Analysis of Hepatitis B virus (HBV) mutations in patients from Western Saudi Arabia with chronic disease

Sherif Aly El-Kafrawy¹, Ghazi Abdulatif Jamjoom¹, Hisham Othman Akbar², Hind Ibrahim Baker Fallatah², Mai Mohamed El-Daly¹, Yousef Abdulfattah Qari³, Abdullah Saeed Alghamdi³, Mohamed Babatin⁵, Mohammed Abdullah Alsaedi¹, Norah Abdulhamid Othman¹, Tagreed Lafi Al-Subhi¹, Mohamed Abdel-Hamid⁶, Esam Ibraheem Azhar¹,⁸

¹ Special Infectious Agent Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia
² Unit of Gastroenterology and Hepatology, Department of Internal Medicine, King Abdulaziz University, Jeddah, Saudi Arabia
³ Department of Medicine, King Abdulaziz University Hospital, Jeddah, Saudi Arabia
⁴ Gastroenterology unit, King Fahad Hospital, Jeddah, Saudi Arabia
⁵ Department of Medicine, King Fahad hospital, Jeddah, Saudi Arabia
⁶ Viral Hepatitis Research Laboratory, National Hepatology and Tropical Medicine Research Institute, Cairo, Egypt
⁷ Department of Microbiology and Immunology, Faculty of Medicine, Minia University, Minia, Egypt
⁸ Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia

Abstract
Introduction: Extensive research has provided a link between HBV variants and the clinical complications of liver diseases. This study was performed to further investigate the relationship between HBV variants in preS, S and BCP/PC regions and disease progression in chronic hepatitis B (CHB) cases in Jeddah, Saudi Arabia.

Methodology: 182 CHB patients were recruited for this study. HBV DNA was amplified by PCR in the PreS, S, and BCP/PC regions. Sequences were generated from 31 and 26 treated cases in PreS and S regions respectively and from 72 cases in the BCP/PC region.

Results: The majority of cases (86.7%) were genotype D. Mutations at preS1-A2922C, X-A1624C and PC-G1887A were detected only in cases with either a high fibrosis score or hepatocellular carcinoma (HCC), while mutations at positions PC-C1982A, PC-G1951T, X-C1628T and X-A1630G were detected more frequently in HCC cases, without reaching statistical significance. Seven deletions were detected in the PreS region. No deletions were detected in the CCAAT box.

The accumulation of mutations per sample in the preS1-2 and S regions were associated with elevated ALT (p < 0.001, 0.001 and 0.001; respectively) and increased fibrosis (p = 0.018, 0.02 and 0.013; respectively). The accumulation of mutations per sample in the BCP/PC region is associated with high viral load. Occult hepatitis B infection (OBI) was identified in 5 samples.

Conclusion: Our results add to the knowledge about HBV genotype-D variants. The accumulation of mutations per sample and OBI seem to play a role in the progression of HBV infection. G1896A was associated with the HBeAg negativity. The preS deletions did not play a role in liver disease progression.

Key words: HBV variants; chronic HBV; Saudi Arabia; sequencing.

(Received 08 July 2017 – Accepted 03 February 2018)

Introduction
The availability of an effective vaccine against hepatitis B virus (HBV) has reduced the prevalence of the virus but the infection still forms a major health challenge worldwide [1]. The liver disease caused by the virus ranges from asymptomatic self-resolved hepatitis to chronic hepatitis with complications that might result in cirrhosis and hepatocellular carcinoma (HCC). The treatment available for chronic HBV infection is mostly based on nucleos(t)ide analogues which inhibit the viral polymerase activity (entecavir, tenofovir and lamivudin) [2]. These antiviral regimens do not affect cccDNA molecules, which make it difficult to attain viral clearance.

HBV is an enveloped member of the Hepadnaviridae family of viruses and is classified into 10 genotypes. The viral genome is partially double-stranded and has four overlapping open reading frames:
the Polymerase (P), Surface (S), Core (C), and X genes [3]. The HBV replicates through an RNA intermediate and has a high degree of genetic variability \((1.4 \times 10^{-3} - 6.6 \times 10^{-4} \text{ substitutions/site/year})\) [4] due to the lack of proofreading of the viral RNA dependent DNA polymerase enzyme and the high replication rate of the virus.

Increasing research has been performed on the HBV strains and variants that may contribute to the clinical complications of liver diseases, especially to HCC. Some mutation patterns of HBV, such as the precore (preC) mutation at nucleotide 1896 (G1896A) or the double mutation in the basal core promoter (BCP) region at nucleotides 1762 (A/T) and 1764 (G/A), have been widely studied in relation to clinical severity [5,6]. Recently, several naturally occurring mutations have been described that are related to clinical severity. These include mutations in the pre-S, S and X regions, specifically, the preS1 and preS2 start codon deletion [6], W4P/R in preS1 region [7,8], W182 in S region [9], and V5M in the X region [10].

The introduction of mandatory vaccination in early childhood has reduced the prevalence of HBV in Saudi Arabia from about 4% before vaccination was started in 1990 to 1.5% currently, as indicated by comparing the prevalence in citizens older than 25 years and below 25 years [11,12].

Several studies have been done on the epidemiological aspects of HBV [13,14]. Genotype D is reported to be the most prevalent genotype among CHB cases in Saudi Arabia [14]. However, only two studies have investigated the genetic characterization of HBV. The first study reported the association of enhancer II, core promoter and precore mutations in HBV genotype D1 with the development of HCC in Saudi Arabia [15]. That study found that the mutations T1673/G1679, G1727, C1741, C1761, A1757/T1764/G1766, T1773, T1773/G1775 and C1909 in HBV/D1 were associated with HCC development. After adjusting for age and gender, stepwise logistic regression analysis indicated that mutations G1727, A1757/T1764/G1766 and T1773 are independent predictors of HCC development. Another study investigated HBV variants in the S region [16]. The authors detected 48 mutations in the viral strains of 20 cases from Saudi Arabia, 24 of which were nonsynonymous. The “a” determinant did not show the vaccine escape mutant G145SR, while the two mutations F130L and S135F in the same region were detected. No relationship was detected between the mutation/mino acid change and clinical presentation.

In two previous reports [17,18], we explored the genetic variants in the PreS and S regions of HBV in treatment naïve CHB cases from Jeddah, Saudi Arabia. We observed no significant association of the detected mutations in either the PreS or the S regions with HCC. In continuation to our previous work, we extend our work here to investigate variants in the HBV PreS, S and BCP/PC regions in treated and untreated CHB cases.

**Methodology**

The study population included 182 CHB infected individuals presenting at King Abdul-Aziz University Hospital (KAUH) and King Fahd General Hospital, Jeddah, Saudi Arabia, between 2014 and 2015. All CHB cases attending the hepatology clinics were approached and subjects who agreed to participate in the study donated 10 mL of blood. The study included CHB subjects over 18 years of age, whose HBV status was confirmed by a positive result for HBsAg, HBeAg and/or HBV DNA. Cases with HCV, HDV or HIV coinfection were excluded from the study. Sera were separated and stored at -80°C until tested. Serologic markers for HBV, HCV, HIV infections and AFP were analyzed using commercially available ELISA kits. All tests for serologic markers were performed according to manufacturers’ instructions.

DNA was extracted from serum samples using QIAamp DNA Blood mini kit (QIAGEN, Hilden, Germany) according to manufacturer’s protocol. The extracted DNA was amplified in the different HBV regions according to Chen and Kao [19,20]. PCR was performed using GoTaq Green Master Mix (Promega, Mannheim, Germany) on a Veritti 96 well thermal Cycler (Life technologies, Carlsbad, CA, USA). PCR amplicons were electrophoresed on 2% agarose gel with ethidium bromide staining and visualized under UV. The PCR product sizes for the three regions were 491, 399 and 497 base pairs (bp) for the pre-S, S and BCP/PC regions, respectively. Positive PCR products with expected sizes were gel purified using Qiagen gel extraction kit (Qiagen, Hilden, Germany) according to manufacturer’s protocol. The PCR products were subjected to cycle sequencing in both forward and reverse directions on an ABI 3500 Automatic Sequencer (Life technologies, Carlsbad, CA, USA) using the Bigdye Terminator V3.1 Reaction Cycle Kit (Life technologies, Carlsbad, CA, USA) according to manufacturer’s instructions. The generated sequences were assembled, annotated and analyzed for the mutations using the Geneious software version R8 [21]. Variant calling was detected using the “find
variation/SNP" command in Geneious. Alignment was performed using ClustalW software (V1.83) (www.clustal.org). Phylogenetic analysis was performed using the MEGA software (v.6) (www.megasoftware.net) [22] with the Neighbor-Joining method of the Maximum Composite Likelihood model, gamma-distributed rates among sites with 1,000 bootstrap replicates. Sequences from this study were deposited in the Genbank with the accession numbers KY631547-KY631572 for the S sequences, KY631573-KY631603 for the PS region and KY631604-KY631675 for the BCP-PC region.

We have assessed the liver fibrosis using transient elastography (Fibroscan 502, Echosens, Paris, France, 2005). The examination was performed by a well-trained certified Fibroscan technician and each test result was verified by one of two expert hepatologists. All included patients should have at least 10 valid readings, success rate of at least 70% and IQR of less than 30. The stiffness score was measured in Kapa. We defined the fibrosis stages as follow: F1 < 7, F2 7-9.5, F3 9.5-12.4 and F4 > 12.5 [23].

χ² or Fisher’s exact test were used as appropriate to compare data. Two-sided p value of < 0.05 was used as a predictor for statistical significance. Statistical analysis was performed using SPSS version 21 (IBM corporation, USA).

Results

Out of the 182 cases recruited for the study, 155 met the inclusion criteria. Their mean age was 45.7 years (19-85 years) with a male:female ratio of 1.31:1. The study included 47 cases who were under treatment that included Enticavir (Paraclude) and Lamivudine (Zefix). There were 9 HBV related HCC cases in the study group, confirmed by CT scan or MRI and/or AFP levels > 400 IU/mL. The demographic, biochemical and serological data of the recruited subjects are presented in Table 1.

All cases were positive for anti-core antibody (HBcAb), confirming exposure to HBV, while 138 cases (92%) were positive for HBsAg and 6 cases (4.2%) were positive for HBeAg. HBeAb positive results were found in 68 cases (93.15%). The median

Table 1. Characteristics of the recruited cases.

<table>
<thead>
<tr>
<th></th>
<th>Frequency (%)</th>
<th>CHB</th>
<th>HCC</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>155</td>
<td>146</td>
<td>9</td>
<td>ND</td>
</tr>
<tr>
<td>Age (years)</td>
<td>45.7 (19-85)</td>
<td>44.79</td>
<td>60.56</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>88/67</td>
<td>81/65</td>
<td>7/2</td>
<td>NS</td>
</tr>
<tr>
<td>*HBeAg Pos</td>
<td>6 (4.2)</td>
<td>6</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>Neg</td>
<td>137 (95.8)</td>
<td>133</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>*HBsAg Pos</td>
<td>138 (92)</td>
<td>135</td>
<td>3</td>
<td>0.012</td>
</tr>
<tr>
<td>Neg</td>
<td>12 (8)</td>
<td>9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HBcAb Pos</td>
<td>155 (100)</td>
<td>146</td>
<td>9</td>
<td>ND</td>
</tr>
<tr>
<td>Neg</td>
<td>0 (0)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>*HBeAb Pos</td>
<td>68 (93.15)</td>
<td>68</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Neg</td>
<td>5 (6.85)</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>**Genotype A</td>
<td>5 (4.35)</td>
<td>5</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>B</td>
<td>2 (1.74)</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>5 (4.35)</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>100 (86.95)</td>
<td>91</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>3 (2.61)</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Nationality Saudi</td>
<td>114</td>
<td>109</td>
<td>5</td>
<td>0.03</td>
</tr>
<tr>
<td>Non-Saudi</td>
<td>41</td>
<td>37</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>ALT (NR: &lt; 30U/L)***</td>
<td>35.1a</td>
<td>32.81</td>
<td>71.14</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AST (NR: &lt; 37U/L)***</td>
<td>30.1a</td>
<td>22.68</td>
<td>149.00</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Alb (NR:35-50g/L)***</td>
<td>35.98a</td>
<td>36.62</td>
<td>25.86</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>T Bil (NR:&lt; 17μmol/L)***</td>
<td>12.8a</td>
<td>9.99</td>
<td>58.14</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>GGT (&lt;85U/L)***</td>
<td>51.9a</td>
<td>30.59</td>
<td>393.14</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AFP (&lt;5.4IU/L)***</td>
<td>2.8b</td>
<td>2.7b</td>
<td>2256b</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HBV DNA (IU/ml)</td>
<td>40b</td>
<td>4.3×10^3-b</td>
<td>22b</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fibrosis Score F1</td>
<td>43</td>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>F2</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F4</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Samples that are not tested are due to insufficient serum quantity; ** Genotyping was performed on samples that were amplified in one of the three regions; *** NR= normal range; * mean; ^ median; ND: Not done; NS: Non-significant.
AFP value was 2.81U/mL and the median HBV viral load value was 40 IU/mL.

Of the 12 HBsAg negative samples, 5 had detectable HBV-DNA either in the PS- or the S-regions, confirming occult HBV infection (OBI). These cases did not receive antiviral treatment and were HBsAg negative at a minimum of two time points 6 months or more apart. Three of these samples were from HCC cases and one had a fibrosis score of F4.

The progression of liver disease was evaluated by the elevation in liver function tests (LFTs), fibrosis score > F2, or the development of HCC; progression was correlated with the different HBV variants. We found the fibrosis score to be associated with age, negative HBsAg result, elevated AST and low albumin (p values 0.015, 0.025, 0.011 and < 0.01; respectively) while HCC was associated with HBsAg negative result, elevated levels of ALT, AST, GGT, total bilirubin (T Bil), low levels of albumin (Alb), and non-Saudi nationality (p values 0.012, < 0.01, < 0.01, < 0.01, < 0.01 and 0.03; respectively).

From the 155 patients meeting the inclusion criteria, we amplified three regions in the HBV genome, PreS region (spanning nt2813-156), S region (nt201-599) and BCP/PC region (nt1624-2020). Sequences were generated from 31 and 26 treated cases in the PreS and S regions respectively and 72 cases in the BCP/PC region (and the overlapping X region). The remaining samples from the preS and S regions were from treatment naïve cases who were the subject of earlier publications [17,18]. Seventeen variants were detected, most frequently in CHB cases with elevated fibrosis score and elevated LFTs or in HCC cases (Table 2).

**S-Region Mutations**

The S gene was mainly used for genotyping since it has a lower mutation rate than the other PS or BCP regions. Phylogenetic analysis of the sequences generated in the S-region is shown in Figure 1. In case we could not amplify the S-region, the sample genotype was elucidated from the PS sequences (8 cases) or the PC sequences (2 cases). The results showed that HBV genotype D was most prevalent (86.7%) followed by genotype C (4.7%), genotype A (3.8%), genotype E (2.9%) and genotype B (1.9%).

The P120T/S was detected in 2 samples. In the ‘a’ determinant region of the S region, we detected nucleotide substitution mutations in 32 cases, 17 of which were nonsynonymous. The G145R was not detected in any of the samples sequenced in this study.

**Pre-S Region Mutations**

The preS deletion mutations were categorized as one of seven different deletions, as follows:

a) A 33-nucleotide deletion from position 2815-2848 was detected in all genotype D sequences (100) (preS1 aa1-11);

b) A 57-nucleotide deletion from position 2995-3051 (preS1 aa50-68) plus a 6-nucleotide deletion from position 3071-3076 (preS1 aa76-77) were detected in one sample of genotype D;

c) A 29-nucleotide deletion from position 1-29 (preS2 aa5-14) plus a 13-nucleotide deletion from position 43-55 (preS2 aa19-22) were detected in one sample of genotype A

d) A 25-nucleotide deletion from position 29-53 (preS2 aa14-22) was detected in three samples of genotype D;

e) A 33-nucleotide deletion from position 24-56 (preS2 aa12-23) was detected in one sample of genotype D;

f) A 27-nucleotide deletion from position 25-51 (preS2 aa13-21) was detected in one sample of genotype D’

g) An in-frame shift of 3 nucleotides (1 aa) from position 2815-2817 was detected in two samples of genotype E.

No deletions were detected in the CCAAT box; only four substitution mutations were detected in eight samples with four patterns (CCATT, CCAGT, CCART and CCAAG).

**BCP/PC Region Mutations**

The BCP A1762T/G1764A combined mutations were detected in 11 cases; while the G1764A single mutation was detected in five additional cases. Two samples had an insertion in the BCP and the PC regions; one sample had a 9-nucleotide insertion from position 1828-1836 and the other had a 5-nucleotide insertion from position 1868-1872. These two samples were CHB cases with genotype D, had positive HBsAg, negative HBeAg and normal LFT. The PC G1896A mutation was detected in 56 HBeAg negative and 1 HBeAg positive sample (p value 0.008). This mutation was also detected in 26 HBeAb positive cases and was not detected in HBeAb negative samples. Mutations at positions A1624C, T1753A, A1772T, C1817T, C1946G, T1971A and A1981T were detected in cases with increased fibrosis scores. Variants at positions A1624C, A1630G, A1757G, G1887A, G1899A, G1951T, A1981T and C1982A were detected either solely or more frequently in HCC cases.
Table 2. Mutations detected more frequently in CHB cases with fibrosis, HCC* or elevated LFT.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Number</th>
<th>Abnormal LFTs</th>
<th>Fibrosis score</th>
<th>HCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-A1624C</td>
<td>1</td>
<td>NS</td>
<td>NS*</td>
<td>NS</td>
</tr>
<tr>
<td>X-C1628T</td>
<td>3</td>
<td>&lt; 0.01</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>X-A1630G</td>
<td>6</td>
<td>&lt; 0.01</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>X-G1658C</td>
<td>2</td>
<td>&lt; 0.01</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>PC-T1753A</td>
<td>2</td>
<td>NS</td>
<td>NS*</td>
<td>NS</td>
</tr>
<tr>
<td>PC-A1757G</td>
<td>28</td>
<td>NS</td>
<td>NS</td>
<td>NS*</td>
</tr>
<tr>
<td>PC-A1772T</td>
<td>2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>PC-C1817T</td>
<td>2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>PC-G1887A</td>
<td>1</td>
<td>NS</td>
<td>NS</td>
<td>NS*</td>
</tr>
<tr>
<td>PC-G1899A</td>
<td>26</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>PC-T1909C</td>
<td>6</td>
<td>&lt; 0.01</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>PC-G1951T</td>
<td>9</td>
<td>0.002, 0.011</td>
<td>NS</td>
<td>NS*</td>
</tr>
<tr>
<td>PC-C1946G</td>
<td>2</td>
<td>NS</td>
<td>NS*</td>
<td>NS</td>
</tr>
<tr>
<td>PC-T1971A</td>
<td>4</td>
<td>NS</td>
<td>NS</td>
<td>NS*</td>
</tr>
<tr>
<td>PC-A1981T</td>
<td>4</td>
<td>NS</td>
<td>NS*</td>
<td>NS*</td>
</tr>
<tr>
<td>PC-C1982A</td>
<td>3</td>
<td>&lt; 0.01</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>PreS1-A2922C</td>
<td>1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Variants that are found more frequently with high fibrosis score but did not reach statistical significance; **NS=Not Significant (Variants that are found more frequently with HCC but did not reach statistical significance); *A1762T/G1764A were detected in 1 HCC case and in 10 CHB cases and showed no significant association with HCC.

Figure 1. Phylogenetic tree of the HBV S region of the recruited cases using the Neighbor-Joining method. Red solid squares for HCC cases. CHB cases are represented by solid circles black for genotype D, blue for genotype A, green for genotype B, brown for genotype C and pink for genotype E; reference sequences of the same genotypes might be collapsed to save space and are represented by bold letters. The bootstrap consensus tree inferred from 1000 replicates and the evolutionary distances were computed using the Maximum Composite Likelihood method. Evolutionary analyses were conducted in MEGA6 [22].
From the mutations in Table 2, A1630G, A1757G and G1951T were found to accumulate in two samples with high viral loads (1.7×10^7 and 3.8×10^6 IU/mL); these mutations were only combined in these two samples, with no significant difference. Figure 2 is a schematic presentation of the mutations in Table 2 and the corresponding viral loads of the samples harboring these mutations.

The accumulation of mutations per sample (total number of mutation per sample in a specific region) was correlated to the clinical data of the cases (Table 3). The total number of mutations in the preS and S regions was found to be significantly associated with elevated ALT and increased fibrosis. The accumulation of mutations per sample in the BCP/PC region was associated with high viral load. The accumulation of mutations per sample in the three regions was associated only with elevated ALT.

**Discussion**

Increasing evidence suggests that mutations in the HBV PreS, S, BCP and PC regions of the viral genome are linked with liver disease progression and poor outcome [20,24]. In two previous reports [17,18], we described the genetic characterization of the PreS and S regions in CHB treatment-naive cases from Saudi Arabia. In these studies, we could not detect any association between the HBV genetic variants in these regions and the risk of HCC. We also detected two novel mutations in the C242A (Q187K) and the synonymous C479A. In the present study, we extend our genetic characterization of HBV in Saudi Arabia by investigating HBV variants in preS, S and BCP/PC region in CHB cases in treated patients.

In the present study, HBV genotype was significantly correlated with nationality of the recruited subjects (p = 0.01). As Saudi nationals were mainly (91.6%) genotype D together with Middle Eastern nationals (80.8%), other Asian nationals (Pakistanis and Filipinos) were either genotype C (50%), B (25%) or A (25%). These results are consistent with the geographic distributions of HBV genotypes in the different regions of origin of these subjects.

In the preS1 region, the deletion pattern resulting in a deletion of 11 amino acids (nucleotides 2815-2848) is a typical characteristic of HBV genotype D [25-27], and was also detected in cases with severe forms of CHB genotype C in Thailand [28]. In our study, this deletion was detected only in genotype D samples. Most of the preS deletions were located in the preS2 region (deletions c, d, e, f and g); only deletions “a and b” were observed in the 5'-terminus of preS1. Another deletion (h) was detected in two samples of genotype E and resulted in an in-frame deletion of 3 nucleotides (1aa). This mutation has been reported in genotype E strains from South-Western Africa [29].

While preS deletions are associated with severe liver disease, some studies have reported no or little association with HCC [30]. These studies included cases of HBV genotypes other than genotype D. Our results are in accordance with the findings of Urone et al. [31], who found that the preS deletions in Italian genotype D cases limited their role in influencing the evolution toward chronic infection. PreS2 protein was reported by earlier studies to be inessential for the virus infectivity, secretion and assembly [32,33]. Since most of the preS deletion mutations in this study were found in the preS2 region, we expect that this result and the

**Table 3.** Association of clinical characteristics with the accumulation of mutations per region in the recruited subjects.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean number of mutations in abnormal/normal cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PS1</td>
</tr>
<tr>
<td>Elevated ALT</td>
<td>2.3/1.6</td>
</tr>
<tr>
<td>Increased fibrosis</td>
<td>6.6/1.6</td>
</tr>
<tr>
<td>High viral load</td>
<td>1.9/1.84</td>
</tr>
</tbody>
</table>
genotype D of our cases to be possible reasons for the lack of association between these deletions and the elevation of LFTs and fibrosis score reported from other studies [6,19,34].

The CCAAT-promotor box was found to be the main activating element of the S promotor [35]. It has been reported that HBV mutants with a defect in the CCAAT box could lead to the retention of S proteins in the endoplasmic reticulum in vitro, which might aggravate HBV-associated liver disease [36,37]. In our study, the most prevalent mutation detected was CCAGT, found in five samples. One sample harbored both the wild-type and the CCAGT variant, and one sample each had one of the CCAAG and CCATT variants. All of the cases with the CCAAT variants were of CHB genotype D, were positive for HBsAg, and had normal LFTs. There was no deletion in the CCAAT-promotor box. The CCAAT mutations detected in our study appeared to have no effect on the progression of liver disease. CCAAT variants are not well studied in HBV genotype D. As such, our data do not necessarily contradict the existing data about CCAAT, but do point to the need for further research regarding the effect of these mutations in genotype D cases.

The preS2 start codon mutations included ATG>CTG in one genotype D sample, and ATG>ACG in two samples (1 genotype D and 1 genotype E). These mutations lead to abolishing the translation of the preS2 protein which might lead to overproduction of “large” S protein in hepatocytes, associated with fulminant hepatitis in the mouse model and in people [32,38]. In our study, the samples with preS2 start codon mutation had normal LFT and were HBsAg positive, HBeAg negative at the time of enrolment. These cases had been infected for more than 10 years; one of them remembered having jaundice when he was infected while the other two did not remember such a sign.

Mutations in the S gene can lead to vaccine escape and inability to detect the viral surface antigen in serologic assays [39]. This is expected, since the HBV vaccine is derived from the S region of the viral genome [40]. One of the most reported mutations in the S gene is the P120T/S [41], which is known to cause vaccine escape and failure of HBsAg serologic detection [42]. In our study, this mutation was detected in only two cases of genotype D; both were HBsAg positive and had a fibrosis score of F1, indicating that this mutation was of no significance to their clinical status.

Another mutation in the S gene is the G145R, which is reported to play an important role as a vaccine escape mutant. This mutation is located in the “a” determinant region (aa124-147) and is known to be the most virulent mutant in breakthrough infections [43,44]. In our study, only the wild type was detected in all of the sequenced cases. A similar finding was reported from Riyadh, Saudi Arabia [16] where this variant was also not detected in their cases.

In this study, there were no differences in the preS and the S regions between treated and untreated cases, as we reported earlier [17,18]. The treatment included Entecavir (9 patients) and Lamivudine (7 patients) but the drugs used were not specified in 29 other patients. This lack of association might be due to the short segment of reverse transcriptase (RT) gene overlapping the S gene fragment that we amplified. A more detailed study is needed to investigate the HBV RT gene, where most of the antiviral resistant mutations are reported [45].

OBI is defined as “the presence of HBV DNA in the liver (with detectable or undetectable HBV DNA in the serum) of individuals testing negative for HBsAg” [46]. Another finding of the current study is the identification of OBI in 3 HCC cases and 2 CHB. OBI was significantly associated with HCC (p value < 0.001), elevation in LFT (p values 0.01, 0.005, 0.039 and < 0.001 for ALT, ALB, AST and GGT, respectively) and age (p value < 0.001). Our results are in accordance with those reported by Yuki et al. [47] and Norman et al. [48] describing the clinical relevance of OBI. They reported the development of liver fibrosis and elevation of LFT in OBI cases upon long-term follow-up of cases with acute hepatitis B cases. In addition, Norman et al. [48], in another long-term follow-up study of army veterans who received HBV contaminated vaccines in 1942, noticed a slight excess mortality in HCC cases with subclinical HBV infection. The association of OBI with the development of HCC was reviewed by Pollicino and Saitta [49]. Although little information is available about the relation of OBI to severe liver disease in HBV genotype D, it has been reported to be associated with HCC in regions of predominantly genotype D, such as Egypt [50] and with more severe clinical and biochemical liver profiles in Northern India [51]. Interestingly, a very low mutation frequency was detected in OBI samples in the preS and S regions. Only five mutations were found in two HCC OBI samples, which fits with the findings of Zhang et al. [52], who found low evolution rate and mutation frequency for OBI cases. Another long term study of acute cases reported no change in sequence during more than 30 years of follow-up in OBI cases [53]. In all OBI samples in our study, the HBV viral load was below the detection limit of the kits used (< 12 IU/mL). Furthermore, we were not able to amplify the BCP/PC
region any of the five samples, possibly because of their very low viral load.

Elevated LFTs were significantly associated with PC-C1982A (ALT, AST, T Bil, p < 0.01), X-C1628T (AST, GGT, p < 0.01), X-A1630G (AST, GGT, p < 0.01), PC-G1951T (AST, p-0.002, T Bil, p = 0.011), PC-T1909C (T Bil, p < 0.01), and X-G1658C (ALT, p = 0.046).

Our study detected mutations in the BCP/PC region at positions A1624C, A1630G, A1757G, G1887A, G1899A, G1951T, A1981T and C1982A. These mutations were detected either solely or more frequently in HCC cases, but the small HCC sample size may explain that lack of statistical significance. These results are in accordance with those of Khan et al. [15], who reported that the G1899A and the A1757G mutations were associated with HCC in Saudi patients. Similarly, these mutations were reported from India [54] and in an international meta-analysis [5] to be related to an increased risk of HCC. The A1981T mutation has been reported to be associated with increased risk of HCC [55].

The G1896A mutation produces a stop codon that is reported to abolish the production of HBeAg. In our study, HBeAg negativity was significantly associated with the presence of G1896A in the BCP/PC region (p 0.008), but it was not associated with elevated LFTs, fibrosis score or HCC. This is comparable to the findings of Khan et al. [15] among Saudi patients. Although HBeAg negativity and HBV-DNA have been correlated to the severity of liver disease, we only found HBeAg negativity to be associated with low viral load (p value of <0.01).

We detected the double mutation BCP-A1762T and BCP-G1764A in one HCC case and in 10 CHB cases; they showed no significant association with HCC or the severity of liver disease. Earlier reports linked these mutations with severe liver disease including HCC development [24]. On the other hand, Khan et al. found no association of these mutations with HCC in Saudi cases [15].

In the BCP/PC region, the following variants were detected mainly in cases with increased fibrosis scores: A1624C, T1753A, A1772T, C1817T, C1946G, T1971A and A1981T. The low frequency of these mutations did not allow for assessment of statistical significance. The T1753A and C1817T variants were reported by Pourkarim et al. [56] to be associated with cirrhosis in CHB patients with different ethnic backgrounds. The two insertions detected in this region among our cases did not seem to have a role in the progression of infection, since they were detected in two samples of genotype D with normal LFTs and AFP, HBsAg positive and HBeAg negative.

The accumulation of mutations in the recruited subjects appeared to play a role in the progression of HBV infection. The accumulation of mutations in the preS1, PreS2 and the S regions was found to be associated with abnormal levels of ALT (p values < 0.001, 0.001 and 0.001 for preS1, preS2 and S, respectively). Mutations in these three regions were also associated with increased fibrosis in the recruited subjects (p values 0.018, 0.02 and 0.013 for preS1, preS2 and S, respectively). This is consistent with the findings of Marschenz et al. [57] who found that accumulation of mutations along the HBV genome including the preS and S regions were associated with the development of liver cirrhosis. Accumulation of mutations in the BCP/PC region was significantly associated with high viral load in the studied population. Of note is that mutations at positions A1630G, A1757G and G1951T, which were combined in two samples with high viral load, were not significantly associated.

Adding to the previous knowledge about HBV genetic variants in Saudi Arabia [15-17], the present study describes the genetic variants of HBV in CHB cases from Jeddah, Saudi Arabia in three different regions of the viral genome (PreS, S and BCP/PC). To the best of our knowledge, this is the first study to investigate the viral mutations in these three regions (preS, S and BCP/PC) from this part of the world. The findings of this study add more information to the genetic characterization of HBV viral strains circulating in this location. Due to the small number of HCC cases recruited, further studies are needed to confirm the effect of the described variants on HCC.

**Conclusion**

We can conclude that genotype D is the most prevalent HBV genotype in Saudi Arabia. The accumulation of mutations in the preS and S regions were associated with worsening of disease progression (elevated ALT and increased fibrosis). Mutations at preS1-A2922C, X-A1624C and PC-G1887A were detected only in cases with either high fibrosis scores or HCC, but a statistically significant association could not be demonstrated perhaps due to the small sample. Mutations at positions PC-C1982A, PC-G1951T, X-C1628T and X-A1630G were significantly associated with elevated LFTs and were detected more frequently in HCC cases. Mutations at these positions need to be investigated further in a larger case control study. The preS deletions detected in our study did not seem to play
a role in the progression of liver disease or development of HCC, in contrast to what has been reported in other studies [58]. OBI seems to play a role in worsening the prognosis of the infection; this issue needs further research in genotype D cases but should be taken into account in case management. HBeAg negativity was found to be associated with the PC-G1896A variant. Genotyping of HBV among different types of patients can provide information that is relevant for clinical management and prognosis.

Ethical approval
Ethical approval was obtained from the Ethical Research Committee of King Fahd Medical Research Center, King Abdulaziz University (approval#: 011-CEGMR-06-ETH). Informed consent was obtained from all individuals participating in the study. The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients to be included in the study.

Funding
This project was funded by the National Plan for Science, Technology, and Innovation (MAARIFAH) – King Abdulaziz City for Science and Technology, the Kingdom of Saudi Arabia – award number (12-BIO2247-03). The authors also acknowledge with thanks the Science and Technology Unit, King Abdulaziz University for technical support.

References


Corresponding author
Sherif A. El-Kafrawy, PhD
Special Infectious Agent Unit, King Fahd Medical Research Center, King Abdulaziz University, University Street, Jeddah, 21589, Saudi Arabia.
Phone: +966567772698
Email: saelkfrawy@kau.edu.sa

Conflict of interests: No conflict of interests is declared.