Hepatitis B virus genotype, HBsAg mutations and co-infection with HCV in occult HBV infection

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Running title: Characterization of OBI

Abstract

Background: The association between mutations in the Hepatitis B Surface antigen (HBsAg) gene and the occurrence of occult HBV (OBI) in patients has not been studied adequately to determine if the two are correlated. The current study was aimed to investigate HBsAg mutations, the genotype of HBV and co-infection with HCV in OBI in the central part of Iran to determine any possible associations.

Material and methods: In this study 3700 plasma samples were examined for the presence of HBsAg, anti-HBc and HBV-DNA. All $HBsAg^-$/anti-HBc^+/$HBV\text{-}DNA^+$ samples were regarded as OBI. The genotype of HBV was identified using Gap-PCR and RT-PCR was used to determine possible co-infection with HCV. Finally, direct sequencing was performed to analyse mutations within the Surface antigen gene of HBV in occult versus acute HBV infection.

Results: Of the 3700 patient samples analysed, 352 (9.5%) cases were determined to be $HBsAg^-$/anti-HBc^+ in which HBV-DNA was detected in 57 (16.1%), these latter patients were classified as OBI. All of the patients studied carried the D genotype. Direct sequencing of the S-gene from occult and acute HBV patients revealed one silent and one Glycine to Arginine mutation but the acute HBV patients showed an additional mutation (Alanine to Threonine). All the mutations were outside the range of the α-determinant. Furthermore, none of the OBI patients were co-infected with HCV.

Conclusions: The absence of conformational mutations in the α-determinant of HBsAg confirmed that this antigen could be detected by commercial ELISA kits and therefore was not responsible for false negatives during blood screening. However, it can be concluded that suitable amounts of HBsAg were not expressed by HBV in the OBI patients to be detected by ELISA. Low level expression of HBsAg might be related to the D genotype of the virus. Furthermore, our results suggest that OBI is not related to co-infection with HCV.

Key words: Occult hepatitis B infection, Genotype, HBsAg, Mutation, HCV.

Introduction

Iran is classified as having low endemicity for hepatitis B infection [1]. Despite all blood and blood components being screened in Iran for the Hepatitis B surface antigen (HBsAg, also referred to as the S-gene), some cases of post-transfusion hepatitis B have been reported [2]. Occult HBV infection (OBI) is a clinical form of hepatitis B in which, despite undetectable serum levels of HBsAg, HBV-DNA is present in the serum of patients [3-5]. This type of hepatitis creates severe problems for blood transfusion services worldwide as it creates an avenue for post-transfusion infection despite the efforts of these services to screen for potential carriers of the disease. Studies showed that one of the main causes of post-transfusion hepatitis B infection could be OBI [6], however, the mechanism(s) responsible for progression of OBI has yet to be clarified. Recently we reported OBI in Isfahan and Kerman, two of the central provinces of Iran [7, 8]. The α-determinant of HBsAg is a highly conformational, immunodominant antigenic determinant and is common to all HBsAg subtypes. It is located at amino acid positions 124 – 147 within the major hydrophilic region (MHR) of HBsAg (see figure 1). Several investigators from different parts of the world suggested that mutations within the α-determinant of the S-gene of HBV may change the epitope making it undetectable by routine ELISA methods, hence, these samples are inappropriately being considered as HBsAg negative

[9-13]. In contrast, some researchers failed to report mutations in the α-determinant of HBsAg in OBI patients [12, 14-16]. Therefore we decided to carry out additional evaluations of possible mutations in the α-determinant which, if correlated to the disease, could be a useful tool to identifying the mechanisms of OBI progression. However, other studies have reported all the current HBV genotypes in OBI patients [9, 15-18] and some investigators suggested that, in OBI, mutations of the S-gene are genotype specific [15], thus, we also determined the genotype of HBV in the patients that were studied in this report. In addition to these complications, some investigators have indicated that low expression of HBsAg in OBI patients may also render it undetectable by ELISA [12, 19]. One mechanism that may be responsible for low HBsAg expression is co-infection with Hepatitis C (HCV) [19, 20]. Previous studies showed that the HCV core protein can act as a gene-regulatory protein which leads to decreased function of translation machinery of hepatocytes [20]. In fact, HBsAg can not be expressed in hepatocytes co-infected with both HCV and HBV.

Due to the critical role of HBsAg in detection and clinical diagnosis of HBV infection, this project aimed to investigated the mutations of the S-gene (including the α-determinant), the genotype of HBV and co-infection with HCV in a subset of Iranian OBI patients.

Material and methods:

Subjects

Peripheral blood samples were collected from 3700 volunteer blood donors attending the Rafsanjan Blood Transfusion Services (Kerman, Iran) and placed in 5 ml tubes without anticoagulants. The samples were centrifuged at 370 x g for 4 minutes and the sera collected. All sera were separated within 24 hours of collection. If needed, serum samples were stored at -20ºC for a maximum of 2 months or at -70ºC, where longer storage times were required, for further processing. All of the samples were screened for HBsAg, anti-HBc and HBV-DNA as described previously [5]. In addition, 57 samples were collected from OBI patients ($HBsAg^-$/ant-HBc^+/$HBV\text{-}DNA^+$) as well as 100 acute HBV infected patients. The study protocol was approved by the ethical committee of the Rafsanjan University of Medical Sciences .

Prior to sample collection, all of the participants of this study filled out and signed the informed consent form which was designed based on the aims and objectives of the study.

Sequencing of the S gene

HBV-DNA PCR product was sequenced directly using an automated ABI (Applied Biosystems Incorporation) 0377 sequencer at the sequencing unit of the Cinnagen Company, Iran. HBV reference genome sequences were downloaded from NCBI GenBank.

RNA extraction

0.1ml of blood plasma were added to phenol and vortexed and centrifuged for 5 min at 12000rpm. The upper aqueous phase was transferred to a fresh 1.5ml tube and 0.2ml chloroform was added and mixed vigorously for 15 seconds. The sample was then incubated at room temperature for 5 min, followed by centrifugation at 12000rpm for 15 min at 4°C. The upper aqueous layer (containing RNA) was transferred to a fresh 1.5ml tube and 0.5ml isopropanol was added to the isolated aqueous layer. This mixture was incubated at room temperature for 10 min and the RNA was precipitated by centrifugation at 12,000rpm for 10 min at 4°C. The pellet was washed with 1ml of 75% (v/v) ethanol and centrifuged at 7500rpm for 5 min at 4°C. The resultant pellet was then air-dried for 10-15 min and dissolved in 25-50µl DEPC-treated water (the added volume depended on the size of the pellet) and heated at 60°C for 10 min.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To make cDNA, a reverse transcription reaction was performed using the following protocol: 4µl of 5x first strand buffer [125mM tris-HCl pH 8.3, 188mM KCl, 7.5mM $MgCl_2$ 25mM DTT]; 1µl of each dNTPs (10mM in DEPC-treated water); 4µl oligo-dT (125µg/ml); 1µl RNA (1µg/ µl); 4µl DEPC–treated water; 1.5µl M-MLV reverse transcriptase enzyme. After

addition of M-MLV-reverse transcriptase and RNase inhibitor, samples were mixed and incubated for 45 min to 1 hour at 37°C. To amplify cDNA species, a PCR reaction mixture was prepared by addition of the following reagents to a 0.2ml micro-centrifuge tube on ice: 5µl of 10x PCR buffer; 1.5µl $MgCl_2$ (stock concentration 1.5mM); 1µl of dNTPs (10mM); 2µl of each forward and reverse primers (25ng/µl); 4µl cDNA; and sterile double distilled water to a final volume of 50µl. Primers were design for the polyprotein gene of HCV. The sequence of forward and reverse primers is given in table 1. The PCR thermocycler was programmed as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 40 seconds, 63°C for 40 seconds and 72°C for 45 seconds. During the last 45 seconds of the first stage 1µl of *Taq* polymerase was added to the mixture. The presence of a 354bp fragment in the PCR products indicated a positive result. Positive samples, which were confirmed by ELISA and western blotting methods, were used as positive controls along with a commercial negative control for the analysis of all samples (Cinnagen, Iran).

Genotyping

HBV genotyping was performed as described by Amini et al., 2008 [21]. The sequences of forward and reverse primers used in this section are shown in table 1.

Results

In the present study we found that all patients were negative for HBsAg, only 352 (9.5%) samples were positive for anti-HBc whereas HBV-DNA was detected in 57 of those 352 samples (16.1% of the $HBsAg^-$/anti-HBc^+ pool). Therefore, current results indicated that 16.1% of $HBsAg^-$/anti-HBc^+ samples had detectable HBV-DNA and were regarded as OBI patients. Overall, 1.54% of all donated samples were OBI carriers.

The results of this study demonstrated that there was no mutation in α-determinant of the S-gene of HBV in occult and acute HBV infected patients but one silent, G369A (Thr123Thr), and a Glycine to Arginine mutation, G553A (Gly185Arg), was observed outside the range of the α-determinant in both groups as well as a mutation at +580 of HBV S-gene, G580A (Ala194Thr), in acute HBV infected patients (Figure 1). We submitted this altered sequence of the S-gene of OBI patients in the NCBI GenBank database (accession number EU346890).

We also found that all of the samples analysed in this study were HCV-RNA negative and genotyping of HBV demonstrated that all patients were infected with the HBV D genotype (Figure 2).

Discussion

In OBI, despite undetectable levels of HBsAg, detectable levels of HBV-DNA exist in the peripheral blood of patients [2, 22]. In our previous investigations, we showed a markedly elevated prevalence of OBI in Iranian blood donors compared to some other countries [7, 8]. Our

previous results encouraged us to carry out a set of experiments to analyse the possible mechanisms of OBI in Iranian blood donors. Although, the results of some studies have demonstrated that HCV co-infection may be one of the main reasons for inducing OBI [19, 20], our results showed that OBI patients were negative for both HCV markers (HCV-Ab^-/HCV-RNA^-). Therefore, it seems that HCV does not play a prominent role in inducing OBI in our patients. Although, OBI is reported elsewhere in HCV infected patients [23, 24] our previous investigations showed that there was no occurrence of OBI in HCV infected haemodialysis [3] and thalassemic patients [4]. Considering those results it appeared that HCV infection is not prevalent in Iranian OBI patients. Additionally, Fallahi et al., reported that HCV incidence per 100,000 blood donations was 0.13% in Iran [25]. Suggesting that the incidence of HCV in Iranian blood donors is less than other regional countries [25]. Therefore, it can be assumed that the low HCV incidence in Iranian blood donors is a significant reason for undetectable HCV in our patients [25].

Previous studies showed that genotypes of HBV may influence the clinical presentation of hepatitis B [26]. For Instance, Kao et al., 2003, showed that the clinical presentation is different in patients infected with various types of HBV genotypes [27]. In addition, Yuen et al., 2004, showed that C genotype of HBV is associated with hepatocellular carcinoma [28]. Our results showed that all of the patients studied were

infected by the D genotype of HBV. In agreement with these results, Katsoulidou et al., 2009, Van et al., 2008 and Pinarbasi et al., 2009, also showed that the D genotype of HBV was present in OBI patients from Greece, the Netherlands and Turkey, respectively [29, 30]. In addition to the D genotype other HBV genotypes, including the C genotype [9, 17, 18] as well as A and G genotypes [15, 18, 31] have been reported in the OBI, thus, it is not possible to conclude that the D genotype is uniquely involved in OBI. Clearly there are other factors to be considered and a more complete study is needed in this field.

Other features of HBV variation that have the potential to induce OBI could include mutations in S-gene that have not been revealed in the current study in this report because any mutations were not seen in the α-determinant region. However, Pinarbasi et al., 2009 [16] along with our own continuing studies, have not shown any additional mutations in this region (especially in the α-determinant region). On the other hand, Zaaijer et al., 2008, reported multiple mutations in this region of the HBV genome in OBI patients [10]. Some mutations in S-region of HBV in OBI patients were also reported by Awerkiew and colleagues [11]. Banerjee et al., 2007, detected some mutations in the S-gene of HBV in OBI patients [13]. However, the combined data suggests that S-gene mutations are not involved in inducing OBI in our geographical region, hence, it is likely that HBsAg has normal conformational structure and can be detected by

standard ELISA protocols being used for blood screening and using more sensitive tests may improve HBsAg detection. Moreover, it would appear that virus related parameters such as mutations in the S-gene or co-infection with HCV do not play prominent role in establishing OBI in our studied patients. Furthermore, we have speculated that possible failures of the immune system to respond during the process of HBV viral clearance could be the main reasons of OBI in our patient population.

Finally, due to the complexity of OBI, different aspects of the disease must be examined. In our future work we will study the expression and polymorphisms within important molecules involved in the immune system of OBI patients. Our preliminary studies suggested that polymorphisms in exon 9 of the vitamin D receptor is associated with OBI [5], suggesting that we focus on genes that regulate immune responses of patients with viral infections.

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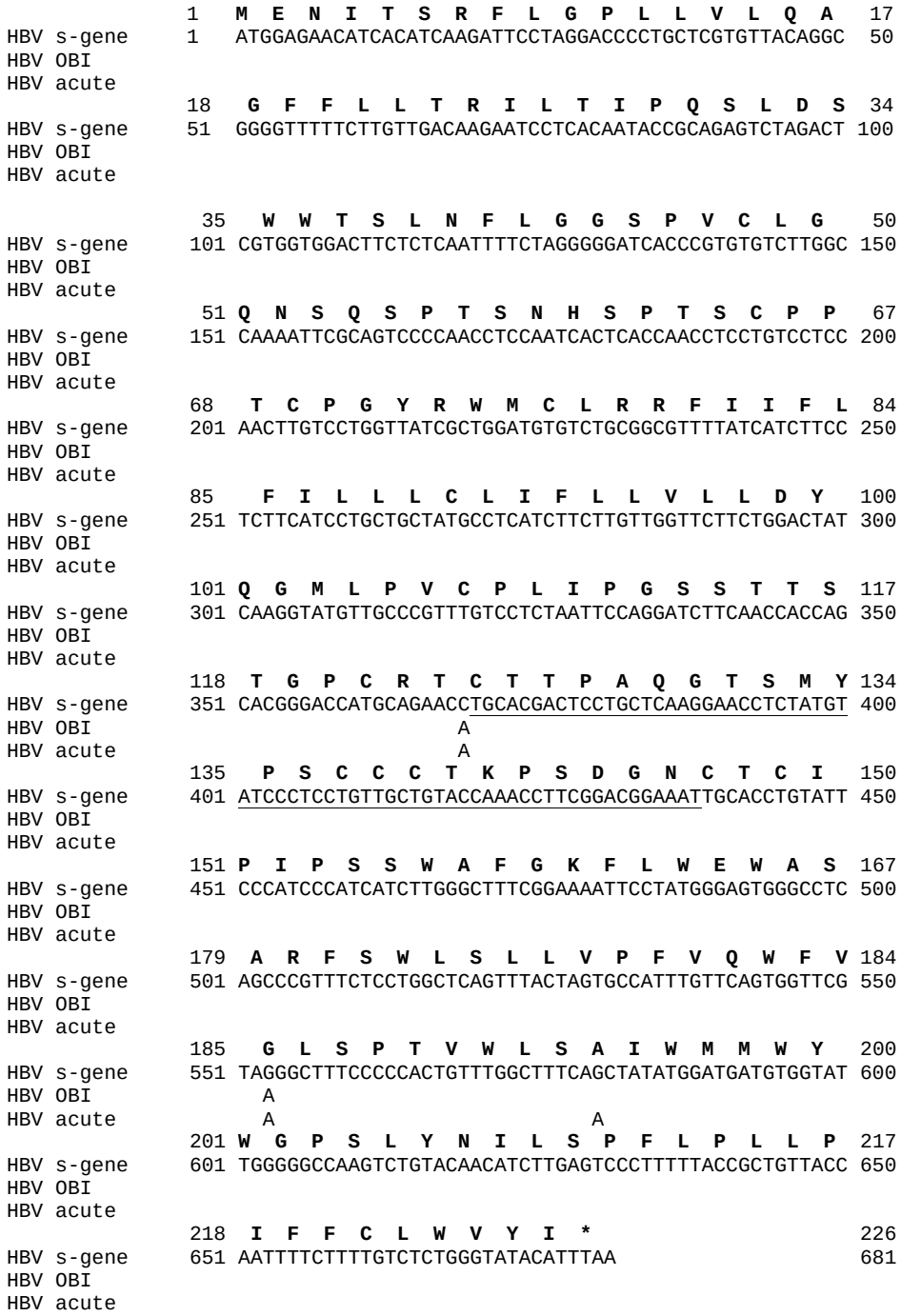

Figure 1. The nucleotide and peptide sequences of the Hepatitis B virus S-gene. The top line of the sequences shows the protein sequence of the reference protein. The second line shows the nucleotide sequence of the reference gene (labeled 'HBV s-gene'). The lines below show the nucleotide sequence of the s-gene sequences from patients with occult (labeled 'HBV OBI') and acute (labeled 'HBV acute') hepatitis infections, blank spaces indicate homology with the reference sequence. Both occult and acute infected patients carried mutations G369A (Thr123Thr) and G553A

(Gly185Arg). In addition to these mutations, the acute patients also carried G580A (Ala194Thr). The α-determinant region is shown underlined.

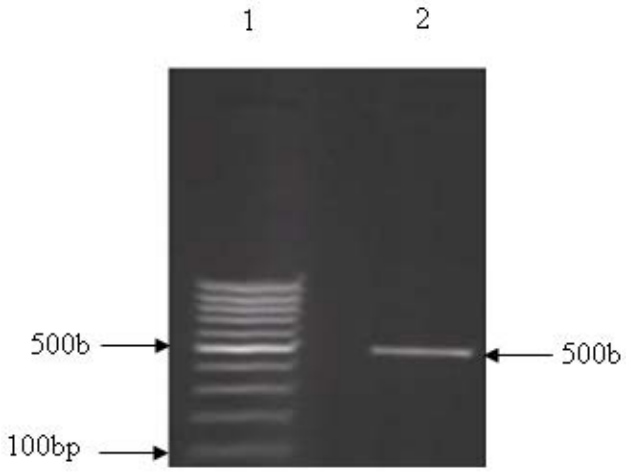

Fig. 2 Gap-PCR based HBV genotype analysis. An ethidium bromide stained agarose gel of DNA amplicons obtained from PCR based HBV genotype analysis of acute and occult HBV infected patients. Lane 1: DNA ladder, 2: amplification of a pre-S gene region of D genotype (500bp). All of patients carried D genotype.

Gene Name	Primers (5` to 3`)	AT (°C)	MT (°C)	Fragment Size

S (HBV) **F** **R**	 TCGTGGTGGACTTCTCTC ACAGTGGGGGAAAGCCC	55	93	500bp
Pre-S1 (HBV) **F** **R**	 TGGGAACAAGAGCTACAGCATGG CAACTGGTGGTCGGGAAAGAATC	65	94	533bp (non-D genotype) 500bp (D genotype)
Poly protein (HCV) **F** **R**	 ATCCCAGCTTCCGCTTAC TGCAGTCCTGAACTGTC	94	63	354bp

Table 1. Primers used in the study.

The table shows sequences of primers, the amplified fragment size and temperatures that were used in HBV-DNA and HCV-cDNA amplification. AT= annealing temperature; MT= melting temperature; F= forward primer; R=reverse primer. PCR fragment sizes of 533bp indicate the virus is not the D genotype whereas amplicons of 500bp indicate the D genotype.