Detection of Hepatitis B Virus X Gene and PreC Promoter Mutations from Chronic Hepatitis B Patients in the South of Turkey

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Abstract—Hepatitis B virus (HBV) infection is a global health problem with more than 2 billion infected individuals. HBV infection leads to diverse outcomes ranging from acute to chronic hepatitis, which may result in severe complications as liver cirrhosis and hepatocellular carcinoma (HCC). HBV is one of the most important human DNA viruses having strong oncogenic potential. Recently, many studies have reported on HBV X gene and PreC promoter mutations associated with HCC. In order to detect the prevalence of HBx gene and PreC promoter mutations possibly related to HCC, we have analyzed sera samples collected from 61 patients with chronic hepatitis B. We have detected T1653 mutation in 1 of 61 (1.63%), A1896 mutation in 10 of 61 (16.39%), and T1762–A1764 dual mutation in 4 of 61 (6.55%). T1653 and T1762–A1764 dual mutations were suggested significantly related to HCC in earlier reported studies. Our findings demonstrate that HBx gene and PreC promoter mutations related to HCC are present in our region and prospective clinical chord studies would be useful for better patient management and of early diagnosis of possible HCC cases.

Keywords: hepatitis B virus, chronic hepatitis B, X gene mutations, PreC promoter mutations, hepatocellular carcinoma

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1. INTRODUCTION

Hepatitis B virus (HBV) infection is a health problem threatening people living all over the world. HBV infection leads to a wide spectrum of liver disease ranging from acute to chronic hepatitis, cirrhosis, and hepatocellular carcinoma [1]. More than 2 billion people are infected with HBV around the world. Of these 350 million people are chronically infected with HBV and one million deaths occur each year due to active hepatitis, cirrhosis or primary liver cancer [2]. HBV, liver cirrhosis, exposure to aflatoxin B1, alcohol consumption, and diabetes have etiological roles in the development of hepatocellular carcinoma (HCC) [3]. Among these, HBV is the major risk factor for HCC and associated with more than 50% of cases of HCC [4].

HBV genome contains overlapping open reading frames (C, S, P, X) regulated with 4 promoter (Basal core promoter-BCP, Pre-S1, PreS2/S and X), two enhancers (EnhI and EnhII) and negative regulatory elements [5].

From the acute to chronic phase of HBV infection different structural, non-structural or regulatory region based mutation profiles may occur [6]. In fact, the main reason of the mutations is the lack of proofreading activity of HBV polymerase. All possible single base changes can be produced per day because the HBV genome is only ~3200 base pairs. However, mutated variants can be selected and mutations arise increasingly under the pressure of host defense and antivirals [7].

There has been increasing evidence of an association between molecular alteration and the development of HCC in patients with HBV infection [6]. Mutations in the basal core promoter (BCP) region at nucleotides (nt) 1762 and 1764 (T1762/A1764) and in the precore region at nt 1896 (A1896) are associated with HBV e antigen (HBeAg) seroconversion and persistent viral replication. Double mutations in the BCP at nt 1762 and nt 1764, resulting in T1762–A1764, have been described in patients with chronic HBV, fulminant hepatitis, or who are immunosuppressed. This double mutation results in a decrease in HBeAg production by 70% and may increase viral replication [8].

It is noteworthy that both BCP and precore mutations are often found in patients with advanced liver disease such as HCC [6]. The T1762/A1764 mutations alter HBeAg production at the transcriptional level,
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and the A1896 mutation in the precore region terminates the translation of precursor protein, abrogates HBeAg production, and results in seroconversion. At the stage of chronic HBV infection, T1653 mutation in EnhII in X gene region is important to the contribution of HCC risk [9]. T1762–A1764 dual mutation in BCP region [10, 11] T1753C/A/G mutations in C promoter [12] could also be important to contribute to HCC risk [13–15].

In our study, we aimed to detect X/PreC (EnhII, BCP, Precore) mutation patterns in chronic HBV patients without HCC in Mersin (a southern costal of Turkey).

MATERIALS AND METHODS

Sera Samples

Sera samples were provided from the stored samples of 61 patients, priory diagnosed as chronic hepatitis B in Mersin University Hospital, Department of Gastroenterology. All sera samples were HBsAg (+) and HBV DNA (+).

Extraction of HBV DNA

One hundred microliters of serum was mixed with 300 µL of lysis buffer (13.3 mmol/µL Tris–HCl, pH 8.0, 6.7 mmol/µL ethylene-diaminetetraacetic acid, 0.67% sodium dodecyl sulfate, 133 µg/mL proteinase K and incubated at 55°C for 4 h. Two phenol-chloroform extractions were followed by one chloroform extraction, and DNA was precipitated with ethanol. The precipitate was dissolved in 20 µL of TE buffer (10 mmol/µL Tris–HCl, pH 8.0, 1 mmol ethylenediaminetetraacetic acid).

Semi-Nested PCR Amplification

The first round of PCR was performed with 5 µL of DNA, 1.25 U of Taq polymerase (Promega, Madison, WI), 0.2 mM dNTP (each), 1 mM MgCl2, 0.25 mM of each forward and reverse primers, and 1 × PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl). The primers; HBV-X1 (Nt. 1177–1190) and HBV-X2 (Nt. 1961–1979) were used for the first round of PCR. The reaction was allowed to produce at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min in each cycle. We have also used a hot start (at 94°C for 5 min) and a post elongation step (at 72°C for 5 min) before and after the reaction. The amplification was performed for 25 cycles in a thermal cycler (Boeco Germany, TC-PRO). In the second round of PCR, we have used 1 µL of the first PCR product and internal primers (at the same conditions and same thermal cycles described above). The primers; HBX-IS (Nt. 1400–1423) and HBV-X2 (Nt. 1961–1979) were used for the second round of PCR. The primer sequences and PCR product lengths are demonstrated at Table 1.

DNA Cycle-Sequencing of HBV Isolates

The second-round PCR products were 579 bp. These products included X gene region and significant portion of PreC promoter. The PCR products were purified by Wizard PCR prep DNA purification system (Promega). HBX-IS (Nt. 1400–1423) was used as sequencing primer. Purified PCR products were cycle-sequenced by SILVER SEQUENCE(tm) DNA Sequencing System (Promega) on a thermal cycler (Boeco Germany, TC-S) according to the manufacturer’s instructions. The cycle-sequencing reaction was performed for 95°C for 2 min, then 95°C for 30 s (denaturation), 42°C for 30 s (annealing), 70°C for 1 min (extension) for a total of 45 cycles, then 4°C soak. The cycle-sequencing products were separated by electrophoresis through a polyacrylamide gel with urea. The gel was visualized by silver staining according to the recommendation of the manufacturer.

Analysis of Sequences

The sequences of these products were determined and compared with previously reported HBV complete genome (AY721609).

Table 1. Primer sequences and PCR product lengths of HBx gene Core promoter/Precore gene region

<table>
<thead>
<tr>
<th>X gene region</th>
<th>Primer position</th>
<th>Sequences (5’–3’)</th>
<th>PCR product lengths, bp</th>
</tr>
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<tbody>
<tr>
<td>1st Round</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBV X1 sense external (Nt. 1177–01190)</td>
<td>5’TGC CAA GTG TTT GCT GAC GC-3’</td>
<td>802</td>
<td></td>
</tr>
<tr>
<td>HBV X2 (Nt. 1961–1979)</td>
<td>5’-AAG GAA AGA AGT CAG AAG G-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd Round</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBX IS (Nt. 1400–1423)</td>
<td>5’-CTG GAT CCT ACG CGG GAC GTC CTT-3’</td>
<td>579</td>
<td></td>
</tr>
<tr>
<td>HBV X2 (Nt. 1961–1979)</td>
<td>5’-AAG GAA AGA AGT CAG AAG G-3’</td>
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