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Assessment of CXCL12 (SDF-1α) Polymorphisms and Its Serum Level in Posttransfusion Occult HBV-infected Patients in Southeastern Iran

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Background. Occult hepatitis B infection (OBI) is defined as a form of hepatitis in which, despite absence of detectable HBsAg, HBV-DNA is present in peripheral blood of patients. The main aim of this study was to determine an association between polymorphisms in +801 of CXCL12 (SDF-1α) and its serum level in OBI patients.

Methods. In this experimental study, plasma samples of 3700 blood donors were tested for HBsAg and anti-HBc by ELISA. The $HBsAg^-$/anti-HBc^+ samples were selected and screened for HBV-DNA by PCR. HBV-DNA positive samples assigned as OBI cases and PCR-RFLP techniques were performed to examine the CXCL12 (SDF-1α) polymorphisms. The serum level of CXCL12 (SDF-1α) was also analyzed by ELISA.

Results. Of 3700 blood samples, 352 (9.5%) were HBsAg/anti-HBc^+ and HBV-DNA was detected in 57/352 (16.1%) of $HBsAg^-$/anti-HBc^+ samples. Our results showed a significant difference in genotypes and alleles of +801 region of CXCL12 (SDF-1α). However, the serum level of CXCL12 (SDF-1α) was decreased in OBI patients but was not significant. Our results also showed that the alleles of +801 region of CXCL12 (SDF-1α) were also not associated with serum level of the chemokine.

Conclusions. The polymorphisms in +801 region of CXCL12 (SDF-1α) are possibly related to OBI.

Key Words: Occult hepatitis B infection, CXCL12 (SDF-1α), Polymorphism.

Introduction

Although HBsAg is undetectable in serum of occult HBV-infected (OBI) patients, HBV-DNA is observed in patient serum (1). This type of hepatitis is one of the main challenges of blood transfusion services and even though all donated blood and blood components are screened for HBsAg, some cases of posttransfusion hepatitis B are reported (2). One of the primary causes of posttransfusion hepatitis B infection is OBI (3), which we found in our previous investigations in Isfahan and Kerman, the two central provinces of Iran (3,4). However, the responsible mechanism(s) for progression of OBI are yet to be clarified but some investigators suggested the key roles of genetic and immunological parameters in resistance of some individuals and sensitivity of some patients (5,6). Chemokines play important roles in appropriate immune response to viral infections (7–9). Among this group of cytokines, CXCL12 (SDF-1α) is the subject of interest in viral infections and immune surveillance (10). CXCL12 (SDF-1α) is an 8-kDa protein that was initially identified in a bone marrow stromal cell line ST-2 (10) and subsequently by expression cloning as a pre-B cell stimulating factor for stromal cells in the generation of B cells *in vitro* (11). CXCL12 (SDF-1α) is a main chemokine in homing of immune cells to liver and also induction of cellular immunity against viral infections, especially HBV (10). Therefore, genetic factors that affect expression of this chemokine enable the immune system to decrease eligible

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immune response against viral infections. Previous studies showed that the secretion of CXCL12 (SDF-1α) can be affected by its polymorphism in +801 region (12) and CXCL12 (SDF-1α) is known as one of the main chemokines involved in immune response against HBV infection (13). Therefore, the aim of this study was to investigate the functional polymorphisms in CXCL12 (SDF-1α) (+801) as well as serum level of this chemokine in OBI patients.

Materials and Methods

Patients

Peripheral blood samples were collected from 3700 volunteer blood donors from the Rafsanjan Blood Transfusion Services (Kerman, Iran) on EDTA pre-coated 5.5 mL tubes. The samples were centrifuged at 370 × g for 4 min. All sera were separated within 24 h following collection. If needed, serum samples were stored at −20°C for a maximum of 2 months or at −70°C (in case of longer time) for further use. For analysis of polymorphisms, a 2-mL sample was collected from patients with OBI (57 cases) and 100 healthy controls ($HBsAg^-/HBV\text{-}DNA^-/anti\text{-}HBc^+$). Healthy control cases were selected from an age-, gender-, and socioeconomic-matched Rafsanjanese population (Table 1) following approval by the Rafsanjan University of Medical Sciences Ethics Committee. Written informed consent was obtained by both patients and controls and their blood collection was performed.

Detection of Serological HBV Markers

HBsAg screening test was performed by enzyme-linked immunosorbent assay (ELISA) (Behring, Germany). Anti-HBc screening test was also performed by a manual microplate enzyme immunoassay using anti-HBc commercial kit (RADIM, Milan, Italy). The present method is based on a competitive enzyme immunoassay (EIA). All 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 min and then cooled down to 4°C for 5 min in 200 µl proteinase K (200 µg/ml). Following phenol/chloroform extraction (1:1), viral DNA was precipitated with ethanol and the pellet was redissolved in DNase-free, deionized water and stored at −20°C for further use.

HBV-DNA PCR and Gel Electrophoresis

To amplify the S gene of HBV-DNA a PCR reaction mixture was made up of addition of the following reagents to a 0.2 mL micocentrifuge tube on ice: 2.5 µl of *Taq* DNA polymerase buffer (10X), 0.5 µl of $MgCl_2$ (stock concentration 1.5 mM), 0.5 µl of each dNTP (dATP, dCTP, dGTP, dTTP) stock concentration of 10 mM), 1 µl of each primer [Table 2 (forward and reverse), stock concentration of 25 ng/µl], 5 µl of prepared DNA and sterile double-distilled water to a final volume of 25 µl. The amplification was performed with the following program: one cycle of 93°C for 2 min, 93°C for 1 min (denaturation), 1 min at appropriate annealing temperature for annealing of different targets (Table 2), 72°C for 40 sec (elongation) followed by 30 cycles of 93°C for 20 sec, 55°C for 20 sec and 72°C for 40 sec. During the last 45 sec of first stage 0.3 µl of *Taq* DNA polymerase was added to the mixture. In addition to appropriate annealing and melting temperatures, Table 2 indicates the size of products that should be achieved by PCR. HBV-DNA was purchased from Cinnagen (Tehran, Iran) and used as positive control. PCR product (10 µl) was run on a 1% agarose gel after adding 4 µl of loading buffer. The presence of a 500-bp fragment indicated a positive result. In parallel with samples, a 100-bp DNA ladder was also run to estimate the molecular size of DNA fragments.

Table 1. Demographic and socioeconomic conditions of OBI patients and controls

Variable	Control	Patient
Age (years)	28 ± 8	28 ± 6
Sex	*n* (%)	*n* (%)
Female	3 (3%)	2 (3.5%)
Male	97 (97.8%)	55 (96.5%)
Socioeconomic status	*n* (%)	*n* (%)
Weak	22 (22%)	12 (21%)
Medium	47 (47%)	28 (49%)

Note: No significant differences were seen regarding demographic and socioeconomic conditions in OBI patients compared to controls.

Detection of Polymorphism

CXCL12 (SDF-1α) gene polymorphism (+801) was analyzed by PCR-RFLP method. PCR of this gene was performed as described in HBV-DNA amplification except 1 µl of prepared DNA and using 57°C annealing temperature.

Table 2. Sequences of primers

Genes	Primers	Product size (bp)
S gene (HBV)	F: TCGTGGTGGACTTCTCTC	500
	R: ACAGTGGGGGAAAGCCC	
CXCL12 (SDF-1α)	F: CAGTCAACCTGGGCAAAGCC	302
	R: AGCTTTGGTCCTGAGAGTCC	

Note: Table shows the sequences of primers and fragment size of PCR production.
F: forward primer, R: reverse primer and bp: base pair.

The sequence of forward and reverse primer is shown in Table 2. The amplified PCR product of CXCL12 (SDF-1α) gene covers the +801 region with a molecular size of 302 bp. The *S*ac-1 (Fermentas, Helsinki, Finland) has merely a restriction site on this region; thus, the fragment will be digested into two 202- and 1000-bp fragments following digestion. In case of the heterozygotic form (A/G), three different fragments with 302, 202 and 100 bp is then visible. In homozygotic form a 302-bp fragment [without any digestion (A/A)] or two 202 and 100 bp [digesting both alleles (G/G)] was then observed. The digested products were run on a 2.5% agarose gel after adding 4 μl of loading buffer (Cinnagen) and studied on a UV transilluminator after staining with ethidium bromide.

Chemokine Level Assay

Serum levels of CXCL12 (SDF-1α) were measured by ELISA (R&D Systems, Abingdon, UK) in patients and healthy controls immediately after blood collection. Assays were performed according to manufacturer's guidelines. The sensitivity of kits was 2 pg/ml and inter- and intra-assay assessments of reliability of the kit were conducted.

Liver Enzyme Evaluation

SGPT (serum glutamic-pyruvic transaminase), SGOT (serum glutamic oxaloacetic transaminase), direct and total bilirubin (DB and TB, respectively) and ALP (alkaline phosphatase) of OBI patients were measured using MAN Ltd Kits (Iran).

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 determined by χ^2, t-test and ANOVA using EPI 2000 and SPSS software v.13 with test power of 90%; p value <0.05 was considered significant.

Results

This study was performed on 3700 collected blood samples in Rafsanjan blood transfusion services. All samples were found to be negative for HBsAg and HCV, HTLV-1 and HIV antibodies. Of 3700 samples, 352 (9.5%) cases were positive for anti-HBc and HBV-DNA was detected in 57/352 (16.1% of HBsAg negative but anti-HBc positive). Results of this study indicated that 16.1% of HBsAg negative but anti-HBc positive samples had detectable HBV-DNA, representing 1.54% (57/3700) of total collected samples. The mean age in patients and control groups was 28 ± 6 and 28 ± 8, respectively. There was no significance between groups (Table 1). Three (3%) control group subjects were female and 97 (97%) were male, whereas two of the patients (3.5%) were female and 55 (96.5%) were male. There was no significant difference regarding the age of controls and patients or socioeconomic status between groups (Table 1). Evaluation of the serum level of CXCL12 (SDF-1α) showed that concentration of this chemokine was decreased in OBI patients (48.74 ± 11.35 pg/ml) in comparison to controls (58.54 ± 9.79 pg/ml), but this difference was not significant ($p > 1.0$) (Figure 1).

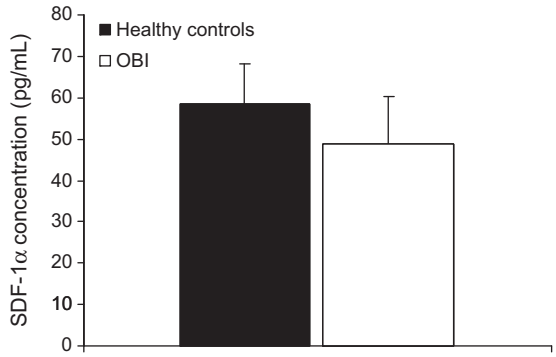

Figure 1. CXCL12 (SDF-1α) serum level in OBI patients and controls. No significant difference was observed in CXCL12 (SDF-1α) serum level ($p > 1.0$). Data are shown as mean ± SE.

Evaluation of the polymorphisms in +801 region of CXCL12 (SDF-1α) by *Sac*-1 restriction enzyme showed that the prevalence of *A/A* genotype was 6 (10.5%) in patients and 11 (11%) in controls. Our results also revealed that the frequency of *A/G* genotype was 20 (35%) and 45 (45%) in patients and controls, respectively. The frequency of the *G/G* genotype in patients was 31 (28%) and in controls was 44 (44%) (Table 3). Statistical analysis of our data confirmed a significant difference between groups ($p < 0.001$). The frequency of *A* allele was 32 (43%) and 67 (33.5%) in patients and controls, respectively. Eighty two (72%) cases of *G* allele were observed in patients but the frequency of this allele was 133 (66.5%) in controls. Statistical analysis of alleles also exhibited a significant difference between patients and controls ($p < 0.001$) (Table 3). Our results also showed that all OBI patients had normal serum levels of ALT, SGOT, SGPT, DB and TB (Table 4).

Table 3. Frequency of polymorphisms of CXCL12 (SDF-1α) gene in OBI patients and controls

Genotype	Patient *n* (%)	Control *n* (%)	*p* value
A/A	6 (10.5%)	11 (11%)	<0.001
A/G	20 (35%)	45 (45%)	
G/G	31 (28%)	44 (44%)	
	Alleles		
A	32 (43%)	67 (33.5%)	<0.001
G	82 (72%)	133 (66.5%)	

Table 4. Average of serum level of liver factors of controls and OBI patients

Factors	Control	OBI patients	*p* value
ALP	120 U/l	130 U/l	=1.0
SGOT	15 U/l	20 U/l	=0.8
SGPT	22 U/l	19 U/l	=1.0
DB	0.2 mg/dL	0.19 mg/dL	=1.0
TB	0.8 mg/dL	1 mg/dL	=0.8

Note: No significant differences were seen regarding serum levels liver factors in OBI patients compared to controls.

Discussion

It is now well established that following viral infection, some pro-inflammatory mediators including chemokines such as CXCL12 (SDF-1α) contribute to the recruitment of immune cells to the liver and produce an anti-viral immune response (13). It is also stated that distinct chemokines and chemokine receptors are related with different stages of the chronic hepatitis B virus infection and associated liver disease (13). CXCL12 (SDF-1α) may also play a key role in liver regeneration, fibrosis and other viral infection-related clinical presentations (13). To our knowledge this is the first report on the involvement of CXCL12 (SDF-1α) with OBI to date. Shi et al. showed elevated levels of CXCL12 (SDF-1α) in carcinoma patients in comparison to patients with liver cirrhosis, hepatitis and healthy donors (14). Interestingly, similar to our findings, Shi et al. could not find a significant difference between healthy donors and hepatitis and cirrhosis patients. In agreement with our results constitutive, but not elevated, levels of CXCL12 (SDF-1α) have been demonstrated in the non-inflamed biliary epithelium of the liver in association with CXCR4 expressing lymphocyte recruitment (15), which possibly shows that OBI is comparable with noninfectious liver disease. Liver-infiltrating lymphocytes (LIL) express CXCR4 [the CXCL12 (SDF-1α) receptor], and it is thought that cells entering the noninflamed liver may be attracted to, and retained at, the biliary epithelium where they can provide immune surveillance against pathogens such as viral particles entering via the biliary tract (16). Immunohistochemical studies have revealed decreased levels of CXCL12 (SDF-1α) protein in hepatocellular carcinoma when compared with other chronic liver diseases such as hepatitis C (17,18). Our previous *in vitro* results on expression of CXCL12 (SDF-1α) in isolated and cultured hepatocytes and under external stimuli of heat shock encouraged us to investigate the effects of an internal stimuli such as viral particles (*in vivo*) on liver CXCL12 (SDF-1α) production (10). It is not well defined by what means OBI patients are unable to completely overcome the viral contamination. However, it seems that chemokines play key roles in clearance of HBV (19). This is the first report on the relation of CXCL12 (SDF-1α) 3′A polymorphism with OBI to date and probably confirms the role of this polymorphism in the pathogenesis of OBI. The low protein level of CXCL12 (SDF-1α) in OBI patients compared to controls may also be due to the 3′A polymorphism, which may affect protein level. Although polymorphism in +801 region of CXCL12 (SDF-1α) gene is believed to be involved in the regulation of CXCL12 (SDF-1α) expression (20), Nahon et al. reported that CXCL12 (SDF-1α) sera levels and its known CXCL12 (SDF-1α) 3′A polymorphism were not related with the risk of death and/or hepatocellular carcinoma (21).

In hepatitis B, the expression pattern of chemokines is changed and CXCL12 (SDF-1α) is increased (22). Studies showed that polymorphisms in specific regions of this chemokine also influence the pattern of CXCL12 (SDF-1α) expression (12).

Interestingly, in contrast to our findings, Wald et al. showed that serum and intra-liver level of CXCL12 (SDF-1α) was increased in chronic hepatitis B (13). The following probable reasons could partially describe the decreased amount of CXCL12 (SDF-1α) in OBI patients: Our patients are different in clinical aspects from those studied by others because we studied OBI patients, whereas Wald and colleagues investigated chronic hepatitis B patients. The two clinical and immunological aspects of OBI are quite different from the clinical presentation of hepatitis B and probably not comparable to chronic hepatitis B infection. Therefore, there are insufficient studies in the database to compare the serum level of CXCL12 (SDF-1α) and its related polymorphisms.

If this study was performed with a wider sample size, the difference between groups may possibly be significant. Furthermore, due to the low rate of frequency of OBI in the population, detecting OBI cases is difficult and time consuming. It should also be noted that it is important to check the RNA level of CXCL12 (SDF-1α) and the functionality of this polymorphism in further studies.

Finally, due to the complexity of OBI, other aspects of the disease need to be examined. It is recommended to study the expression and polymorphisms of other important related cytokines and their receptors in OBI patients as a future investigation.

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