ABSTRACT

This study investigated the prevalence of the precore G1896A mutation in Chinese patients with hepatitis B e antigen (HBeAg) negative HBV infection and its relation to serum HBV pre-S1 antigen. The overall prevalence of the precore G1896A mutation was 72.6% in HBeAg-negative Chinese patients with detectable serum HBV DNA. The prevalence of the precore G1896A is significantly higher in Chinese HBeAg-negative patients with chronic hepatitis B than that in inactive HBV carriers with detectable serum HBV DNA. Serum pre-S1 and the precore G1896A mutation were simultaneously detected in most of Chinese HBeAg-negative patients.

Key words: Hepatitis B virus, precore mutation, pre-S1

Hepatitis B virus (HBV) infection is a global public health problem, with more than 350 million individuals chronically infected (18, 20). The manifestations of HBV infection ranges from the asymptomatic carrier state to chronic hepatitis B, liver cirrhosis, and hepatocellular carcinoma (18). During the initial phase of chronic HBV infection, serum HBV DNA levels are high and Hepatitis B e antigen (HBeAg) is present. The majority of carriers eventually loses HBeAg and develop antibody to HBeAg (anti-HBe). Seroconversion of HBeAg, whether spontaneous or after antiviral therapy, reduces the risk of hepatic decompensation and improve survival (21). However, a portion of patients after HBeAg seroconversion are in the state of HBeAg-negative chronic hepatitis B. These patients are more likely to have reactivation of HBV replication and exacerbations of hepatitis; thus, serial testing is necessary to determine if an HBsAg-positive, HBeAg-negative carrier is truly in the inactive state (13, 19, 21). Sensitive PCR assays are commonly employed in such serial tests. However, these assays are often expensive and time consuming. Simple and cost effective assays are needed for distinguishing HBeAg-negative hepatitis B from the inactive HBsAg carrier state.

In most instances, HBeAg-negative chronic hepatitis B is a result of HBV variants in the precore or core promoter region. The HBeAg is encoded by the core gene, which is divided into the precore (PC) region and the core region by two in-frame initiating ATG codons. Transcription of these open reading frames produces the pregenomic RNA that is essential for HBV replication and the PC RNA that translates into HBeAg, which is secreted into the serum (22). Variations
in the PC region affect HBeAg synthesis without appreciably affecting HBV replication. The PC mutants and the basal core promoter (BCP) mutants are two major types of HBV core gene variations frequently occur that affect the synthesis of HBeAg (1). The most prevalent PC mutation is the 1896 guanine (G)-to-adenine (A) mutation, which creates a TAG stop codon in the PC open reading frame and abolishes HBeAg synthesis at the translational level (1). Most HBeAg negative carriers with high levels of serum HBV DNA have precore variants (4), and all the cases of transmission from HBeAg negative health care workers to patients and from HBeAg negative mothers to their babies have involved in precore mutants (12, 29).

Serum pre-S1 antigen reflects active viral replication in chronic HBV infection (17), and almost all symptomatic anti-HBe patients carry pre-S1 antigen (8). The presence of pre-S1 antigen appears to correspond with the presence of HBV DNA, indicating hepatitis B virus replication (30). In the present study, we investigated the prevalence of precore mutation in Chinese patients with HBeAg-negative HBV infection using a molecular-beacon assay (31), and its relationship to serum pre-S1 antigen.

From 2003 to 2007, 496 patients attending Dalian Central Hospital and Dalian No. 6 Hospital (a hospital for infectious diseases) were recruited into this study. Patients were all HBsAg positive for at least six months and HBeAg negative at presentation. Patients were excluded from the study if they had any of the following conditions: 1) treatment for HBV infection previously, 2) co-infection with hepatitis A, C, D and E virus or HIV, 3) a history of alcohol or drug abuse, and 4) other possible causes of chronic liver damage. In the present study, the inactive state was defined as: 1) HBsAg positive for at least six months, 2) serum HBV DNA < 10000 copies/ml, and 3) persistently normal ALT/AST levels (21). For patients with serum HBV DNA > 10000 copies/ml and persistently normal ALT/AST levels, liver biopsy was performed to confirm whether they are chronic hepatitis B or inactive carriers. Of the 496 patients, 296 were chronic hepatitis B patients and 200 were inactive HBV carriers. Among the inactive HBV carriers, 192 had serum HBV DNA levels below 10000 copies/ml and ALT levels below the upper limit of normal (ULN). The ULN was set as 30 IU/L. Eight patients that had serum HBV DNA levels above 10000 copies/ml but ALT levels under 1 × ULN were confirmed by liver biopsy and had no necroinflammation in the liver. In addition, four patients with serum HBV DNA levels above 10000 copies/ml but persistent normal ALT levels were confirmed by biopsy and had moderate necroinflammation in the liver, and these patients were considered to be chronic hepatitis B. The demographic data, HBV DNA levels and liver biochemistry of the 496 HBeAg-negative patients were summarized in Table 1. Serum samples were collected and stored at -70°C until use.

Table 1. Demographic data for the study population on presentation

| Number of patients                | 496   |
|----------------------------------|-------|
| Male: female ratio              | 388: 108 |
| Median age, years (range)       | 43 (27 – 56) |
| HBeAg+ (%)                      | 0     |
| Anti-HBe+ (%)                   | 100   |
| ALT levels (Mean ± SD, IU/L)    | 65.8 ± 60.1 |
| HBV DNA (Median, log_{10} copies/mL) | 4.56 (undetectable to 8.77) |
| Chronic hepatitis: inactive carrier | 296: 200 |
Hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb), anti-HBe and hepatitis B core antibody (anti-HBc) were measured with commercially available reagents (Kehua Biotech, Shanghai, China) according to manufacturer’s instruction. Alanine aminotransferase (ALT) activity was determined kinetically on Hitachi 7600 automatic biochemistry analyzer with commercial kits (Roche Diagnostics, Penzberg, Germany). HBV DNA levels were tested using real-time PCR reagents (Fosun Diagnostics, Shanghai, China) on ABI Prism 7500 PCR System (Applied Biosystems Inc, Foster City, CA). All reagents were approved by State Food and Drug Administration of China for in vitro diagnostic use.

The G1896A precore mutation was detected by a molecular-beacon assay described by Wartz et al (31). In brief, two molecular beacons containing the wild-type G1896 and mutant A1896 sequences were constructed to hybridize to their targets within a 104-bp fragment that was amplified by the PB primers. HBV DNA was extracted from 200 µl of serum with a QIAamp DNA mini kit (Qiagen, Chatsworth, CA, USA) and eluted in 50µl of elution buffer. The reaction was carried out in a final volume of 25µl of PCR consisting of PCR mix, 0.25µmol of TET wild-type molecular beacon, 0.25µmol of FAM mutant molecular beacon, and 5 ng of HBV DNA. The amplification was performed on ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA) with the procedure consisting of 95ºC for 10 min followed by 40 cycles of 95ºC for 20 s, 62ºC for 30 s, and 72ºC for 30 s. The fluorescence spectra of the molecular beacons were detected measured during the annealing step of the PCR cycle.

Serum pre-S1 antigen was measured with commercially available enzyme immunoassay (EIA) kits (Fosun Diagnostics, Shanghai, China) according to manufacturer’s instruction. In brief, microtiter plates with 48 wells were coated with monoclonal antibodies. For the test, 200 µl of serum samples or controls were added into each well and incubated at 37°C for 30 min. Then the wells were emptied and washed three times before adding peroxidase-conjugated monoclonal antibody in to the wells. After incubated at 37°C for another 30 min, enzyme reaction was performed by adding the substrates into the wells. The reaction was stopped after 10 min with stopping buffer. The light absorbance at 492 nm was determined by a microplate reader (Labsystems, Shanghai, China). The kit was proved by the Chinese Food and Drug Administration for in vitro diagnostic use.

Statistical analyses were performed with the Statistical Program for Social Sciences (SPSS 13.0 for Windows, SPSS, Chicago, IL). Continuous variables were tested using the t-test. Statistical significance was denoted as a $P$ value less than 0.05.

Of the 496 patients, 380 (76.6%) had detectable serum HBV DNA by real-time PCR. The median HBV DNA level of these patients was $5.44 \log_{10}$ copies/ml (range 3.02 to 8.77 $\log_{10}$ copies/ml). Of the 380 patients with detectable HBV DNA, 276 patients (72.6%) had precore G1896A mutation. Patients with precore mutations had significantly higher serum HBV DNA levels ($5.90 \pm 1.49 \log_{10}$ copies/ml, Mean ± SD) than those with wild-type virus infection ($4.04 \pm 1.05 \log_{10}$ copies/ml, $P < 0.01$). Precore G1896A mutants were found in 248 (83.8%) of 296 patients with CHB, while only 28 (33.3%) of 84 inactive HBV carriers with detectable HBV DNA had precore G1896A mutation (Table 2).

| Table 2. Clinical parameters in patients with or without precore G1896A mutation |
|--------------------------------|-------------------------------|-----------------------------|
|                                | G1896A mutants                | Wild-types $P$              |
| HBV DNA (Mean ± SD, $\log_{10}$ copies/mL) | 5.90 ± 1.49                   | 4.04 ± 1.05<0.01            |
| ALT (Mean ± SD, IU/L)          | 91.6 ± 67.3                   | 43.7 ± 36.0<0.01            |
| Chronic hepatitis (%)          | 248 (83.8)                    | 48 (16.2)                   |
| Inactive carriers (%)          | 28 (33.3)                     | 56 (66.7)                   |
ALT levels in the serum of HBsAg positive, HBeAg negative, and HBV DNA detectable patients infected with the wild-type HBV was 43.7 ± 36.0 IU/L (mean ± SD) compared with 91.6 ± 67.3 IU/L in the serum of the patients with precore G1896A mutant HBV infection. The ALT levels in patients with detectable HBV DNA were significantly higher than that in HBV DNA undetectable carriers (24.1 ± 2.8 IU/L, P < 0.01).

A total of 272 (54.8%) patients were positive for pre-S1 antigen in serum. Serum pre-S1 antigen was detected in 248 (83.8%) of the 296 patients with HBeAg-negative CHB compared with 24 (12%, P < 0.01) of 200 inactive carriers. Serum pre-S1 was found in 264 (69.5%) of 380 patients with detectable HBV DNA. The mean HBV DNA level of pre-S1 antigen positive patients was significantly higher than that of pre-S1 antigen negative patients (6.11 ± 1.35 log_{10} copies/ml vs 3.75 ± 0.68 log_{10} copies/ml, P < 0.01). Eight patients with positive pre-S1 antigen had undetectable HBV DNA. The mean ALT level of pre-S1 antigen positive patients was 67.6 ± 8.2 IU/L, compared with 29.5 ± 12.8 IU/L in pre-S1 negative patients (P < 0.01).

Of the 272 patients with serum positive pre-S1 antigen, 248 (91.2%) had precore G1896A mutation. However, of the 224 patients with serum negative pre-S1 antigen, only 28 (12.5%) had precore G1896A mutation. Of the 296 patients with HBeAg negative CHB, serum pre-S1 antigen and precore G1896A mutation were simultaneously detected in 232 (78.4%) patients. Among the 48 pre-S1 antigen negative patients with HBeAg-negative CHB, 16 (33.3%) had precore G1896A mutation. Among the 200 inactive HBV carriers, 176 (88%) were pre-S1 antigen negative (108 with undetectable HBV DNA, 56 with wild-type precore sequence, and 12 with precore G1896A mutation (Table 3).

Table 3. Relationship between precore G1896A mutation and pre-S1 antigen

| Chronic hepatitis B (n=296) | Inactive HBsAg carriers (n=200) |
|-----------------------------|---------------------------------|
|                             | G1896A | Wild-type | RR | G1896A | Wild-type | HBV DNA undetectable | RR |
| Pre-S1 antigen positive     | 232 (93.5%) | 16 (33.3%) | 2.81 | 16 (57.1%) | 0 (0%) | 8 (6.9%) | 5.67 |
| Pre-S1 antigen negative     | 16 (6.5%) | 32 (66.7%) |    | 12 (42.9%) | 56 (100%) | 108 (93.1%) |    |

In the present study, we examined the prevalence of naturally occurring HBV precore G1896A mutation in Chinese patients with HBeAg-negative HBV infection. We found that precore mutation can be frequently detected in Chinese patients with HBeAg-negative HBV infection. In addition, the mutation is more often found in HBeAg negative patients with active CHB. Patients with precore G1896A mutation have significantly higher serum HBV DNA and ALT levels than those with wild-type virus infection. Moreover, serum pre-S1 antigen is more often detected in patients with precore G1896A mutation.

The influence of precore G1896A and basal core promoter mutations on the virulence of HBV infection remains controversial. The precore mutation may be associated with viral persistence in anti-HBe positive patients with ongoing chronic hepatitis B (11) and fulminant hepatitis (2, 5, 9, 24, 33). Precore G1896A mutation was more frequent in patients with fulminant than acute self-limited hepatitis and was independently associated with the fulminant outcome (25). Jardi et al (16) reported that among HBeAg negative CHB, patients with precore G1896A mutation and elevated ALT showed highest HBV DNA, and simultaneous
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The presence of the main BCP and precore mutations was associated with the degree of histological injury. The precore mutant correlated with high levels of HBV DNA in genotype D HBeAg negative CHB, but its distribution was similar in HBeAg negative CHB patients and inactive carriers; thus it appears to have little value as a single marker for predicting disease activity (28). Other studies showed that the precore mutation has also been found in patients with inactive HBV carriers (3, 23), and not necessarily associated with progressive liver damage (6, 7, 32). The percentage of the precore G1896A mutant in total virus is negatively associated with HBV DNA and ALT (26). Our results showed that precore G1896A mutation does exist in inactive HBV carriers with detectable serum HBV DNA. However, this mutation is more frequently found in patients with CHB than in inactive carriers (83.8% vs 33.3%, P < 0.01). Moreover, patients with precore mutation had much higher serum HBV DNA and ALT levels than patients without precore mutation (5.90 ± 1.49 log10 copies/ml vs 4.04 ± 1.05 log10 copies/ml, P < 0.01 and 91.6 ± 67.3 IU/L vs 43.7 ± 36.0 IU/L, P < 0.01, respectively). This implies that precore mutant HBV still actively replicates in HBeAg-negative patients. The controversial results suggest that the role of precore mutation may be much more complex. Long-time follow-up studies are needed for further exploration of the role of precore mutations.

The prevalence of precore G1896A mutation varies geographically and depends on the genotypes of HBV (14). The G1896A mutation is found predominantly in HBV genotypes B, C, and D, due to a genotype-specific nucleotide difference upstream from rt1896 that can critically alter the secondary conformation of the HBV encapsidation signal (27). The median prevalence of the G1896A mutation in HBeAg-negative patients is 50% in Asia, 92% in the Mediterranean, and 24% in America (15). Our results showed that the overall prevalence of precore G1896A mutation in Chinese HBeAg-negative, serum HBV DNA detectable population was 72.6%. The prevalence of G1896A mutation is significantly higher in HBeAg-negative patients with CHB than in HBeAg-negative inactive carriers. The relative risk (RR) of CHB in patients with precore mutation versus patients without precore mutation was 1.95. These results may support the suggestion that the mutants are selected by immune pressure during HBeAg seroconversion, which is favorable to mutants with incapacitated HBeAg production (32).

Serum pre-S1 antigen levels are related to the degree of viral replication (10, 15). In the present study, we found that serum pre-S1 antigen was detected in 83.8% of HBeAg-negative CHB patients but only 12% in inactive carriers. Most patients with precore G1896A had detectable serum pre-S1 antigen. These results support the suggestion that precore G1896A mutation may lead to evade immune clearance of the virus. As a marker of HBV replication, serum pre-S1 antigen is not affected by the precore mutation. Thus serum pre-S1 antigen may be a potential indicator for active hepatitis in HBeAg-negative patients. However, further studies are needed to determine the significance of serum pre-S1 antigen for distinguishing CHB patients from inactive carriers.

In conclusion, the prevalence of precore G1896A mutation in HBeAg-negative CHB patients is much higher than that in inactive HBV carriers. Serum pre-S1 antigen is detectable in most of the patients with the precore mutation.

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