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Hepatitis B virus basal core promoter mutations A1762T/G1764A are associated with genotype C and a low serum HBsAg level in chronically-infected HBeAg-positive Chinese patients

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ABSTRACT

The present study was aimed to obtain baseline information of basal core promoter A1762T/G1764A and precore G1896A mutations of hepatitis B virus (HBV) in 192 HBeAg-positive chronically-infected Chinese patients, who were potential candidates for antiviral treatment. The detection of these mutations (including minor mutant subpopulations) was achieved by direct sequencing, whose sensitivity for minor mutant subpopulations identification was confirmed by clone sequencing. Patients enrolled were infected with either genotype B (46.35%) or C (53.65%) HBV identified by routine tests in our laboratory. The A1762T/G1764A or G1896A mutations were detected in 125 specimens (125/192, 65.10%), in which 77 (77/125, 61.60%) existed as subpopulations. The A1762T/G1764A mutations were found to be more prevalent in genotype C than that in genotype B HBV [62.14% (64/103) vs. 20.22% (18/89), $P < 0.0001$]. There is no statistically significant link between G1896A and genotypes. The emergence of A1762T/G1764A mutations was also found to be associated with an older age, an elevated ALT/AST level, and a lower HBsAg level in serum [wild-type vs. mutant: 4.57 (3.46–5.42) vs. 3.93 (2.51–5.36), $P < 0.0001$]. In conclusion, HBV basal core promoter mutations A1762T/G1764A are associated with genotype C and a low serum HBsAg level in chronically-infected HBeAg-positive Chinese patients.

1. Introduction

Hepatitis B virus (HBV) remains an important pathogen in China (Lu et al., 2010, 2011). Understanding the subtle molecular characteristics of this virus is still attractive and crucial in terms of the control of HBV infection. Within its life cycle, HBV replicates and generates RNA intermediates via its reverse transcriptase lack of proofreading activity, thus constituting an overwhelming genetic diversity which plays important roles in viral survival, immune escape, transmission and pathogenesis (Ganem and Prince, 2004). In the present study, the attention is paid to the mutations occurring within the basal core promoter (BCP) and precore (preC) regions, which regulate the replication of HBV genomic DNA and preC mRNA synthesis, respectively. PreC mRNA is responsible for a secreting hepatitis B e antigen (HBeAg). This antigen has been suggested to be an immune tolerant antigen important for HBV persistence in the host (Seeger and Mason, 2000; Ganem and Prince, 2004). The nucleotide mutations in BCP/preC regions have been detected in HBV infected patients at various disease stages and may have impact on HBV replication, HBeAg expression, viral interaction with the host and the outcome of the infection (Chu et al., 2003; Yuen et al., 2004).

BCP/preC mutations in various HBV infected populations have been studied extensively. One of the most commonly detected preC variants contains a nucleotide (nt) 1896 guanine (G)-to-adenine (A) point mutation (G1896A), which results in a stop codon that prevents the translation to a full length preC protein, thus abolishing production of HBeAg (Carman et al., 1989; Scaglioni et al., 1997). It is also acknowledged that the occurrence of G1896A mutation is restricted to HBV genotypes with a thymine (T) at nt1858 (Alestig et al., 2001; Tong, 2007; Kramvis et al., 2008). The BCP region adjacent upstream to preC is crucial for HBV replication initiation. The most common BCP variant involves a paired A–T at nt1762 and G–A at nt1764 (A1762T/G1764A) mutations (Chu et al., 2003; Yuen et al., 2004). *In vivo* and *in vitro* studies showed that A1762T/G1764A mutations suppressed the synthesis of preC mRNA

Abbreviations: HBV, hepatitis B virus; BCP, basal core promoter; preC, precore; nt, nucleotide; G, guanine; A, adenine; T, thymine; HBeAg, hepatitis B e antigen; HCC, hepatocellular carcinoma; NA, nucleos(t)ide analogues; HBsAg, hepatitis B surface antigen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CMIA, chemiluminescent microparticle immunoassay; nPCR, nested polymerase chain reaction; bp, base pairs; W, A/T; R, A/G.

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and downregulated HBeAg production, but enhanced viral replication and core protein production (Buckwold et al., 1996; Scaglioni et al., 1997; Laras et al., 2002). Since A1762T/G1764A and G1896A mutations decrease or abolish HBeAg production, they are found more frequently in HBeAg-negative patients than in HBeAg-positive patients (Chu et al., 2003; Yuen et al., 2004). It is also known that the occurrence of A1762T/G1764A and G1896A mutations have a genotype preference (Chan et al., 1999). Our previous study showed that the most prevalent genotype B and C HBV strains in China were prone to have preC G1896A mutation and BCP A1762T/G1764A mutations, respectively, especially in HBeAg-negative patients (Du et al., 2007).

The clinical relevance of BCP mutants has been studied widely and its association with the development of HBV-related hepatic decompensation, liver cirrhosis and hepatocellular carcinoma (HCC) has been suggested (Yotsuyanagi et al., 2002; Chen et al., 2005; Du et al., 2007; Chen and Yang, 2011; Kusakabe et al., 2011; Lee et al., 2011). Ren et al. in China also found that chronically-infected patients with genotype B strains with BCP/preC mutants were more likely to develop hepatitis B-related acute-on-chronic liver failure than those with wild-type infection (Ren et al., 2010). The G1896A mutant has been reported to be found in inactive carriers, fulminant hepatitis, chronic hepatitis B, liver cirrhosis and HCC patients, respectively (Yotsuyanagi et al., 2002; Du et al., 2007; Ren et al., 2010; Xiao et al., 2011). However, its contribution to the development of end-stage liver diseases has been argued rather than confirmed.

In addition, some previous studies have showed that chronic hepatitis B patients with BCP/preC mutations might be at a high risk of developing antiviral nucleos(t)ide analogues (NA) resistance due to the replication compensation of BCP/preC mutations to drug resistance mutants. The *in vitro* studies by Chen and Tacke et al. have both revealed that the preC mutations could increase the deficient replication capacity of lamivudine-resistant mutants (Chen et al., 2003; Tacke et al., 2004; Heipertz et al., 2007). Furthermore, the *in vitro* study by Amini et al. also revealed that the BCP/preC mutations could increase the deficient replication capacity of rtA194T tenofovir-resistance mutant (Amini-Bavil-Olyaee et al., 2009). More attention should be paid on this latter point in the antiviral era. Antiviral NAs are wildly used for chronic hepatitis B patients worldwide including China. It is noteworthy that many of the candidates for NA therapy are HBeAg-positive chronic hepatitis B patients. However, carefully designed studies on the prevalence and molecular characteristics of BCP/preC mutations in the HBeAg-positive Chinese chronic hepatitis B patients remain insufficient. Therefore, the present study was aimed to obtain baseline information of BCP A1762T/G1764A and preC G1896A mutations in 192 Chinese HBeAg-positive chronic hepatitis B patients, who were potential candidates for antiviral treatment. In this study, the detection of these mutations (including minor mutant subpopulations) was achieved by direct sequencing approach. The sensitivity for minor mutant subpopulations identification was confirmed by clone sequencing. The correlations of A1762T/G1764A and G1896A mutations with HBV genotype B/C and patient ages were analyzed. Furthermore, the demographic, biochemical and virological characteristics among the patients with the wild-type, A1762T/G1764A and G1896A mutant HBV infections were investigated, respectively.

2. Materials and methods

2.1. Patients

One hundred and ninety-two chronic hepatitis B patients with defined HBV genotypes (B = 89; C = 103) were selected from a database in the Reference Laboratory for Viral Hepatitis of Peking University, Beijing, China. Their blood samples were kept at −80 °C from previous studies (Liu et al., 2010; Yang et al., 2010; Li et al., 2012) and from routine clinical tests. The clinical diagnosis of chronic hepatitis B was according to EASL, 2009 guideline (EASL, 2009). The two genotype groups were matched for the sex and mean ages (Table1). The patients enrolled had positive serum hepatitis B surface antigen (HBsAg), HBeAg and plasma HBV DNA. None of them had a history of antiviral treatment and detectable naturally occurring antiviral resistance mutations. The values of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (40 U/L as cut-off) were available from the database. HBV genotypes and antiviral resistance mutations were defined by the well-established direct sequencing approach in our laboratory (Liu et al., 2010; Yang et al., 2010).

2.2. Tests of HBV serological markers and plasma HBV DNA

Serum HBsAg, HBeAg, anti-HBs and anti-HBe were detected by chemiluminescent microparticle immunoassay (CMIA) using ARCHITECT i2000 analyzer (Abbott Diagnostics, North Chicago, IL). The quantification of serum HBsAg was archived by ARCHITECT HBsAg kit (Abbott Diagnostics) with a detection range of 0.05–250 IU/mL. A sample with HBsAg concentration higher than the upper limit of detection was diluted with ARCHITECT HBsAg Manual Diluent (Abbott Diagnostics). The quantification of plasma HBV DNA was performed by TaqMan® 48 automatic florescence quantitative PCR kits using Roche COBAS® AmpliPrep®/COBAS® TaqMan® 48 Analyzer (Roche Diagnostics, Mannheim, Germany). The limit of detection was 12 IU/mL (∼70 copies/mL) of HBV DNA in plasma. The dilutions were made using nucleic acid testing dilution matrix (AcroMetrix, Benicia, CA).

2.3. Detection of BCP/preC mutations

HBV DNA was extracted from 200 μL serum samples using QIAamp DNA Blood Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instruction. BCP/preC mutations were detected by nested polymerase chain reaction (nPCR)-based direct sequencing. External primers were as follows: 5′-TCGCATGGAGACCACCGTGAAC-3′ (forward, nt 1604–1625) and 5′-GCTTGCCTGAGTGCCGTATG-3′ (reverse, nt 2073–2054); internal primers were 5′-GAGACCACCGTGAACGCCC-3′ (forward, nt 1611–1629) and 5′-GTGCTGTATGGTGAGGTGAAC-3′ (reverse, nt 2063–2043).The first round PCR reaction was performed in a 25 μL reaction mix containing 8 μL of DNA extract, 0.25 μL of each external primers (20 μmol/l) (Sangon Bioengineering, Shanghai, China), 0.25 μL of the Ex Taq Plus polymerase (5 U/μL) (Takara Biotechnology, Dalian, China), 2.5 μL of 10× DNA polymerase buffer, 0.5 μL of dNTP mixture (200 μM, Takara) and 13.25 μL sterilized ddH_2O. The reaction was performed with 35 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s, then with a final extension step at 72 °C for 7 min. The second round PCR reaction was performed in a 30 μL reaction mix containing a 5 μL of 50-fold diluted 1st round PCR product, 1 μL of each internal primers (20 μmol/l), 0.5 μL of Ex Taq Plus DNA polymerase (5 U/μL), 3 μL of 10× DNA polymerase buffer, 0.6 μL of dNTP mixture and 19.7 μL ddH_2O. The reaction was performed with 35 cycles at 94 °C for 30 s, 64 °C for 30 s and 72 °C for 30 s, then with a final extension step at 72 °C for 7 min. A PCR fragment with approximate 453 base pairs (bp) in length was visualized on 1.5% agarose gel, then purified and sequenced commercially (Invitrogen, Beijing, China). The sequence was obtained using the internal primer pairs and confirmed in both directions.

Clone sequencing was performed for four specimens (no. 418, 523, 611 and 1305) to verify the subpopulation sequences determined by direct sequencing. The PCR products were first purified