Clinical Significance of Hepatitis B Virus (HBV) Genotypes and Precore and Core Promoter Mutations Affecting HBV e Antigen Expression in Taiwan

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To assess the prevalence and clinical significance of hepatitis B virus (HBV) genotypes and precore and core promoter mutations in Taiwan, a cohort of 200 Taiwanese chronic hepatitis B patients was analyzed. The HBV genotypes and sequences of the precore and the core promoter regions were determined in 66 asymptomatic carriers and 134 patients who had liver biopsy-verified chronic hepatitis and liver cirrhosis. The HBV e-antigen (HBeAg)-negative patients had a higher frequency of mutations at core promoter nucleotides 1753 and 1773 and precore nucleotides 1846, 1896, and 1899 than HBeAg-positive patients. Among the 200 patients, the frequencies of genotype C, T1762 and A1764, C1753, T1766 and A1768, and A1896 mutations increased and the frequencies of T or G1752, T1773, G1799, and C1858 mutations decreased with advancing liver diseases. These factors were different between those with HBeAg-positive status and those with HBeAg-negative status. Based on multiple logistic regression analysis, the risk factors of liver cirrhosis for 200 patients were the presence of T1762 and A1764 mutations (odds ratio [OR] = 11.11; 95% confidence interval [CI] = 3.91 to 31.25; P < 0.001), age ≥35 years (OR = 3.42; 95% CI = 1.33 to 8.77; P = 0.011), and genotype C (OR = 2.87; 95% CI = 1.21 to 6.81; P = 0.017). Further categorical analysis found that 62.1% of patients with genotype C, T1762 and A1764 mutations and age ≥35 years had liver cirrhosis. None of the 55 patients infected with the genotype B, A1762 and G1764 wild type and age <35 years showed liver cirrhosis. In conclusion, our data suggest that pathogenic differences between HBeAg-positive and -negative patients may exist. In Taiwan, HBV genotype C and the T1762 and A1764 mutations may play a role in HBV-related liver cirrhosis, and these could serve as molecular markers for prediction of the clinical outcomes of chronic HBV patients.

Worldwide, hepatitis B virus (HBV) infection is a major health problem. Chronic HBV infection is associated with a wide range of clinical manifestations, from an asymptomatic carrier status with a normal liver histology to severe and chronic liver disease, including cirrhosis and hepatocellular carcinoma (HCC) (11, 13, 25). The natural history of chronic HBV infection can be divided into three phases: immune tolerance, immune clearance, and residual or integrated phases (11, 13). In addition, a fourth reactivation phase has also been proposed. During the early phase of chronic hepatitis B (CHB) patients are positive for hepatitis B e antigen (HBeAg) and have frequent acute flares characterized by substantial increases in serum alanine aminotransferase (ALT) levels. Spontaneous HBeAg seroconversion during the course of chronic HBV infection usually correlates with the disappearance of biochemical markers of hepatitis and a drastic decrease in viremia. However, hepatitis recurred in 15 to 33.2% of patients, which may develop cirrhosis and HCC (12, 18).

HBeAg-negative CHB patients suffer from active liver disease. They have increased ALT levels and detectable serum HBV DNA by classical hybridization techniques (17). HBeAg-negative CHB is frequently associated with precore and core promoter mutants. The predominant precore variation is a G-to-A change at nucleotide (nt) 1896 (A1896), which creates a premature stop codon and which abolishes the synthesis of HBeAg (1, 4, 8). The most common core promoter mutations involve a two-nucleotide substitution: A-T at nt 1762 and G-A at nt 1764 (T1762 and A1764) (23, 34). Several transfection studies show that the 1762 and A1764 mutations decrease the level of pre-C mRNA by 50 to 70% and lead to reduced HBeAg synthesis (7, 16). Several studies have shown that HBeAg may be a target antigen on HBV-infected hepatocytes (39). Failure to produce a target antigen may be a means of evading immune clearance. Nevertheless, the significance of additional mutations in the precore and core promoter regions other than nucleotides 1762 and 1764 and nucleotide 1896 for HBeAg production is unknown.

Although A1896 has previously been reported to be associated with severe forms of chronic liver disease (6, 38), the real significance of A1896 is still unclear because this mutation is frequently detected among patients with mild liver disease (2, 36). In addition, mutations in T1762 and A1764 have frequently been reported to be associated with advanced liver disease and an increased risk for HCC (19, 28, 41), and the mutations have also been found in patients with nonadvanced liver disease (23). Thus, the real significance of the T1762 and...
A1764 mutations is still unclear. Furthermore, another two basal core promoter (BCP) mutations at nt 1766 (C to T) and nt 1768 (T to A) have previously been reported in patients with fulminant hepatitis (3), and the C1753 mutation was reported to be associated with the progression of liver disease (42). However, it is unclear whether other mutations in the precore and core promoter regions were associated with the progression of liver disease.

HBV can be classified into eight genotypic groups (genotypes A to H) (33, 35, 40). Genotypes B and C are the most prevalent variants in Taiwan, and it has been reported that the genotype C variant is associated with liver diseases more severe than those with the genotype B variant is associated (20, 24). Several previous studies have indicated that genotype C has a higher frequency of T1762 and A1764 mutations than genotype B (26, 29). Thus, it is unclear whether different clinical outcomes between genotype B and genotype C infections correlate with the T1762 and A1764 mutations.

To assess the prevalence and clinical significance of HBV genotypes and mutations in the precore and core promoter regions, we compared the frequencies of HBV genotypes and mutations in the precore and core promoter regions to different clinical stages of chronic hepatitis B patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Asymptomatic carriers (n = 66)</th>
<th>Chronic hepatitis patients (n = 95)</th>
<th>Liver cirrhosis patients (n = 39)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>31.7 ± 10.2</td>
<td>36.5 ± 11.3</td>
<td>45.3 ± 11.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex (no. of males:no. of females)</td>
<td>35:31</td>
<td>76:19</td>
<td>33:6</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>ALT level (U/liter)</td>
<td>23.8 ± 9.8</td>
<td>186.6 ± 187.1</td>
<td>122.8 ± 128.6</td>
<td>NS</td>
</tr>
<tr>
<td>HBV DNA concn (log pg/ml)</td>
<td>2.4 ± 1.1</td>
<td>6.1 ± 3.6</td>
<td>6.4 ± 3.4</td>
<td>NS</td>
</tr>
<tr>
<td>HAI inflammation score</td>
<td>3.6 3.4</td>
<td>6.1 3.6</td>
<td>6.4 3.4</td>
<td>NS</td>
</tr>
</tbody>
</table>

HAI score (HAI-F; score range, 0 to 4) were analyzed independently. Patients were classified into three groups: asymptomatic carriers, patients with chronic hepatitis, and liver cirrhosis patients. We also analyzed whether these factors were different between HBeAg-positive and -negative CHB patients. In addition, we investigated the relationship between precore and core promoter mutations and HBeAg.

**MATERIALS AND METHODS**

**Patients.** Between 1998 and 2001, 200 HBeAg-positive patients who had undergone long-term follow-up at Kaohsiung Chang Gung Memorial Hospital were analyzed. They included 66 patients (28 HBeAg-positive and 38 HBeAg-negative CHB carriers) who had persistently normal serum ALT levels, as detected by routine testing and confirmed by repeated tests every 3 to 6 months; who had undergone periodic ultrasound examinations showing a normal liver every 6 months for at least the preceding 3 years; and who were inferred to be asymptomatic carriers. In addition, the study included 134 CHB patients who had had abnormal liver functions for at least 6 months and who had received consecutive liver biopsies for the diagnosis of inflammation or fibrosis or for evaluation before lamivudine or interferon treatment. Among the latter group of patients, 95 patients had chronic hepatitis and 39 had liver cirrhosis. The histological grading of chronic liver disease was based on a modified Knodell histology index in association to the degree of hepatic inflammation and fibrosis (HAI score) (15). The necroinflammatory score (HAI-INF; score range, 0 to 18) and the fibrosis score (HAI-F; score range, 0 to 4) were analyzed independently. Patients were classified into three groups: asymptomatic carriers, patients with chronic hepatitis, and liver cirrhosis patients. HBV DNA levels were evaluated in patients with chronic hepatitis and cirrhosis at the time of the biopsy. The sera were frozen at −70°C until use.

**Serology.** The presence of HBSAg, HBeAg, anti-HCV antibodies, and anti-HDV antibodies were determined with commercial assay kits (HBsAg, enzyme immunoassay [EIA], Abbott, North Chicago, IL; HBeAg, ELA, Abbott; anti-HCV, EIA 3.0, Abbott; anti-HDV, radioimmunoassay, Abbott). HBV DNA was determined by use of a hybridization capture kit (HBV test, Hybrid Capture II; Digene Corp., Gaithersburg, MD) with a detection limit for the standard test of 0.5 pg/ml (141,500 copies/ml).

PCR amplification and direct sequencing of BCP and precore regions. DNA was extracted from 10 μl serum with a QIAamp DNA Mini kit (QIAGEN Inc., Hilden, Germany), according to the manufacturer's recommendations. For sequence analysis, the precore and core promoter regions were amplified by nested PCR, and the primers described previously (9) were used. First-round PCR was performed with 5 μl of DNA extract in a 50-μl reaction mixture containing 10× buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl, 15 mM MgCl2, 1% Triton X-100), 2.5 mM deoxynucleoside triphosphates, 1 U Taq polymerase, and 20 μM external primers. PCR was performed as follows: 96°C for 2 min; 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min for 30 cycles; and finally, 72°C for 10 min. For the second-round PCR, 1 μl of the first-round PCR product was reamplified by use of the same reaction mixture composition and PCR conditions used in the first-round reaction, except that internal primers were used. The sensitivity of this method is 100 copies/ml.
RESULTS

Characterization of HBV in CHB patients. Among the 200 patients, there were 144 men and 56 women; the mean age was 36.7 ± 12 years. The clinical features, HBV DNA levels, and prevalence of HBV genotypes and mutants in the precore and core promoter regions with various stages of chronic liver disease are presented in Table 1. The mean age and male-to-female ratio were significantly increased with advancing clinical stages of liver disease. HBV DNA levels were significantly higher for chronic hepatitis patients than for liver cirrhosis patients. Overall, the frequencies of genotype C, C1753, T1766 and A1768, T1762 and A1764, and A1896 mutations had apparent increasing trends with advancing clinical stages; and the frequencies of T or G1752, T1773, G1779, and C1858 mutations had decreasing trends with advancing clinical stages. In addition, the frequency of the T1762 and A1764 mutations increased with advancing liver diseases in both genotype B- and genotype C-infected patients (P < 0.001 and P < 0.001, respectively) (Fig. 1).

HBcAg status. Our study population included 103 HBcAg-positive and 97 HBcAg-negative patients. Comparisons of the clinical features, HBV genotypes, HBV DNA levels, and levels of liver inflammation between HBcAg-positive and -negative patients are shown in Table 2. Compared with HBcAg-negative patients, the HBcAg-positive patients with chronic hepatitis and cirrhosis were significantly younger and had higher HBV DNA levels and lower fibrosis scores. Furthermore, these data were compared in relation to various stages of chronic liver diseases. The HBcAg-positive patients were younger (P = 0.005) and had higher HBV DNA levels (P < 0.001), if they were in the chronic hepatitis stage, than the HBcAg-negative patients.

The frequencies of age, male-to-female ratios, and infection with genotype C virus were significantly increased with advanced stages of liver disease in HBcAg-positive and -negative patients (for age, P = 0.001 and P < 0.001, respectively; for male-to-female ratios, P < 0.001 and P < 0.05, respectively; for genotype C, P < 0.01 and P < 0.005, respectively).

HBV mutations and HBcAg status. The HBcAg-negative patients had a significantly higher frequency of mutations at core promoter nucleotides 1753 and 1773 and precore nucleotides 1846, 1896, and 1899 than HBcAg-positive patients. The frequencies of age, male-to-female ratios, and infection with genotype C, C1753, T1766 and A1768, T1762 and A1764, and A1896 mutations had decreasing trends with advancing clinical stages; and the frequencies of T or G1752, T1773, G1779, and C1858 mutations had increasing trends with advancing clinical stages; and the frequencies of A1764 and C1858 mutations had decreasing trends with advancing clinical stages. In addition, the frequency of the T1762 and A1764 mutations increased with advancing liver diseases in both genotype B- and genotype C-infected patients (P < 0.001 and P < 0.001, respectively) (Fig. 1).

![FIG. 1. Prevalence of T1762 and A1764 core promoter mutations in 200 genotype B and genotype C hepatitis B virus-infected patients with various liver diseases.](image-url)
(45.4%) of the HBsAg-negative patients (borderline significance [P = 0.06]). In HBsAg-positive patients, the frequencies of the A1762 and A1764 mutations and the T1766 and A1768 mutations increased and the frequencies of the T or the G1752 and C1799 mutations decreased with advanced clinical stages of liver disease. In contrast, in HBsAg-negative patients, the frequencies of the T1762 and A1764, C1753 and A1896 mutations increased and the frequencies of the C1799 and C1858 mutations decreased with advanced clinical stages of liver disease.

**HBV mutations and genotypes and clinical features.** T1762 and A1764 mutations were found in 77 patients (38.5%), among whom 75 had T1762 and A1764 mutants, 7 had T1762 mutants alone, and 1 had T1764 mutant alone. Compared with wild-type virus, patients infected with the T1762 and A1764 mutants alone, and 1 had T1764 mutant alone. Compared with wild-type virus, patients infected with the T1762 and A1764, C1753 and A1896 mutations increased and the frequencies of the C1799 and C1858 mutations decreased with advanced clinical stages of liver disease.

**HBeAg and clinical status**

<table>
<thead>
<tr>
<th>HBeAg and clinical status</th>
<th>No. (%) of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>HBeAg positive</td>
<td></td>
</tr>
<tr>
<td>Asymptomatic carriers</td>
<td>28</td>
</tr>
<tr>
<td>Chronic hepatitis patients</td>
<td>59</td>
</tr>
<tr>
<td>Liver cirrhosis patients</td>
<td>16</td>
</tr>
<tr>
<td>Subtotal</td>
<td>103</td>
</tr>
<tr>
<td>HBeAg negative</td>
<td></td>
</tr>
<tr>
<td>Asymptomatic carriers</td>
<td>38</td>
</tr>
<tr>
<td>Chronic hepatitis patients</td>
<td>36</td>
</tr>
<tr>
<td>Liver cirrhosis patients</td>
<td>23</td>
</tr>
<tr>
<td>Subtotal</td>
<td>97</td>
</tr>
</tbody>
</table>

*P = 0.05*,

*b P = 0.06*,

*c P = 0.044*,

*d P = 0.001*,

*e P < 0.001*,

*f P = 0.001*,

*g P = 0.004*,

*h P = 0.012.*
genotype C infection. However, the prevalence of T1762 and A1764 mutants was significantly higher in genotype C-infected patients than in genotype B-infected patients only in the chronic hepatitis stage \( (P = 0.003) \) (Fig. 1).

**Risk factors for liver cirrhosis.** Stepwise multiple logistic regression was performed for the 200 patients by using the factors of age, gender, infecting HBV genotype, and precore and core promoter mutations (Table 4). It was found that the significant risk factors for liver cirrhosis were the presence of the T1762 and A1764 mutations (odds ratio \( [\text{OR}] \) = 11.11; 95% confidence interval \([\text{CI}]\) = 3.91 to 31.25; \( P < 0.001 \)), age \( \geq 35 \) years \( (\text{OR} = 3.42; 95\% \text{ CI} = 1.33 \text{ to 8.77}; P = 0.011) \), and infection with genotype C \( (\text{OR} = 2.87; 95\% \text{ CI} = 1.21 \text{ to 6.81}; P = 0.017) \). However, the risk factors for liver cirrhosis for HBeAg-positive patients were the presence of the T1762 and A1764 mutations \( (\text{OR} = 16.95; 95\% \text{ CI} = 3.4 \text{ to 83.33}; P = 0.001) \) and age \( \geq 35 \) years \( (\text{OR} = 4.41; 95\% \text{ CI} = 1.03 \text{ to 19.0}; P = 0.046) \); and the risk factors for HBeAg-negative patients were the presence of the T1762 and A1764 mutations \( (\text{OR} = 11.36; 95\% \text{ CI} = 2.99 \text{ to 43.48}; P < 0.001) \) and infection with genotype C \( (\text{OR} = 4.01; 95\% \text{ CI} = 1.3 \text{ to 12.37}; P = 0.016) \). When the patients were categorized according to these determinants, the rate of liver cirrhosis was the highest \( (18 \text{ of 29} [62.1\%]) \) among patients who were infected with a genotype C, T1762 and A1764 mutant and who were \( \geq 35 \) years of age. In contrast, none of the 55 patients who were infected with HBV genotype B, A1762 and G1764 wild type and who were <35 years of age had liver cirrhosis.

**DISCUSSION**

Ever since the discovery of HBeAg by Magnius and Espmark in 1972 (32), its function has remained an enigma. There is still no solid evidence about the events that lead to seroconversion from HBeAg to anti-HBe status, but a strong contender has been the mutations in A1896 and in T1762 and A1764, which could prevent and reduce the production of HBeAg \( (1, 4, 8, 23, 34) \). In the present study, the HBeAg-negative patients had a significantly higher frequency of core promoter nucleotides C1753 and T1773 and precore nucleotides T1846, A1896, and A1899 than HBeAg-positive patients. Many studies have shown that the A1896 mutant could be detected in 0 to 80% of HBeAg-positive patients \( (5, 31) \). However, precore mutant ratios tended to correlate with anti-HBe seroconversion, but high precore mutant ratios were associated with persistent hepatitis after anti-HBe seroconversion \( (14) \). Those findings are compatible with those of the present study. In addition, our data showed that A1899 mutation was found only in HBeAg-negative patients \( (10.3\% ) \) and was associated with the A1896 mutation. This finding is compatible with that of a previous study \( (30) \). A previous study also found that the frequency of T1864 increased after HBeAg clearance during follow-up \( (30) \). However, it remains unclear whether T1864 could affect HBeAg secretion.

The T1762 and A1764 mutations have been reported to reduce HBeAg production by approximately 50 to 70% \( (7, 16) \). However, it is controversial whether the T1762 and A1764 mutations lead to the HBeAg-negative phenotype \( (28, 29) \). Our study found the frequency of the T1762 and A1764 mutations had a borderline difference between HBeAg-positive and -negative patients. This suggests that the T1762 and A1764 mutations appear to be insufficient to lead to the HBeAg-negative phenotype, as approximately one-third of the HBeAg-positive patients had this mutation. In addition, our study also found a higher frequency of C1753 and T1773 in HBeAg-negative patients. Parekh et al. \( (37) \) demonstrated that mutations at nt 1753, 1762, 1764, and 1766 conferred lower levels of HBeAg expression than mutations at nt 1762 and 1764 alone.

Several factors that lead an increased risk of advanced liver diseases for patients with CHB have been identified. These include age, male gender, repeated episodes of severe acute exacerbation, and HBV reactivation after HBeAg seroconversion \( (10, 12, 18, 24) \). In this study, our results showed that the frequency of mutation at nucleotides 1752, 1753, 1766 or 1768, differences between HBeAg-positive and -negative patients. Age, male gender, infection with genotype C, and the presence of the T1762 and A1764 mutations were the same risk factors in both HBeAg-positive and -negative patients. Takahashi et al. found that C1753 is a common mutation in genotype C patients \( (>85\% ) \) and is more closely associated with the progression of liver disease in HBeAg-positive patients \( (42) \). In our study, C1753 was a less common BCP mutation and was found in only 5.2% of all patients \( (10.7\% \) of genotype C-infected patients), and it was associated with the progression of liver disease only in HBeAg-negative patients. Either or both of the T1766 and A1768 mutations were previously reported in a patient with fulminant hepatitis \( (3) \). These changes were detected in 7% of our patients and were associated with the progression of liver disease, especially among HBeAg-positive patients. A previous study demonstrated that the G1752 and the G1799 mutations were linked to genotype B but not to the progression of liver disease \( (19) \). Our study showed that G1752 and G1799 appeared together with genotype B infection and that the frequency was significantly decreased with advanced clinical stages of chronic liver diseases.

Previous studies have indicated that the T1762 and A1764 mutations were more predominant in HBV genotype C-infected patients \( (26, 29) \). However, it is unclear whether both the T1762 and A1764 mutations and genotype C infection were
independent factors for advanced liver disease. In this study, the frequency of the T1762 and A1764 mutant increased with advanced liver disease in both genotype B- and genotype C-infected patients. These findings were compatible with those of a previous study (19). By using multiple logistic regression analysis, our results consistently showed that old age, genotype C infection, and the presence of the T1762 and 1764 mutation were independent risk factors for liver cirrhosis. The T1762 and A1764 mutations were found in the dominant viral species at the late HBeAg-positive phase and the anti-HBe stages of HBV infection. Thus, CHB patients infected with T1762 and A1764 mutant may have a longer duration of active replication. Previous studies found that HBV genotype C infection was associated with later HBeAg seroconversion and multiple episodes of acute exacerbation without HBeAg seroconversion than genotype B HBV infection (21, 22). The delayed HBeAg seroconversion may prolong the inflammation process and subsequently result in more severe liver damage. In addition, our study also showed that patients with genotype C infection or T1762 and A1764 mutation had more severe hepatic necroinflammation. Therefore, the detection of HBV genotype C and the T1762 and A1764 mutant may be useful as markers for the identification of immunonavtivation.

In summary, the predictive factors for the progression of chronic liver disease were different between HBeAg-positive and -negative patients, and these findings suggest that pathogenic differences between the two groups may exist. Several nucleotide mutations in the precore and the core promoter regions might contribute to reduced HBeAg production and were associated with advanced liver disease. In Taiwan, genotype C and the T1762 and A1764 mutant may play a role in HBV-related liver cirrhosis and could serve as molecular markers for prediction of the clinical outcomes for patients with chronic HBV infection.

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