Are Fas Ligand Polymorphisms Associated With Occult HBV Infection?

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Abstract

Background: Occult hepatitis B infection (OBI) is a form of hepatitis in which there is an absence of detectable HBsAg, despite the presence of HBV-DNA in the peripheral blood of patients. The main aim of this study was to investigate the relationship between polymorphisms in the -844 and IVS2nt-124 regions of the Fas ligand (FasL) gene with OBI.

Materials and Methods: The plasma samples from 3700 blood donors were tested for HBsAg and anti-HBs by enzyme-linked immunosorbent assay (ELISA). The HBsAg+/anti-HBc+ samples were selected and screened for HBV-DNA by polymerase chain reaction (PCR). HBV-DNA positive samples were assigned as OBI cases, and PCR-RFLP techniques were performed to examine the polymorphisms.

Results: Of 3700 blood samples, 352 (9.5%) were HBsAg+/anti-HBc+, and HBV-DNA was detected in 352 (16.1%) of HBsAg+/anti-HBc+ samples and designated as OBI patients.

Our results showed the patient and control groups had no significant differences regarding the studied polymorphisms.

Conclusion: Based on our results, it can be concluded that the functional polymorphisms in the promoter region of the FasL gene are not associated with OBI.

Keywords: occult hepatitis B infection, FasL, polymorphism, HBsAg, HBV-DNA

Materials and Methods

Patients

Peripheral blood samples were collected from 3700 volunteer blood donors of the Rafsanjan Blood Transfusion Services (Kerman, Iran) and placed in EDTA pre-coated 5.5 mL tubes. The samples were centrifuged at 3700g for 4 minutes and the sera collected. All sera were separated within 24 hours of collection. If needed, serum samples were stored at -20°C for a maximum of 2 months or at -70°C, where longer storage times were required, for further processing. For analysis of polymorphisms, a 2 mL sample was collected from patients with OBI (57 cases) and 100 healthy controls (HBsAg+/HBV-DNA+/anti-HBc+). The study protocol was approved by the ethical committee of the Rafsanjan University of Medical Sciences.

All of the participants of this study completed and signed the informed consent form, which was designed based on the aims and objectives of the study.

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Abbreviations

OBI, occult hepatitis B infection; Fasl, Fas ligand; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; EIA, enzyme immunoassay; HBV, hepatitis B virus; PCR-RFLP, polymerase chain reaction-restricted fragment length polymorphism
Detection of Serological HBV Markers

HBsAg screening tests were performed by enzyme-linked immunosorbent assay (ELISA) (Behring, Marburg, Germany). Anti-HBC screening tests were performed by a manual microplate enzyme immunoassay (EIA) using an anti-HBc commercial kit (Radim, Pomezia, Italy). The present method is based on a competitive EIA. All of the samples were also screened by ELISA (Radim) for possible HCV, HIV, and HTLV-1 infections.

HBV-DNA Extraction From Plasma Samples

Viral DNA was purified from 200 µL of plasma samples. Briefly, each plasma sample was incubated at 72°C for 10 minutes and then cooled to 4°C for 5 minutes in 200 µL proteinase K (200 µg/mL). Following phenol/chloroform extraction (1:1), the viral DNA was precipitated with ethanol, and the pellet was re-dissolved in DNase free, deionized water and stored at -20°C for further use.

HBV-DNA PCR and Gel Electrophoresis

Polymerase chain reaction (PCR) was carried out in a 25 µL mixture containing 10 mM tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 5 units recombinant Taq DNA polymerase, 200 µM of each dNTP, 0.6 µM of each primer, and 5 µL of the DNA extracted from 200 µL of plasma. The sequences of all primers used in this study are shown in Table 1. For HBV analysis the primers are designed to amplify a 500 bp amplicon of the HBV genome. Fast temperature cycling was performed. Polymerase chain reaction amplification was performed using the touch down method, which included 1 cycle of 93°C for 20 seconds, 55°C for 20 seconds, and 72°C for 40 seconds followed by 30 cycles of 93°C for 20 seconds, 60°C to 56°C for 20 seconds, and 72°C for 40 seconds. Hepatitis B virus genomic DNA, provided by the CinnaGen company (Tehran, Iran), and a negative patient sample were used as positive and negative controls, respectively.

Table 1. The Sequence of the Primers Used in This Study, The Appropriate Annealing Temperatures, and Expected PCR Product Sizes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
<th>Annealing Temperature</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S gene</td>
<td>F: TGTTGTGGACTCTCTC&lt;br&gt;R: ACAATGCGGGGAAAGGCCC</td>
<td>60°C</td>
<td>500</td>
</tr>
<tr>
<td>Fast.</td>
<td>FasL -844 F: 5'-CAATGGAAATGACACATGG-3'&lt;br&gt;FasL -844 R: 5'-CCACACTTGAATAGATC-3'</td>
<td>48°C</td>
<td>85</td>
</tr>
<tr>
<td>Fast.</td>
<td>FasL NS2nt-124 F: 5'-GCAATCATCGAATGATTAC-3'&lt;br&gt;FasL NS2nt-124 R: 5'-DCAATGACGCCTGGTTAATGCCC-3'</td>
<td>58°C</td>
<td>230</td>
</tr>
</tbody>
</table>

Table 1

Genomic DNA Extraction

Peripheral blood was collected on EDTA, and genomic DNA was extracted using a commercial kit (Bioneer, Daejeon, Korea) following the recommended procedures. Extracted DNA was aliquoted (for each patient sample) and stored at -20°C for further use.

Detection of Polymorphisms

The gene polymorphisms were analyzed by the PCR-restricted fragment length polymorphism (PCR-RFLP) method. Polymerase chain reaction of the FasL gene was performed in a volume of 50 µL containing 250 ng of DNA templates, 200 µM of each dNTP (CinnaGen), 0.5 U Taq DNA polymerase (CinnaGen), 1× PCR buffer (CinnaGen), 3 mM MgCl₂, and 5 µM of each specific primer (Table 1). The PCR conditions were the following: an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of melting at 95°C for 50 seconds, suitable annealing temperature for 50 seconds (Table 1), and extension at 72°C for 50 seconds, with a final extension step of 5 minutes at 72°C using a thermal cycler (C1000, Bio-Rad, Hercules, CA). The expected amplified PCR products used to detect the -844 and the IVS2nt-124 amplicons of Fasl were 85 and 230 bp fragments, respectively. The DraIII and FokI restriction enzymes (Fermantas International, Ontario, Canada) were used to distinguish the -844 C>T and IVS2nt-124 A>G polymorphisms, respectively, which resulted in 66 and 19 bp fragments in the case of the -844 T allele and 180 plus 50 bp fragments in the case of the IVS2nt-124 G allele. More than 10% of the samples were randomly selected and retested by appropriate PCR-RFLP techniques for confirmation, and the results were 100% concordant. The digested products were run on a 2.5% agarose gel (CinnaGen) and analyzed using a chemi-doc-XRS system (Bio-Rad) after staining with ethidium bromide.

Statistical Analysis

Hardy-Weinberg equilibrium was assessed using genotype data. Allele and genotype frequencies were calculated in patients and healthy controls by direct gene counting. Statistical analysis of the differences between groups was performed using chi-squared test (EPI 2000 and SPSS software version 13 (Chicago, IL)). A P value of less than 0.05 was considered significant.

Results

This study was performed on 3700 blood samples collected from patients attending the Rafsanjan blood transfusion services. All of the samples were found to be negative for HBsAg, anti-HCV, anti-HTLV-1, and anti-HIV antibodies. Out of the 3700 samples, 352 (9.5%) cases were positive for anti-HBc, and HBV-DNA was detected in 57/352 of them.
Results of this study indicated that 16.1% of HBsAg negative but anti-HBc positive samples had detectable HBV-DNA, which is 1.54% (57/3700) of the total collected samples.

The mean age of the patients and control groups was 28 ± 6 and 28 ± 8, respectively, and there was no significant difference in age between the 2 groups (Table 2). Three (3%) of the control group members were female, and 97 (97%) were male, while 2 of the patients (3.5%) were female, and 55 (96.5%) were male. In addition, analysis of socio-economic conditions showed there was also no significant difference between the patient and control groups (Table 2).

Evaluation of the polymorphisms at position -844 of FasL showed that the frequency of the C allele was 72 (63.1%) and 121 (60.5%) in patients and controls, respectively. Forty-two (36.9%) cases of the T allele were observed in patients, and the corresponding frequency of this allele was 79 (39.5%) in the controls. Statistical analysis of these alleles displayed no significant difference between patients and controls (P=0.718) (Table 3).

The data show that the prevalence of the C/C genotype was 21 (36.9%) in patients and 37 (37%) in controls, while the frequency of the T/C genotype was 30 (52.6%) and 46 (46%) in patients and controls, respectively. The frequency of the T/T genotype in patients was 6 (10.5%) and in controls was 17 (17%) (Table 3). Statistical analysis of our data did not show a significant difference between the 2 groups regarding these genotypes (P=0.492).

Our results also showed that the frequency of A allele in the IVS2nt-124 polymorphism of FasL was 94 (82.5%) and 160 (80%) in patients and controls, respectively. Twenty (17.5%) cases of the G allele were observed in patients whereas the frequency of this allele was 40 (20%) in controls. Statistical analysis of the frequency of these alleles did not indicate a significant difference between patients and controls (P=0.656) (Table 4).

The data showed that the prevalence of the A/A genotype was 38 (66.6%) in patients and 66 (66%) in controls. The frequency of A/G genotype was 18 (31.6%) and 28 (28%) in patients and controls, respectively, and the frequency of the G/G genotype was 1 (1.8%) in patients and was 6 (6%) in controls (Table 3). Statistical analysis of our data showed that the differences between groups regarding these genotypes was not significant (P=0.395) (Table 4).
Discussion

It is now well established that, following hepatitis viral infection, some of the infected hepatocytes express the Fas and FasL system. It is also reported that the rate of expression of FasL is related with the kind of clinical presentation and also correlates to the different stages of the HBV infection and associated liver disease. Furthermore, FasL may play a key role in infected hepatocyte apoptosis. However, despite evidence suggesting a potential correlation between FasL and disease status, our results showed the frequency of evaluated alleles and genotypes was not different between OBI patients and healthy controls. Therefore, based on our results it can be concluded that these polymorphisms are not associated with OBI. Previous studies showed the rate of expression of Fas and FasL was associated with the clinical pattern of the disease. For example, Bortolami and colleagues reported more expressions of Fas and FasL in HBV-infected hepatocytes from patients with cirrhosis than in chronic hepatitis patients. Studies also reported that polymorphisms in the promoter regions of Fas and FasL influence the pattern of their expression. Based on the fact that OBI patients are unable to clear HBV completely, it could be suggested the expression of Fas and FasL in OBI patients may be compromised by mechanisms other than polymorphisms within the promoter region.

Despite evidence linking a functional role for Fas and FasL to viral hepatic disease progression, our data showed that the polymorphisms, which are known to influence FasL expression levels, were not statistically different between OBI patients and healthy controls. This data suggests that if FasL participates in OBI it is highly unlikely that the reported polymorphisms are linked to the disease’s progression. Future studies must be performed on the expression of FasL at the protein level to confirm this. To our knowledge, this is the first report to date investigating the involvement of Fas and FasL polymorphisms during hepatitis C infection. One reason for the discrepancy between our results and these studies could be explained by the different types of hepatitis infection, and there may also be genetic differences in populations studied. In addition, there may also be subtle differences in the type of disease from our studied population. It is not clear by what means OBI patients are unable to completely overcome the viral contamination, however, based on the current studies it seems the polymorphisms within the promoter region of the FasL gene were not related with OBI.

Finally, due to the complexity of OBI, other aspects of the disease must be examined. For instance, our previous study showed the serum level of IL-17, an inflammatory cytokine, was increased, whereas the serum level of IL-10 decreased in OBI patients. Therefore, our future studies will explore polymorphisms and the expression levels of these and other important cytokines and their receptors within the OBI patients.

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