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T Helper and B Cell Escape Mutations within the HBc Gene in Patients with Asymptomatic HBV Infection: A Study From the South-Eastern Region of Iran

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SUMMARY

Background: Escape mutations potentially allow viruses to avoid detection and clearance by the host immune system and may represent a mechanism through which infections may persist in some patients. The association of the mutations in the HBcAg gene with Hepatitis B asymptomatic carriers (ASC) has not been studied adequately. The current study was aimed to investigate HBcAg18-27 CTL epitope mutations in ASC patients in the South-Eastern region of Iran.

Methods: 100 ASC patients were selected for this study and screened for HLA-A2 using flow cytometry. HBV-DNA was extracted from the HLA-A2 positive patients and the HBc gene was amplified using PCR. Direct double sequencing was performed to analyse mutations in the HBc gene of HBV isolates from patients with ASC.

Results: Overall, 25 (25%) of individuals were HLA-A2 positive. Direct double sequencing indicated no mutations in the HBcAg18-27 epitope. However, four mutations within the T helper and three mutations within the B cell epitopes of ASC patients were identified.

Conclusions: The lack of mutations within the HBcAg18-27 epitope suggests that the antigenicity of this region is not altered in HBV isolates of our patients and therefore antigen presentation would occur normally to the patient’s immune system through HLA-A2. However, in the course of this study we revealed some novel mutations within the T helper and B cell epitopes that may affect the efficiencies of immune response of ASC patients against these novel HBV epitopes.

INTRODUCTION

Asymptomatic HBV infection, HLA-A2, HBcAg, mutation

Iran is known to have a low endemicity for hepatitis B infection [1]. Those patients that have been identified and characterized appear to only carry the HBV genotype D strain [2]. Patients considered as HBV asymptomatic carriers (ASC) are considered to have a clinical
form of hepatitis B in which HBV is not eradicated completely from hepatocytes despite a lack of symptomatic liver disease [3]. On the other hand, these patients have inactive HBV but are HBsAg carriers [4]. Some studies have suggested that ASC could be one of the main causes of cirrhosis and hepatocarcinoma [5]. However, the mechanism(s) responsible for the progression of ASC to initiate these pathologies has yet to be clarified. Some investigators believed that genetic parameters of HBV may be responsible for the difference between individuals with resistant HBV strains and those who can clear the virus [6]. Mutations within the antigenic sites of HBV are considered a possible mechanism for the virus to escape detection by the host’s immunological surveillance by attenuating the T and B lymphocyte responses. Mutations such as these are described as ‘escape mutations’ [7]. Previous studies showed that the HBcAg18-27 epitope of HBcAg is immunodominant and can be presented to cytotoxic T CD8+ lymphocytes (CTL) through HLA-A2 [8]. Several investigators from different regions of the world suggested that mutations in the HBcAg18-27 CTL epitope of HBV leads to a reduced or complete absence of presentation of these mutated HBcAg peptides by HLA-A2+ antigen presenting cells (APCs) which subsequently leads to a lack of activated cytotoxic T CD8+ cells that would normally exist after presentation of foreign peptides to naive T cells [8,9]. In ASC patients carrying HBcAg CTL epitopes that are processed normally by APC there may be alternative mutations within the HBcAg gene that can attenuate the host immune response. It has been proposed that mutations in other immunodominant epitopes of the HBcAg gene, such as the T helper (Th) and B cell epitopes, may have crucial roles in reducing host immunological responses [8,9]. This may lead to these isolates of HBV “escaping” the immune surveillance of the host and thereby persisting within the patient. Therefore, the evaluation of mutations within the HBcAg18-27 CTL and other epitopes in HLA-A2 positive ASCs could be a useful tool in helping to understand HBV pathogenesis. Based on the critical role of CTL, Th and B cell epitopes in HBV eradication from hepatocytes, the aim of this project was to investigate the mutations of the HBc gene in a subset of Iranian HLA-A2+ ASC patients.

MATERIALS AND METHODS

Subjects: Peripheral blood samples were collected from 100 asymptomatic HBV infected patients from Kerman (South-Eastern region of Iran) in 5.5 mL tubes. Patients with HBV and HCV co-infection or with detectable HIV antibody were excluded from the study. The diagnosis of ASC was performed by using the “Guide of Prevention and Treatment in Viral Hepatitis” [8]. Patients were recruited for the study based on assessment of their previous clinical record and as a result all suitable patients were involved in the current study. The samples were centrifuged at 3500 rpm for 4 minutes, and the plasma was separated within 24 hours after collection. The plasma samples were stored at -20°C for a maximum of 2 months or at -70°C when longer storage periods were required for analysis. This study was approved by the ethical committee of the Rafsanjan University of Medical Sciences and written informed consent was obtained from all participants prior to sample collection.

Detection of serological HBV markers

All of the samples were screened for HBsAg by ELISA (Behring, Germany), as well as an anti-HBc screening test using a commercial anti-HBc kit (RADIM, Italy). All of the samples were also tested for HCV and HIV by ELISA (Behring, Germany).

Extraction of HBV-DNA from plasma samples

Viral DNA was purified from 200 μL of plasma from HLA-A2 positive patients as described in our previous study [10]. Briefly, each sample was incubated at 72°C for 10 minutes and then cooled at 4°C for 5 minutes in 200 μL proteinase K (200 μg/mL). After phenol/chloroform extraction (1:1), the viral DNA was precipitated with ethanol and the pellet was dissolved in DNase free, deionized water and stored at -20°C.

Amplification of the HBc gene

To amplify the HBc gene, PCR reaction mixtures were prepared in 0.2 mL microcentrifuge tubes, on ice, containing the following reagents: 2.5 μL of 10X PCR buffer, 0.5 μL of MgCl2 (stock concentration 1.5 mM), 0.5 μL of each dNTPs (10 mM), 1 μL of each forward and reverse primers (25 ng/μL), 5 μL of prepared DNA, and sterile double distilled water to achieve a final volume of 25 μL. The sequence of forward primer was: 5′-TTCCGTTCACTCTGCTAATC-3′ and the sequence of reverse primer was: 5′-CGAAGTTGTAAAAGGTTGG-3′. PCR cycling conditions were 94°C for 1 minute, 65°C for 1 minute, 72°C for 2 minutes, for 35 cycles. During the last 45 seconds of the first stage of 0.3 μL of Taq DNA polymerase was added to the mixture. A reference HBV genome was purchased (Cinnagen, Iran) and used as a positive control. After adding 4 μL of loading buffer to 10 μL of the resultant PCR amplicon, the DNA was separated on a 2% agarose gel for primary analysis. The presence of a 500 bp fragment indicated a positive result.

DNA sequencing

HBV-DNA PCR products were sequenced directly using an automated ABI (Applied Biosystems Incorporation) 0377 sequencer by using the forward and reverse primers. HBV reference genome sequences were downloaded from the NCBI GenBank (Ref no: AY371914.1).
Table 1. The table shows the position of mutations found within the ASC patients and the amino acid substitutions they would encode for within the HB core protein. Also shown is the epitope in which the mutation occurs.

<table>
<thead>
<tr>
<th>DNA mutation</th>
<th>Amino Acid substitution</th>
<th>Epitope domain of the substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>A192T</td>
<td>Glu64Asp</td>
<td>T helper</td>
</tr>
<tr>
<td>G198T</td>
<td>Met66Ile</td>
<td>T helper</td>
</tr>
<tr>
<td>C200A</td>
<td>Thr67Asn</td>
<td>T helper</td>
</tr>
<tr>
<td>G205T</td>
<td>Ala69Ser</td>
<td>T helper</td>
</tr>
<tr>
<td>T207C</td>
<td>Silent</td>
<td>T helper</td>
</tr>
<tr>
<td>G205T + T207C</td>
<td>Ala69Ser</td>
<td>T helper</td>
</tr>
<tr>
<td>A220G</td>
<td>Silent</td>
<td>Nil</td>
</tr>
<tr>
<td>A221G</td>
<td>Asn74Ser</td>
<td>Nil</td>
</tr>
<tr>
<td>A220G + A221G</td>
<td>Asn74Gly</td>
<td>Nil</td>
</tr>
<tr>
<td>A231C</td>
<td>Glu77Asp</td>
<td>B Cell</td>
</tr>
<tr>
<td>T239C</td>
<td>Ile80Thr</td>
<td>B Cell</td>
</tr>
<tr>
<td>A260G</td>
<td>Asn87Ser</td>
<td>B Cell</td>
</tr>
<tr>
<td>A289T</td>
<td>Ile97Phe</td>
<td>Nil</td>
</tr>
<tr>
<td>A347T</td>
<td>Lys116Ile</td>
<td>B Cell</td>
</tr>
</tbody>
</table>

**Monoclonal antibodies**
Fluorescence-conjugated monoclonal antibodies (mAb) and their target antigens used in the study were as follows: PE (phycoerythrin) conjugated mouse anti-human HLA-A2 (clone: BB7.2, isotype: mouse IgG2b, κ (BD, USA) and PE conjugated mouse antibody (IgG2b, κ clone; 27-35) (BD, USA) as its isotype-matched negative control.

**Flow cytometry analysis**
For detection of HLA-A2 in peripheral blood mononuclear cells (PBMCs) in ASC individuals, peripheral blood samples were stained with the above mentioned monoclonal antibody and its isotype-matched negative control according to the manufacturer’s guidelines. Briefly, red blood cells from a 100 μL sample were lysed by RBC lysis solution (BD, USA) and PBMCs were washed 3 times with PBS. 20 μL of PE conjugated anti-HLA-A2 was added to the washed PBMCs and after 30 minutes incubation, 1 × 10⁴ cells were analysed using a Partec system model PAS flow cytometer.

**Three-dimensional image**
Three-dimensional (3D) predictions of the mutated Hbc structures have been constructed by the comparative modeling 3D-JIGSAW program [11]: http://bmm.cancerresearchuk.org/~3djigsaw/ based on the Hbc 1QGT X-ray structure and presented by Chimera software [12].

**RESULTS**
100 ASC individuals were selected for this study and screened for HLA-A2 using flow cytometry. 25 HLA-A2⁺ were identified for further analysis. Our results also showed that all HLA-A2⁺ patients were HBV-DNA positive by PCR. HBV DNA was extracted from all of these patients and the HbcAg gene was subsequently sequenced. The sequencing data from all 25 selected patients revealed that all the samples had the same mutation pattern as follows: No mutations were found in the HbcAg18-27 epitopes of the HBV isolates from any of the patients, while the same twelve DNA mutations, leading to 10 amino acid substitutions were found in all patients. Eight of the ten amino acid substitutions occurred in the Th and B cell epitopes (Figure 1 and 2). The mutations were as follows: A192T (Glu64Asp), G198T (Met66Ile), C200A, (Thr67Asn), G205T + T207C (Ala69Ser), A220G + A221G (Asn74Gly), A231C (Glu77Asp), T239C (Ile80Thr), A260G (Asn87Ser), A289T (Ile97Phe) and A347T (Lys116Ile), see Figures 1 and 2 and Table 1. 3D structure analysis was performed by amino acid substitution on the known crystal structure of the HB core protein (Figure 2).
Figure 1. The nucleotide and amino acid sequences of the wild-type (wt) and mutant forms (mut) of the HBc gene identified in HBV from ASC patients. No mutations were detected in the HBcAg18-27 epitope (bold-underlined section), while there were 12 nucleotide mutations which translate to ten amino acid substitutions within the Th and B cell epitopes. The Th epitopes are shown by a double underline, while the B cell epitopes are shown by a dotted underline. Nucleotide substitutions are shown above the wild-type sequence and are highlighted in grey. Amino acid substitutions are shown below the wild-type sequence in red.
Figure 2. A space filled surface model of the Hepatitis B core protein showing the positions of the mutant T helper and B cell epitopes. The surface analysis shows that most of the amino acid exchanges influence the outer surface of the HBc protein. Mutant amino acids are colored black. (A) Shows the outer surface of the mutant monomer HBcAg, while (B) illustrates its contact surface. A mutant HBc dimmer highlighting the localization of exchanges is shown in (C). (D) Demonstrates that exchanges have an influence on the hydrophobicity/hydrophilicity distribution on the surface. Red circles demonstrate increased hydrophobicity, while blue circles show increased hydrophilicity regions. The 3D data was generated by comparative modeling based on the X-ray structure of HBcAg (HBc PDB: 1QGT) using Chimera software.

The figures demonstrate that the amino acid exchanges are mostly localized on the outer surface of the HBc monomer, but not on the dimer contact surface and, therefore, the identified mutations have the potential to influence the hydrophobicity/hydrophilicity distribution on the surface (Figure 2).

The demographic data, HBV-DNA copy number, alanine transaminase (ALT) and aspartate aminotransferase (AST) serum level, status of hepatitis B e antigen (HBeAg) and hepatitis B e antibody (HBeAb) positivity and also duration of the HBsAg positivity are displayed in Table 2.
DISCUSSION

Results of the current study showed that there were no mutations in the HBcAg18-27 epitope of the HBV isolates of HLA-A2 positive ASC individuals. Therefore, based on our results, it can be concluded that the conformation of HBcAg18-27 epitope was not changed in these patients and, hence, in this cohort of patients the HBcAg18-27 epitope could be bound by HLA-A2 and presented to cytotoxic T cells, properly as expected.

Previous studies showed that the HBcAg contains three different epitopes: 1. Th epitopes (HBcAg35-45 and HBcAg49-69 [13,14]), 2. CTL epitopes (HBcAg18-27 and HBcAg91-95 [14,15]) and 3. B Cell epitopes (HBcAg76-87, HBcAg105-116 and HBcAg130-135 [13,16-18]) (refer to Figure 1). Our results showed that there were four amino acid substitutions within the T helper epitopes (Glu64Asp, Met66Ile, Thr67Asn, and Ala69Ser) and four substitutions within the B cell epitopes (Glu77Asp, Ile80Thr, Asn87Ser, and Lys116Ile) (see Table 1). These mutations are mostly localized on the outer surface of the HBc monomer, however, do not appear to disrupt the dimer contact surface. The substitutions may have the potential to change the hydrophobicity/hydrophilicity distribution on the surface of the protein as determined by homology modeling. However, it should be noted that the analysis did not apply any stringent structural integrity analysis such as protein relaxation and, therefore, it cannot be stated whether the identified substitutions can change or disrupt the overall structure of the HB core protein. In a report by Samad Amini-Bavil-Olyaee, et al (2005), they identified the major HBV isolates in Iran and although the isolate described here is similar to isolate IR18, it contains mutations that had not previously been reported in Iran but had been seen in separate isolates from Japan and Poland [2], for example Met66Ile is seen in isolate IR18 but never with Thr67Asn which is seen in isolates from Switzerland and Japan but never in Iran. Likewise, the individual mutations were reported by Sendi et al., but not in the same combination as the isolate reported here [9].

A subsequent BlastN search of the sequence reported here revealed no matches of this isolate in the databases. Based on the presence of mutations within the T helper epitopes, it could be speculated that T helpers cannot detect the HBcAg on the MHC molecules, hence, they cannot secrete suitable cytokines or express co-stimulatory molecules to elicit an effective cytotoxic T cell response, however, this would need to be tested. Although humoral immunity is speculated to be less important than cellular immunity in eradication of HBV infection, the latter is still important in prevention of infection [19]. Our results showed that there are four mutations in B cell epitopes, therefore, humoral immunity may also be affected in ASC individuals.

Previous studies showed that there were some mutations in the HBcAg18-27 CTL epitope [8]. For instance Liu et al. showed that this region of the HBc gene harbored a mutation in which valine was substituted by isoleucine at position 27 (Val27Ile) [8]. Interestingly, they showed that the mutant (Ile27), like the wild type peptide, can bind to the HLA-A2 molecule with high affinity and induce specific cytotoxic T CD8+ cell responses.

Table 2. Main demographic, socioeconomic conditions and clinical parameters of ASC patients.

<table>
<thead>
<tr>
<th>Variant</th>
<th>HLA-A2+ ASC Patients</th>
<th>HLA-A2-ASC Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>36 ±7</td>
<td>40 ±8</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (n (%))</td>
<td>13 (52%)</td>
<td>35 (46.6%)</td>
</tr>
<tr>
<td>Male (n (%))</td>
<td>12 (48%)</td>
<td>40 (53.4%)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>60 ±7</td>
<td>65 ±7</td>
</tr>
<tr>
<td>HBV-DNA copy number in serum</td>
<td>Under 10000 copy/mL</td>
<td>Under 10000 copy/mL</td>
</tr>
<tr>
<td>Mean ALT (U/L)</td>
<td>25 ±8</td>
<td>28 ±9</td>
</tr>
<tr>
<td>Mean AST (U/L)</td>
<td>30 ±4</td>
<td>31 ±6</td>
</tr>
<tr>
<td>#HBeAg positive (n (%))</td>
<td>18 (72%)</td>
<td>45 (60%)</td>
</tr>
<tr>
<td>Mean duration of the HBsAg positivity (year)</td>
<td>6 ±2</td>
<td>7 ±2</td>
</tr>
<tr>
<td>HBCAb positive (n (%))</td>
<td>25 (100%)</td>
<td>75 (100%)</td>
</tr>
</tbody>
</table>

* Significant difference in HBeAg positivity (p<0.002, chi-square, case VS control).
* Significant difference in HBeAg positivity (p<0.001, chi-square, case VS control).
in acutely infected hepatitis B patients [8]. It should be noted that this is a conservative substitution and being so mild in nature may not have been severe enough to change cytotoxic responses. Sendi et al. showed that there are several mutations in T helper, B cell, and CTL epitopes of chronic and ASC patients [9]. Interestingly, they showed that these escape mutations occur at a higher rate in ASC than patients with chronic HBV infection [9]. In summary, our results indicate that the mutations in the T helper epitopes may lead to HBV evasion from immune responses. Therefore, HBV persists in hepatocytes at low levels taking advantage of the reduced immune response caused by the escape mutations. Interestingly, Li et al. showed that HbcAg induces interleukin-10 production and also inhibits HbcAg-specific Th17 responses in chronic hepatitis B patients [20]. Therefore, it may be concluded that in ASC patients, HBV may change its HbcAg to induce more IL-10 production and the subsequent inhibitory effect on Th17 function. Finally, due to the complexity of host-virus interaction in ASC individuals, it is essential to consider other major issues, such as additional mutations in both the virus genome as well as polymorphisms in other components of the host immune system [21-23]. The study of the immune system status of ASC patients demands future attention if we are to make significant headway in the fight against this disease.

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Declaration of Interest:
The authors of this manuscript have no invested interests in products described or used in this article. The authors have no conflicts of interest.

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