.3±15.2	41.3±17.1	0.625
BMI		26.29	24.27	0.419

* P index have been set between patient and control groups.

By performing PCR, a product was obtained with length of 421 bp. Due to enzymatic digestion of PCR product, a fragment of 421 bp was observed as an uncut PCR product in homozygous A individuals, two fragments of 259 and 162 bp in individuals of homozygous C, and three fragments of 421, 259 and 162 bp in individuals of heterozygous (Fig. 1). After genotyping processes, the results of the genotype distribution were compared between cases and control group and the relationship between them was analyzed. The sequencing results also confirmed the RFLP results (Fig. 2).

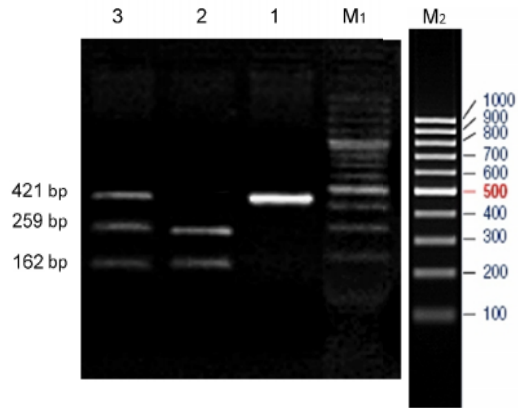


Figure 1. The results of agarose gel electrophoresis of enzymatic digestion product:

- 1) AA homozygous genotype 2) CC homozygous genotype 3) AC homozygous genotype
 M1) Molecular weight marker (GeneRuler 100 bp DNA Ladder)
 M2) GeneRuler 100 bp DNA Ladder Map (Fermentas, Lituania)

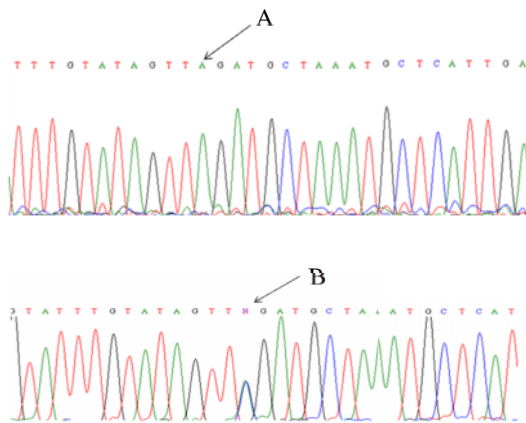


Figure 2. Results of sequencing

- A) The sequence related to the individual of homozygous AA, at polymorphism position (marked with arrows) is seen not only the peak corresponding to nucleotides A
 B) The sequence related to the individual of heterozygous, at polymorphism position both peaks of nucleotides A and C are observed.

Frequency of different genotypes of +1188 polymorphisms in the untranslated end of IL-12 gene in the two studied groups were as follow AA (56.4%), CA (36.4%) and CC (7.1%) for patients and AA (59.3%), CA (33.3%) and CC (7.3%) for the control group. Statistical analysis showed that *p* value equals to 0.487; therefore, there is no significant difference between the patient and control groups in terms of genotype distribution (Table 3).

Table 3. Frequency of genotypes in the studied population

Genotype	Case	Control	<i>p</i> -Value
AA	79 (56.4%)	89(59.3%)	0.487
CA	51 (36.4%)	50 (33.3%)	
CC	10 (7.1%)	11 (7.3%)	

Discussion

In this study, the genotype of a population including over 290 patients with chronic hepatitis B and healthy people was determined according to their +1188 polymorphism of 3' untranslated region of IL-12B gene. Statistical survey of the results and comparison of different genotypes in case and control groups shows that there is no statistically significant difference between these two groups, and this confirms the lack of association of this polymorphism with the risk of chronic hepatitis B infection in the studied population.

IL-12 is a cytokine with critical role against intracellular pathogens, performance of Th₁ cell differentiation induction, cell-dependent cytotoxicity and interferon gamma production whose anti-tumor effects have been also confirmed in numerous studies. For example, Komita et al showed that IL-12 inhibits the growth of hepatocellular carcinoma (HCC) and plays an important role in preventing the recurrence of HCC [9]. Many studies have been published so far on the relationship between +1188 A/C single nucleotide polymorphisms and 3'untranslated region of IL-12B gene (rs3212227), and various diseases and cancers. For example, a study of Chinese population shows that AC and CC genotypes increase the risk of hepatocellular carcinoma, which was not actually statistically significant [10].

The study of Han et al on Korean women with cervical cancer showed a correlation between the increase of allele A and the risk of cervical cancer [7]. The study of Windsor et al on the Australian population showed a significant correlation between the increased frequencies of C allele with the risk of diabetes in the individuals [11]. However, in the study of Hall et al, no significant correlation was observed between this polymorphism and the increase risk of immunogenic diseases of rheumatoid arthritis, MS and LGL syndrome (large granular lymphocyte) in the Greek and English populations [3].

Various studies have been also conducted to examine the relationship between this polymorphism and different types of hepatitis. For example, in the study of Hegazy et al on the English population, a significant correlation was observed between the increase of allele C and reduced risk of hepatitis C infection in the individuals at high risk of infection, who were not affected with hepatitis C [12]. However, in the study of Mueller et al. on German population with chronic hepatitis C, there is no significant correlation between the frequency of different genotypes and increased risk of disease [13]. In the study of Suneetha et al on Indian population, hepatitis

C patients and control group were compared according to the genotypes AA, AC and CC and no statistically significant difference was found between different genotypes in the two groups entered the study [4]. In the study of Liu et al on Chinese population, the frequency of several single-nucleotide polymorphisms was reviewed in IL-12A and IL-12B genes and no significant correlation was found between them and the risk hepatocellular carcinoma arising from hepatitis B infection [6]. Some studies have been also conducted before on the Iranian population regarding this polymorphism and its relationship with several diseases. This includes the study of Shokrgozar et al which reported a significant correlation between AA genotype of MS patients and increased risk of disease [14].

Also, in a comprehensive study conducted in 2005 on the polymorphisms of several cytokines in the Iranian people with myelogenous leukemia, no significant correlation was observed between +1188 polymorphisms in IL-12 gene and risk of disease [15]. However, a study has been recently conducted on occult HBV in southern Iran, the results of which indicate the correlation of CC genotype with a reduced risk of occult infection [16].

Comparison of the results obtained in different studies and the results of this study (Table 4) shows that the frequency of genotype in studied Iranian population is similar to European populations and is different from populations of East Asia. However,

there is a great difference between this result and results of study of Arababadi et al, which is probably due to the differences of race and population characteristics of the statistical community investigated in these studies. In the studies conducted so far, a significant correlation has been observed between the risk of various cancers and immune system disorders and the frequency of genotypes of IL-12B gene. However, no significant difference has been observed so far between affected and healthy individuals regarding the risk of chronic hepatitis virus. Except for the study of Arababadi et al which indicates the correlation between reduced risk of occult HBV and CC genotype [16] and the study of Yin et al in China suggesting that AC genotype was significantly more abundant than the other genotypes in the people who have recovered from hepatitis C infection compared to those with chronic hepatitis [17].

Findings of this study revealed no relationship between +1188 polymorphism of IL-12B gene and increased risk of chronic hepatitis B.

Given that frequency of different genotypes of this gene is different in various populations, and considering the relatively small population studied in this research, it is recommended to conduct more extensive studies with more samples, especially of people who have recovered from hepatitis C, in order to achieve more reliable results with a wider scope.

Table 4. Frequency of genotypes of IL-12B gene in different countries

Studied population	Disease	Genotype distribution			Reference
		AA	AC	CC	
Germany	Control	63	31	6	12
	Chronic Hepatitis C	59	34	7	
England	Control	67.7	31.4	0.9	11
	People in risk of getting Hepatitis C infection	55.3	34.2	10.5	
China	Control	32.2	49.1	18.7	6
	Chronic Hepatitis B	30	50.8	19.2	
	Chronic Hepatitis C	40.7	34.1	25.3	16
	Self restricted Hepatitis C	19	64.3	16.7	
Iran	Chronic Hepatitis B	35.1	64.9	0	15
	Control	36	54	10	
	Chronic Hepatitis B	56.4	36.4	7.1	Current study
	Control	59.3	33.3	7.3	

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