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Original Article

The Association between Interleukin-10 Gene Polymorphisms and Hepatitis B Virus: Evidence in Iran

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Abstract

Background and Aim: Hepatitis B virus (HBV) is a human carcinogenesis agent. Interleukin 10 (IL-10) is a key anti-inflammatory cytokine, and single nucleotide polymorphisms in IL-10 gene promoter are correlated with infections caused by HBV. This research intended to assess the prevalence and genotype of HBV as well as the association between the polymorphisms of -819 and -1082 in the IL-10 gene with HBV in individuals with HBV infection in Qom Province, Iran

Methods: In this cross-sectional research, 360 individuals with chronic HBV infection and control group were involved between July 2018 and March 2019. HBV diagnosis was evaluated using ELISA and nested PCR assays. To determine polymorphisms in the IL-10 gene promoter in HBV positive and control samples, an allele-specific polymerase chain reaction technique was employed.

Results: The constructed phylogenetic trees for the HBsAg gene revealed that all sequences under study belong to genotype D and also, the majority of HBV samples presented similar sequences to the Iranian samples. Genotype frequencies of TT, TC and CC (polymorphism -819) were 82.2%, 11.6% and 6.1% for patients and 85%, 10.5% and 4.4% in control groups, respectively. Also, frequency of genotypes of AA, AG and GG (polymorphism -1082) were 45%, 43.8% and 11.1% for patients and 42.2%, 46.1% and 11.6% in control groups, respectively.

Conclusion: Here, we found no association among IL-10 gene polymorphisms in control and HBV-infected groups. However, more studies about the frequency of chronic HBV infection are necessary to be conducted.

Keywords: Hepatitis B Virus; Nested PCR; Polymorphism; Interleukin-10; IL-10.

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Introduction

Hepatitis B virus (HBV) is important human carcinogenesis since 1993 worldwide (1, 2). It belongs to the *Hepadnaviridae* family and *Orthohepadnavirus* genus (3). HBV genome contains double-stranded DNA; including the pol gene encoding viral polymerase, surface proteins encoding three carboxy-terminal HBV surface (HBs) proteins, and pre-core/core (pre-C/C) that encodes structural core protein. Also, it consists of an extra pre-core sequence (4). The ten genotypes (labeled A to J) and several subgenotypes of HBV have been identified based on virologic and epidemiological properties. In comparison with genotypes B and D, genotype A is associated with considerable loss of HBeAg and HBsAg due to interferon (IFN) therapy (2, 5). Shaving from barbers, tattooing, surgical instruments, perinatal, parenteral (especially via reuse of needles, receiving risky blood products) and sexual routes are the main reasons for HBV transmission (3, 6). Although HBsAg prevalence is decreasing due to vaccination and efficient therapy in some countries it varies geographically. An actual problem is the migration of the population which leads to prevalence changes in countries with known low endemicity (2). Based on the latest information, 350 million patients with HBV have estimated worldwide (7, 8). In Iran, the prevalence of HBV according to anti-HBc

antibodies is 16.4% (9).Some studies mentioned that HBV can stimulate the immune system, which causes major inflammatory responses. A large body of evidence suggests that cytokine-mediated immune response is a key to achieve better clinical outcomes in patients with HBV infection. Several immune factors contribute to HBV infection like classical immune parameters, transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), and interleukins family such as interleukin-6, 23 and 35 (10). The immune system of the host is key for inhibiting inflammation by Interleukin-10 secreted from T-lymphocytes (11). The levels of IL-10 production determine immune regulation and the balance between the cellular and humoral responses (12). Polymorphisms in the regulatory regions of the cytokine genes have an important impact on their expression. Thus the cytokine genes polymorphisms have a major contribution in predicting disease susceptibility and clinical outcome (13). Enhanced secretion of IL-10 contains a protective influence against HBV infection. Nevertheless, IL-10 gene polymorphisms in the proximal promoter region influence secretion of IL-10, which may enhance the HCC risk (hepatocellular carcinoma) (14). Some previous studies reported that different polymorphisms of IL-10 can cause HBV infection (1). Three classic promoter haplotypes; (GCC, ACC, and ATA) in positions of -1082, -819, and -592 in the promoter region of the gene IL10 are identified among populations of the world (15). Several studies reported the impact of these SNPs in the anti-HBV response. In addition, Shu et al. in a recently conducted meta-analysis reported their clinical importance in HBV infection (16). This research aimed to investigate HBV genotype and the relationship among the polymorphisms of -819 and -1082 in the IL-10 gene with HBV infection.

Methods

Participants

In our previous study, 1600 blood samples of HBV-suspected cases were collected from clinical centers of Qom Province (Iran) between July 2018 and March 2019. By considering a 95% confidence interval and an estimated prevalence of 50%, the sample size was calculated using Cochran's formula (9). The control group consisted of 180 healthy individuals according to the number of HBV-positive patients.

HBV detection

The level of HBsAg (hepatitis B surface antigen) of blood samples was detected by an ELISA kit (Pishtazteb, Iran) (3).

Viral DNA extraction and PCR assay

In this research, PCR products of HBV-positive isolates were exposed to direct sequencing to approve the presence of HBV DNA in the blood samples of HBsAg-positive patients. HBV DNA was extracted using a genomic DNA extraction kit (Sinaclon, Iran) by following the protocol presented by the manufacture. The primers were designed for the *HBs* gene of HBV by GenRunner and CLC Sequence Viewer 6 (Table 1).

Table 1. Sequences of applied primers

Gene	Primer sequence	product size
HBs	**Outer primer:** Forward: 5'-GGCGCACCTCTCTTTACG-3' Reverse: 3'-ATAGGGGCATTTGGTGGTC-5' **Inner primer:** Forward: 5'-CACTTCGCTTCACCTCTGC-3' Reverse:3'-CCAAGGCACAGCTTGGAGGCTTGAA-5'	970 bp 303 bp
IL-10 −819	F819T: 5′-GACTGGCTTCCTACAGT-3′ F819C: 5′- GACTGGCTTCCTACAGG-3′ R: 3'GGCAGAGATAAAAATATCACTCG-5'	216 bp
IL-10 −1082	F1082G: 5′-ACACACACACAAATCCAAG-3′ F1082A: 5′-ACACACACACAAATCCAAT-3′ R: 3'-CTCCTTTCATTCCCTGGAGGATA-5'	706 bp

We used blood serum infected with HBV as the positive control. While, we extracted DNA from a HBV-negative serum for the negative control. In table 2, the instructions employed as the first and second round of nested PCR for the *HBs* gene are described.

To approve the existence of the HBsAg gene, we subjected the PCR products of HBV positive samples to direct sequencing.

Moreover, to determine the polymorphisms -819 and -1082 in the IL-10 gene promoter in HBV positive and healthy control cases, the allele-specific polymerase chain reaction (PCR) technique was employed (Table 2).The final volume of all PCR reactions was 25 µL, containing 5 µL of DNA, Master Mix (17 µL), 1 µL of each primer, and 1 µL of Taq polymerase (Life Technologies, Carlsbad, USA). All reaction mixtures (5 µL) run on 1% agarose gel with Safe Stain (GelRed®, California) in a UV transilluminator.

Statistical analysis

Data were analyzed by SPSS (Version 22.0) and Microsoft Excel version 2017. The level of significance in the *t*-test was considered at $p < 0.05$.

Table 2. The protocol for PCR reaction.

Gene	-	Initial denaturation	Subsequent denaturation	Annealing	Extension	Final extension
HBs	First round	95°C 3min 1x	95°C 30s 35x	50°C 30s 35x	72°C 45s 35x	72°C 5min 1x
	Second round	95°C 3min 1x	95°C 30s 35x	53°C 30s 35x	72°C 45s 35x	72°C 5min 1x
IL-10 –819	-	95°C 3min 1x	95°C 45s 35x	47°C 45s 35x	72°C 30s 35x	72°C 10min 1x
IL-10 –1082	-	94°C 5min 1x	95°C 30s 35x	56°C 45s 35x	72°C 1min 35x	72°C 10min 1x

°C: Celsius, min: minute, x: the cycle of reaction, s: second.

Results

In this cross-sectional research, 1600 blood samples collected from HBV-suspected patients (aged from 20 to 67 years) referred to the clinical centers of the Qom Province are tested. A total of 180 (11.25%) ($p < 0.05$) positive cases of HBsAg were identified (121 men (out of 980) and 59 women (out of 692) with the mean age of 32-38 years).

Table 3. Frequency of genotypes in the regions -819 and -1082 of the IL-10 gene promoter in patient (HBV-positive) and in control (healthy) groups (n = 360).

Locus	Genotypes	patient group (n=180)		control group (n=180)	
		N	%	N	%
-819	TT	148	82.2	153	85
	TC	21	11.6	19	10.5
	CC	11	6.1	8	4.4
-1082	AA	81	45	76	42.2
	AG	79	43.8	83	46.1
	GG	20	11.1	21	11.6

Genotype frequencies of TT, TC and CC (polymorphisms -819 in the IL-10 gene) were 82.2%, 11.6% and 6.1% for patients and 85%, 10.5% and 4.4% in control group, respectively. Also, genotype frequencies of AA, AG and GG (polymorphisms -1082 in the IL-10 gene) were 45%, 43.8% and 11.1% for patients and 42.2%, 46.1% and 11.6% in control group, respectively (Table 3; Figures 3 and 4).

The amplification findings of the HBV *HBs* gene using the nested PCR technique are described in Figure 1. The constructed phylogenetic trees for the

HBsAg gene of HBV revealed that all sequences under study belong to genotype D and also, the majority of HBV samples were similar to the Iranian isolates' sequence (Figure 2).

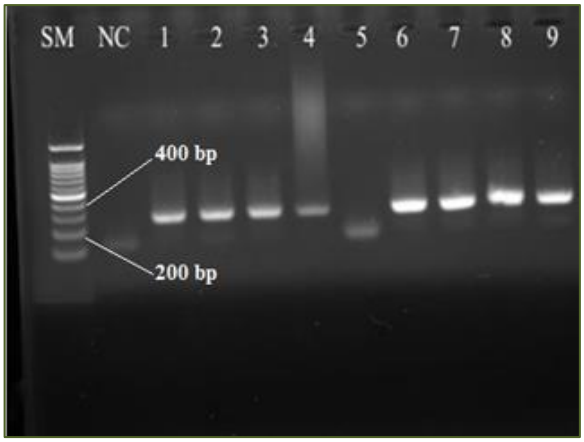

Figure 1. *HBs* gene amplification. Lane SM: 100 bp DNA ladder; Lane NC: negative control; Lanes 1-4 and 6-8: 303 bp positive sample; Lane 5: negative sample; and Lane 9: 303 bp positive control.

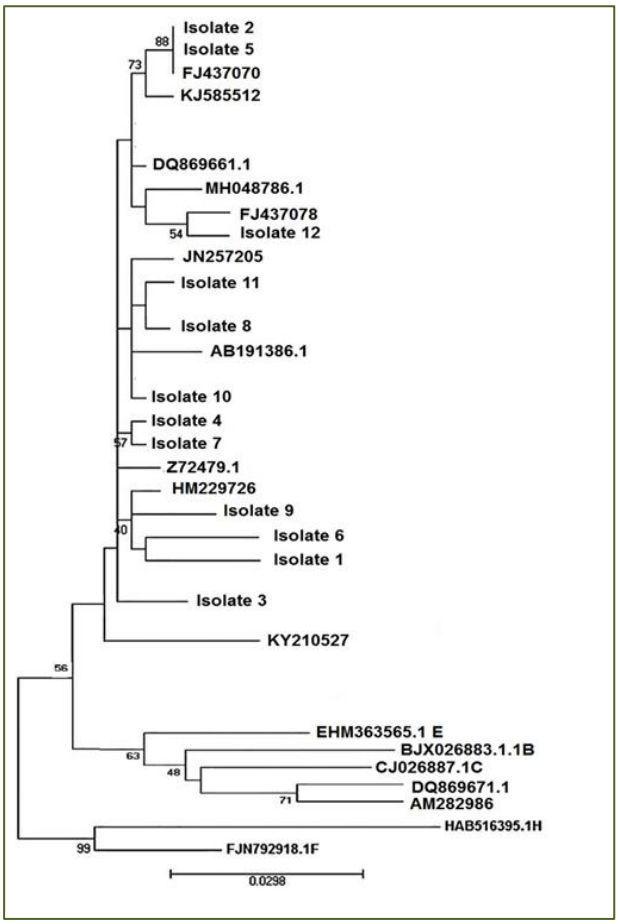

Figure 2. The constructed phylogenetic trees for the *HBsAg* gene of HBV.

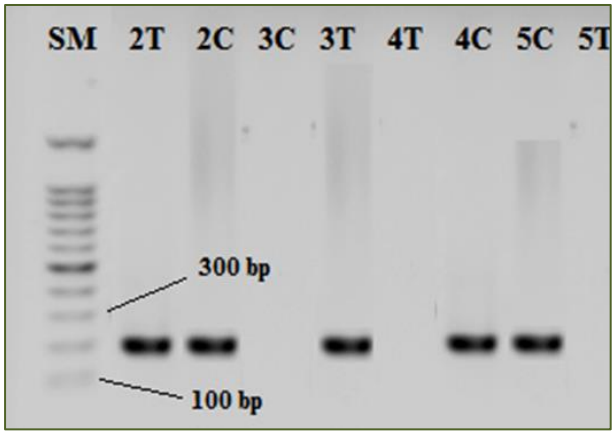

Figure 3. IL-10 Promoter Polymorphism -819 amplification in some samples. Lane SM: 100 bp DNA ladder; Lanes 2T, 2C: heterozygous TC individuals (216 bp fragments); Lane 3C, 3T: homozygous TT individuals (3C without 216 bp fragment); and Lane 4T, 4C, 5C, 5T: homozygous TT individuals (4T, 5T without 216 bp fragment).

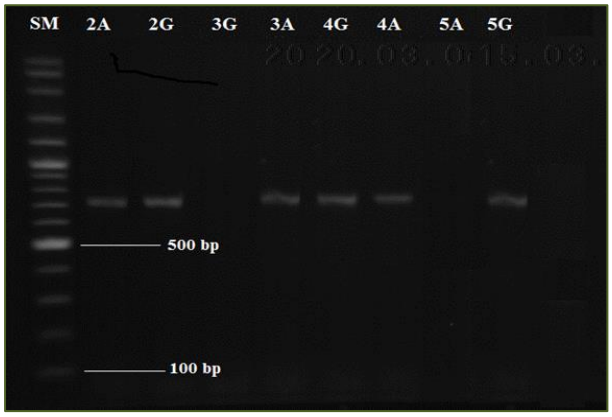

Figure 4. IL-10 Promoter Polymorphism -1082 amplification in some samples. Lane SM: 100 bp DNA ladder; Lanes 2A, 2G, 4G, 4A: heterozygous AG individuals (706 bp fragments); Lane 3G, 3A: homozygous AA individuals (3G without 706 bp fragment); and Lane 5A, 5G: homozygous GG individuals (5A without 216 bp fragment).

Discussion

HBV infection is an important health problem, which causes cirrhosis and hepatocellular carcinoma (17). HBV may cause various asymptomatic and symptomatic states such as progressive chronic HBV infection (18).

The HBV antigen prevalence is estimated at 2.4, 88.8, 37.5, 5.88, 11, and 6.72% in Pakistan, Brazil, Tehran Province (Iran), Bandar Abbas city (Iran), Hormozgan Province (Iran), and in Bushehr Province (Iran), respectively (8, 19-23). However,

in this research that was performed in the province of Qom, (Iran) 180 (11.25%) cases were positive for HBsAg. All sequences under study belong to genotype D and also, the majority of HBV samples were similar to Iranian samples concerning their sequence. Genotypes A, D, and F are reported as the most common genotypes in Brazil (8).

Due to limitations related to the identification method and the sample size, the findings should be generalized with caution. Qom province is the desired destination of many immigrants and travelers from all around the country and African and Asian countries, in many of which several diseases are endemic due to their poor health infrastructures.

Some recently performed studies mentioned particular immuno-modulatory cytokines such as IL-10 as major risk factors that contribute to determining the history of persistent HBV infections (16). Recently, it is well-proved that polymorphism within the IL10 promoter region affects the expression and serum levels of IL-10. Several studies have examined -1082A/G, -819T/C, and -592A/C (24). This study is the first study showing the relationship among polymorphisms of promoter genes of IL-10 (-819 T/C, -1082 A/G) and susceptibility to HBV in the province of Qom (Iran). Allele-specific PCR technique was employed to determine polymorphisms in the IL-10 gene promoter samples infected with HBV (n=180) and healthy controls (n=180). Genotypes frequency of TT, TC and CC were 82.2%, 11.6% and 6.1% for patients and 85%, 10.5% and 4.4% in control group, respectively. Also, genotypes frequency of AA, AG, and GG were estimated at 45%, 43.8% and 11.1% for patients and 42.2%, 46.1% and 11.6% in the control group, respectively. The IL-10-1082 AA and -819 TT genotypes were the most common in the HBV-positive group.

Still, there are discrepancies regarding the relationship between IL-10 gene polymorphism and HBV infection. However, IL-10 polymorphism is reported to be associated with enhanced risk of HCC in Korea, Taiwan, and Chin. In addition, Zhang et al. found a relationship between the IL-10 polymorphism at -1082GA and susceptibility to HBV infection (1, 25, 26).

In the analysis of polymorphisms in the regions -819/-1082, no considerable difference was found between patients and control groups, which is consistent with the studies by Sofian et al. (27), Bineshian et al. (28). However, some studies reported conflicting findings including Mirfakhar et al. (29), Rybicka et al. (24), Truelove et al. (15), Shu et al. (16), Gao et al. (30), Moudi et al. (31), and Shin et al. (32), which can be attributed to the presence of other genotypes on HBV. Also, probably epidemiological and geographical parameters and patients' characteristics affect this association.

The nested PCR method contains two sets of primers that should be used in two successive runs of PCR. The second set accelerates a secondary target through the first run product and limits non-specific products (33). The findings of the present study indicated that nested PCR is a highly sensitive method.

Conclusion

To successful managing of the HBV infection, early diagnosis of disease using nested PCR technique is of crucial importance. Also, no considerable difference was found between patient and control groups concerning the frequency of interleukin 10 gene polymorphism.

Conflict of Interest

The authors declared that they have no conflict of interest.

Acknowledgment

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Ethics

In the present study, both ethical principles and national standards for clinical studies are observed (Approval code: IR.IAU.TMU.REC.1398.064).

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