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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.

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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

Table 1
Main characteristics of the study population.

Characteristics	Total (N = 192)	Genotype B (N = 89)	Genotype C (N = 103)	P-value[a]
Sex (male/female)	148/44	72/17	76/27	0.240
Age (years), median (range)	29.00 (20.00–46.00)	28.00 (20.00–46.00)	30.00 (20.00–42.00)	0.066
ALT (IU/L), median (range)	97.00 (17.00–455.00)	109.00 (35.00–402.00)	84.00 (17.00–455.00)	0.203
AST (IU/L), median (range)	61.50 (18.00–466.00)	63.00 (20.00–466.00)	61.00 (18.00–352.00)	0.843
HBV DNA (Log_{10} IU/mL), median (range)	8.03 (4.39–9.78)	8.05 (5.30–9.78)	8.00 (4.39–9.60)	0.735
HBsAg (Log_{10} IU/mL), median (range)	4.37 (1.83–5.50)	4.45 (2.09–5.49)	4.19 (1.83–5.50)	0.001
HBeAg (S/CO), median (range)	1091.86 (1.55–1784.95)	1149.91 (1.55–1774.99)	1075.40 (3.02–1784.95)	0.557

[a] P-values represented the results of statistical tests between genotype B and C groups, respectively. T-test: age, ALT, AST, HBV DNA, HBsAg and HBeAg; chi-squared test: sex.

with a Sangon gel purification kit (Sangon Bioengineering, Shanghai, China) and were then ligated into pGEM®-T easy vector (Promega, Madison, WI) according to the manufacturer's instructions. The recombinant plasmids were subsequently transformed into *Escherichia coli* DH5α competent cells (Tiangen Biotech, Beijing, China) and white/blue colony selection was used to screen the recombinants according to manufacturer's instructions. Clone sequencing was preformed commercially (Invitrogen).

2.4. Bioinformatics

The nucleotide sequence containing the full-length BCP and preC regions were analyzed using DNAStar version 5.0 (DNAStar, WI) software. The mutations at nt1762, nt1764 and nt1896 sites were identified. The identity of coexisting bases (subpopulations) at one site found in direct sequencing was determined when each base occupied ⩾20% (sensitivity cut-off level of detection) (Yang et al., 2010). Existence of HBV subpopulations were classified as occurrence of mutations at the studied nucleotide sites in HBV genome when analyzing the mutation rates.

2.5. Statistical analysis

Statistical analysis was performed using SPSS software 11.5 (SPSS Inc., Chicago, IL). Statistical differences were evaluated by t-test, chi-squared test or rank sum test where appropriated. All P values were two-tailed. $P < 0.05$ was considered to be statistically significant.

2.6. Sequence accession numbers

The sequences obtained in this study were submitted to GenBank. The accession numbers are JQ811558–JQ811749.

3. Results

3.1. Patients characteristics

The main characteristics of the patients infected with genotype B (89/192, 46.35%) and C (103/192, 53.65%) HBV were compared in Table 1. The mean age of the studied population was 29.42 years (range: 20–46 years) with no significant difference between genotype B and C groups. No statistical differences were found in the male to female ratio, ALT and AST levels and plasma HBV DNA levels between two genotype groups. However, the mean logarithmic titer of serum HBsAg in genotype B group was significantly higher than that in genotype C group ($P = 0.001$).

3.2. Clone sequencing

To validate the direct sequencing results were interpreted correctly in terms of minor mutant subpopulations determination, the clones of four specimens were sequenced. Specimens no. 611 and 1305, both having nt1762–1764–1896 sequence as A/T–A/G–A/G (WRR) according to direct sequencing chromatograms (Fig. 1), were confirmed to harbor nt1762–1764–1896 subpopulations by clone sequencing. Out of 24 and 21 clones from specimens no. 611 and 1305, four sequence combination patterns of nt1762–1764–1896 were found, including A–G–G (wild type), A–G–A (G1896A mutation), T–A–G (A1762T/G1764A double mutations) and T–A–A (A1762T/G1764A and G1896A triple mutations) with different ratios in each specimen, respectively (Fig. 1). In contrast, specimens no. 418 and 523, which showed an nt1762–1764–1896 sequence as A–G–A and A–G–G in direct sequencing, revealed having 94.44% (17/18) A–G–A and 96.00% (24/25) A–G–G sequences in clone sequencing, respectively. The results indicated that the direct sequencing analysis applied for the rest of the specimens was acceptable and the criteria for subpopulation determination used in this study were rational.

3.3. Mutations at nt1762/1764 and nt1896 in patients with genotype B and C HBV infection

HBV BCP/preC sequences were determined in 192 patients by nPCR-based direct sequencing. The A1762T/G1764A or G1896A mutations were detected in 125 specimens (125/192, 65.10%), among which 77 (77/125, 61.60%) existed as subpopulations. Table 2 showed that the overall mutation rate of A1762T/G1764A was found significantly higher than that of G1896A [42.71% (82/192) vs. 32.29% (62/192), $P = 0.035$].

The A1762T/G1764A mutations were found to be more prevalent in genotype C than in genotype B HBV [62.14% (64/103) vs. 20.22% (18/89), $P < 0.0001$] (Table 2). In addition, up to 61.11% (11/18) of A1762T/G1764A genotype B mutants were identified as subpopulations, and this figure was only 34.38% (22/64) in genotype C mutants ($P = 0.041$). As for G1896A mutation, no statistically significant difference was revealed between two genotypes [B vs. C: 39.33% (35/89) vs. 26.21% (27/103), $P = 0.053$] (Table 2). The mutations occurring at nt1896 site were mainly attributed to the mutant subpopulations (51/62, 82.26%) without significant difference in HBV genotypes [B vs. C: 80.00% (28/35) vs. 85.19% (23/27), $P = 0.846$], either.

Among A1762T/G1764A mutation patterns, 92.68% (76/82) were double mutations without HBV genotype difference [B vs. C: 94.44% (17/18) vs.92.19% (59/64), $P = 0.851$]. In addition, 17 HBV strains (8.85%) were found having triple mutations at both nt1762/1764 and nt1896 sites with no genotype preference [B vs. C: 6.74% (6/89) vs. 10.68% (11/103), $P = 0.338$].

3.4. Impact of patient age on A1762T/G1764A and G1896A mutations

To elucidate the age's impact on the occurrence of A1762T/G1764A mutations, the patients were divided into <30 and ⩾30 years of age groups based on a mean age of 29.46 years of

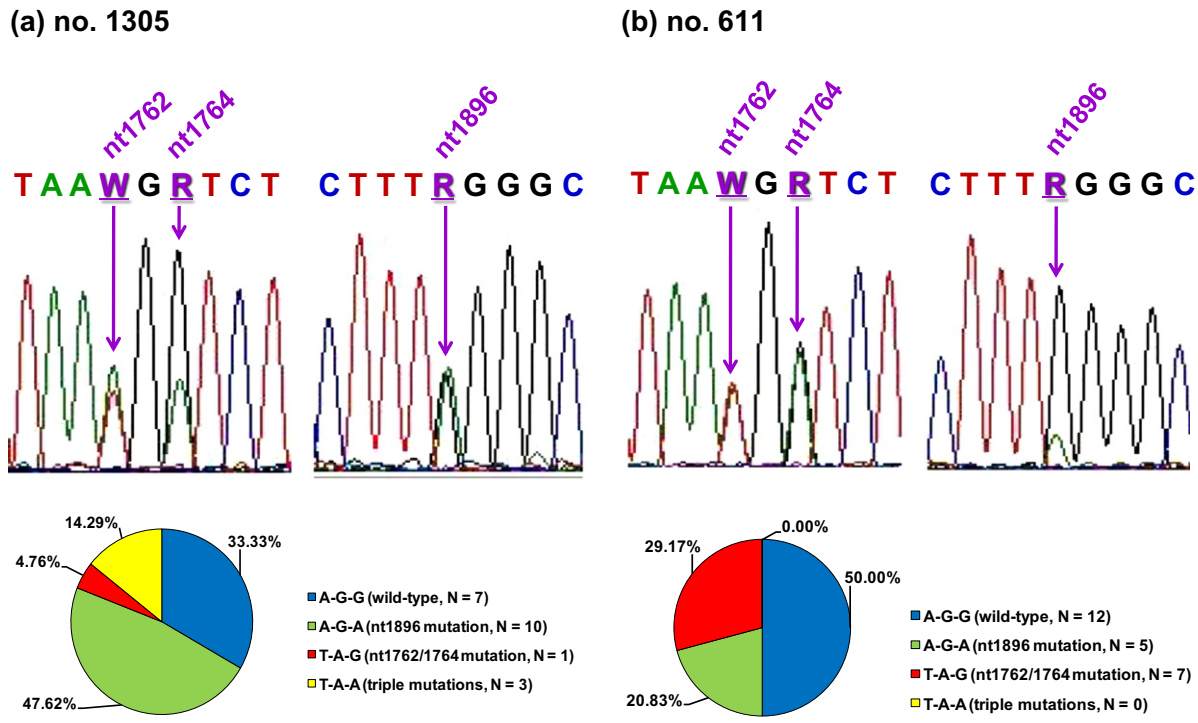

Fig. 1. Sequence analyses of nt1762/1764 and nt1896 by nPCR product-based direct sequencing and clone sequencing. Sequencing chromatogram of direct sequencing: arrow indicated overlapped peaks. Pie graph presented the results of clone sequencing. (a) Specimen no. 1305, sequences from 21 clones available; (b) specimen no. 611, sequences from 24 clones available.

Table 2
Comparisons of A1762T/G1764A and G1896A mutations between patients with genotype B or C HBV infection.

Nucleotide sites in HBVgenome[a]	Total (N = 192)	Genotype B (N = 89)	Genotype C (N = 103)	P-value[d]
nt1762/1764 W/M	110/82[b]	71/18	39/64	<0.0001
nt1896 W/M	130/62[c]	54/35	76/27	0.053

W, wild-type; M, mutant.
[a] Viral subpopulation was classified as occurrence of mutations at the studied nucleotide sites in HBV genome when analyzing the mutation rates.
[b] Thirty-three mutants (33/82, 40.24%) existed as subpopulations [B vs. C: 61.11% (11/18) vs. C: 34.38% (22/64), P = 0.041].
[c] Fifty-one mutants (51/62, 82.26%) existed as subpopulations [B vs. C: 80.00% (28/35) vs. C: 85.19% (23/27), P = 0.846].
[d] P-values represented the results of statistical tests between genotype B and C groups, respectively. Chi-squared tests were used.

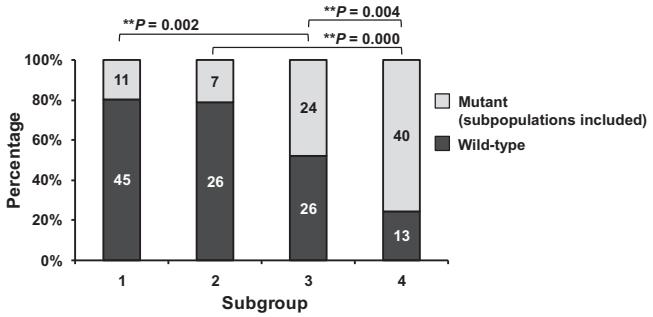

Fig. 2. Distributions of nt1762/1764 wild-type and mutants (subpopulations included) in genotype B and C HBV infected patients with ages <30 years and ⩾30 years, respectively. Subgroups (1) and (2): genotype B infection with <30 and ⩾30 years of age, respectively; subgroups (3) and (4): genotype C infection with <30 and ⩾30 years of age, respectively. The case numbers of patients with wild-type A1762/G1764 (black bar) and mutant T1762/A1764 (gray bar) were indicated in the diagrams. P-value was indicated if a statistically significant difference was identified between two subgroups by chi-squared tests.

the studied population. The age groups were subgrouped by HBV genotype differences. Thus, the four subgroups were: (1) <30 years with genotype B infection (N = 56, 24.64 ± 2.93 years), (2) ⩾30 years with genotype B infection (N = 33, 35.12 ± 4.17 years), (3) <30 years with genotype C infection (N = 50, 24.68 ± 2.62 years) and (4) ⩾30 years with genotype B infection (N = 53, 35.40 ± 3.76 years), respectively. The serum HBeAg and HBV DNA levels showed no significant differences among subgroups. The results in Fig. 2 showed that the ages of the patients influenced the occurrence of A1762T/G1764A mutations only in genotype C but not genotype B HBV. The A1762T/G1764A mutation rate (subpopulations included) was significantly higher in ⩾30 years subgroups (40/53, 75.47%) than that in <30 years group (24/50, 48.00%) of genotype C infection (P = 0.004). Within the same age group, the genotype C infection always showed a significantly higher A1762T/G1764A mutation rates compared with genotype B infection [subgroup (1) vs. (3): 19.64% (11/56) vs. 48.00% (24/50), P = 0.002; subgroup (2) vs. (4): 21.21% (7/33) vs.75.47% (40/53), P < 0.0001]. Since no significant difference of G1896A mutation was observed between two genotypes, the age impact on this mutation was analyzed regardless of HBV genotypes. In contrast to A1762T/G1764A mutations, the results showed that there was no significant difference in G1896A mutation between patient groups <30 years and ⩾30 years [30.19% (32/106), vs. 34.88% (30/86), P = 0.489].

3.5. Characteristics of patients with nt1762/1764 and nt1896 wild-type and mutations

To compare the virtual characteristics of HBeAg-positive patients with nt1762/1764 and nt1896 wild-type and mutant infections, the cases having detectable viral subpopulations, triple mutations (three cases) and single mutation at nt1762/1764 sites (two cases) were excluded in the following analysis. One-hundred

Table 3
Comparisons of main characteristics of the patients with nt1762/174 and nt1896 wild-type-only or mutant-only infections.

Characteristics	Wild-type-only ($N = 67$)	A1762T + G1764A ($N = 35$)	G1896A ($N = 8$)	P-value[a]
Sex (male/female)	54/13	25/10	8/0	0.178
Age (years), median (range)	26.00 (20.00–44.00)	32.00 (21.00–42.00)	30.00 (21.00–42.00)	0.001
ALT (IU/L), median (range)	83.00 (17.00–324.00)	95.00 (34.00–335.00)	168.00 (80.00–402.00)	0.003
AST (IU/L), median (range)	52.00 (18.00–191.00)	75.00 (29.00–317.00)	127.00 (37.00–317.00)	0.000
Genotype B/C	42/25	4/31	6/2	<0.0001
HBV DNA (Log_{10} IU/mL), median (range)	8.13 (5.35–9.78)	7.73 (6.61–9.51)	7.94 (7.25–8.51)	0.044
HBsAg (Log_{10} IU/mL), median (range)	4.57 (3.46–5.42)	3.93 (2.51–5.36)	4.40 (3.69–4.97)	<0.0001
HBeAg (S/CO), median (range)	1313.46 (130.45–1784.95)	791.14 (15.05–1529.09)	118.85 (1.55–1247.08)	<0.0001

[a] P-values represented the results of statistical tests among wild-type-only, A1762T + G1764A and G1896A groups, respectively. T-test: HBsAg; rank sum tests: age, ALT, AST, HBV DNA and HBeAg; chi-squared test: sex and genotype.

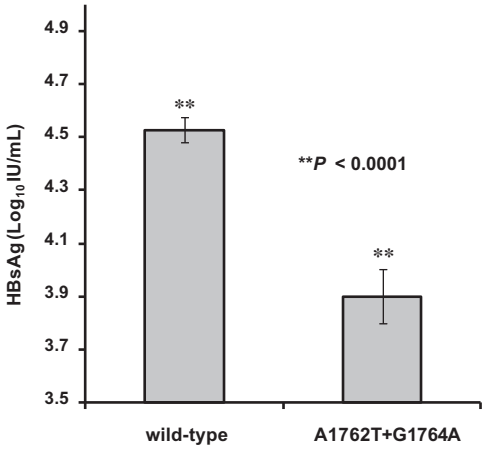

Fig. 3. Comparison of the serum HBsAg levels in the HBeAg-positive chronic hepatitis B patients with nt1762/1764 wild-type ($N = 67$) and mutant ($N = 35$) HBV infection. The subpopulations were not included. P-value showed the statistical test result by t-test.

and ten patients were divided into three groups, which were wild-type-only group (67 cases), A1762T/G1764A double mutation group (35 cases) and G1896A mutation group (eight cases). The results in Table 3 showed that the mutant-only groups showed significantly higher mean ages and serum ALT/AST but significant lower serum HBsAg and HBeAg levels compared with the wild-type-only group, respectively. Of note, A1762T + G1764A mutation group showed the lowest serum HBsAg level. Since this group had an 88.57% (31/35) of genotype C strains, one would speculate that the low level of serum HBsAg was probably attributed to genotype difference as reported previously by Jaroszewicz et al. (2010). However, an univariate analysis showed that the serum HBsAg levels had no significant difference between wild-type genotype B ($N = 42$) and C ($N = 25$) infections [(4.58 ± 0.37) Log_{10} IU/mL vs. (4.44 ± 0.41) Log_{10} IU/mL, $P = 0.176$], neither did serum HBV DNA levels [(7.98 ± 0.63) Log_{10} IU/mL vs. (8.06 ± 0.48) Log_{10} IU/mL, $P = 0.571$], suggesting HBV genotype B and C in themselves seemed not to be a main contributor to the HBsAg level. In addition, the results showed that it was serum HBsAg [(4.44 ± 0.41) vs. (3.84 ± 0.56), $P < 0.0001$] but not HBV DNA [(8.06 ± 0.48) vs. (7.76 ± 0.66), $P = 0.060$] levels had a significant difference between the wild-type ($N = 25$) and A1762T + G1764A ($N = 31$) genotype C HBV infections, respectively. Thus, the observed difference of serum HBsAg levels between the wild-type and A1762T/G1764A groups was very likely due to A1762T/G1764A mutations ($P < 0.0001$, Fig. 3).

4. Discussion

A1762T/G1764A and G1896A mutations have been reported widely in individuals with chronic HBV infection, especially in those with HBeAg loss and seroconversion to anti-HBe (Chu et al., 2003; Yuen et al., 2004). A literature review reported that G1896A mutations was detected in 50% Asia Pacific HBeAg-negative patients, and the median prevalence of A1762T/G1764A mutations among HBeAg-negative patients in the same area was 77% (Funk et al., 2002). However, these mutations have been insufficiently characterized in HBeAg-positive chronic hepatitis B patients, especially those qualified for antiviral treatment. In this study, the prevalence of naturally occurring A1762T/G1764A and G1896A mutations in a cohort of HBeAg-positive chronic hepatitis B patients at treatment baseline were found to be 42.71% (82/192) and 32.29% (62/192), respectively. A total of 65.10% (125/192) of the studied patients harboring HBV A1762T/G1764A or G1896A mutations was somewhat surprising. However, we noticed that 61.60% (77/125) of the mutants existed as subpopulations. This is in line with a previous study by Fukuda et al., whose follow-up study showed that nt1762/1764 existed at various wild-type/mutant ratios as subpopulations before and after HBeAg seroconversion (Fukuda et al., 2001). Clone sequencing in this study revealed that four sequence combination patterns at nt1762/1764 and nt1896 (A–G–G, A–G–A, T–A–G and T–A–A) could coexist with different ratios in different specimens. Considering the roles of nt1762/1764 and nt1896 in HBeAg expression, these results suggested that the different types of sequence combination patterns of nt1762/1764 and nt1896 as a sequence pool might provide a flexible way to help HBV to combat against the changing host immune pressures by adjusting the ratios of different nt1762/1764 and nt1896 sequence combinations. Thus, the regulation of HBeAg expression could be attributed to at least three mechanisms, that is, transcriptional downregulation by A1762T/G1764A mutations, translational blockage by G1896A mutation, and translational level regulation by ratio adjustment of nt1762/1764 and nt1896 subpopulations. This last point could also help to explain why HBeAg seroconversion in HBeAg-positive chronic hepatitis B patients was individualized from the virological point of view. In future, a better understanding of the evolution of nt1762/1764 and nt1896 subpopulations under host immune pressures and antiviral treatments would help to develop good measures to clear completely these subpopulations. In addition, both this study and a previous one by Fukuda et al. (2001) identified a high percentage of BCP/preC subpopulations, a phenomena suggesting that the correlation analysis of nucleotide sequences with clinical and serologic findings should be done with caution.

It was not surprising that the mutation rate of A1762T/G1764A was significantly higher than that of G1896A in the studied population with HBeAg positivity, and these mutations were more prevalent in genotype C than that in genotype B. To this extent, our results were consistent with the findings by Chen et al. (2007) and many others (Huang et al., 2006; Qin et al., 2009). However, of note, the genotypic preferential mutations were discovered only at nt1762/1764 sites but not at nt1896 site in HBeAg-positive patients in the present study. This finding was somehow confusing,

as many reports claimed an association of genotype B with G1896A mutation (Huang et al., 2006; Chen et al., 2007; Qin et al., 2009). Considering the well-known restriction of C1858 on G1896A mutation, the nucleotide at nt1858 of HBV genome was analyzed for the studied cohort. The results showed that there were only 12 C1858 strains among 192 specimens (6.25%), all of which belonged to genotype C and did not have G1896A mutation. This was in contrast to some previous studies which showed C1858 was present in 40% of chronic hepatitis B patients, and T1858 occurred preferentially in HBV genotype B (Chan et al., 1999; Yuen et al., 2003). However, a study on the chronic hepatitis B patients in Taiwan by Chen et al. did report a low frequency of C1858 (<10%) in genotype C HBV (Chen et al., 2007). Kramvis et al. have also reported that T1858 as the consensus nucleotide for subgenotype C2–5 and C1858 was mainly found in subgenotype C1 (Kramvis et al., 2008). In our study the remaining 180 strains with T1858 showed no significant difference of G1896A mutation between genotype B and C strains (35/89, 39.33%, $P = 0.173$). Chen et al. reported that the mutation rate at A1896 apparently increased before but not after HBeAg seroconversion in HBV genotype Ba compared with HBV genotype C (Chen et al., 2007). The genotype B strains in our study were all identified as Ba subgenotype according to the criteria used in Chen's report. The differences in results of this and Chen's studies might reflect the differences in study design, definition for mutation in terms of subpopulations, or the patients enrolled, etc. Nevertheless, the roles of T1858 in genotype C HBV in Chinese patients on G1896A mutation and the viral pathogenesis of G1896A mutant need further investigation.

Our results revealed another interesting phenomenon, that is, the age seemed to play a role just in occurrence of A1762T/G1764A mutations in genotype C but not in genotype B HBV for the enrolled HBeAg-positive chronic hepatitis B patients. In our study, the A1762T/G1764A mutation rate was always about 20% regardless of age in genotype B-infected patients, while it increased from about 50% to 75% in patients infected by genotype C HBV with ages < 30 years or ⩾ 30 years, respectively. Recently, Zheng et al. reported that a 70.59% (72/102) and 80.72% (67/83) mutation rates of A1762T/G1764A were found in older chronic hepatitis B patients (45 ± 8 years) with genotype C infections naïve to antiviral treatment at HBeAg-positive and -negative status, respectively (Zheng et al., 2011). These data were similar to our findings. In addition, they showed that A1762T/G1764A mutations were associated with the cirrhotic HCC development (A1762T/G1764A > 90% in HCC cases), which has been suggested by other studies as well (Chen and Yang, 2011; Kusakabe et al., 2011). Thus, the results supported the hypothesis that A1762T/G1764A mutations might be more important for the pathogenesis of genotype C HBV than that for the genotype B HBV. Of note, the patients enrolled in this study were candidates for antiviral treatment. Tacke et al. showed that A1762T/G1764A and G1896A mutations directly impacted the replication capacity of lamivudine-resistant mutants (Tacke et al., 2004). Thus, it would be worthwhile to study the dynamic changes of nt1762/1764 and nt1896 sequences in relation to treatment response, HBeAg seroconversion and long term clinical outcomes.

Table 3 showed results based on analyses on the strains of so called "wild-type-only" or "mutant-only" in order to diminish the impact of subpopulations on the data interpretation, a problem which has been overlooked by many investigations. We found that serum HBeAg levels decreased dramatically in the mutant-only group compared with the wild-type-only group, though HBeAg was measured by a semi-quantitative assay. These *in vivo* data were in consistent with other *in vitro* and *in vivo* findings showing that A1762T/G1764A and G1896A mutations would downregulate or block the production of HBeAg, respectively (Buckwold et al., 1996; Scaglioni et al., 1997; Qin et al., 2009). The low level of HBeAg detected in the G1896A group might be attributed to the minor wild-type strains in the subpopulations, which were below the detection limit of nPCR. This result hints that a low level of serum HBeAg in HBeAg-positive patients might be a molecular marker of emergence of A1762T/G1764A and G1896A mutations. The quantitative measure of serum HBeAg and identification of cutoff values for prediction of emergence of A1762T/G1764A and G1896A mutations in HBeAg-positive patients would be interesting. In contrast to the tendency of serum HBeAg, the ALT and AST levels increased significantly in the mutant-only groups compared with the wild-type-only group. The results concerning the effect of the A1762T/G1764A mutations on liver injury have been controversial in various investigations. However, the patients in this study were at the immune clearance phase of chronic HBV infection (Lai and Yuen, 2007). It is known that the immune clearance phase of chronic HBV infection is often associated with elevation of serum ALT up to several times the upper limit of normal. Our results suggested that the emergence of A1762T/G1764A or G1896A mutations might be an indicator showing that the patients were experiencing the strong immune clearance. Some of the patients with G1896A mutation may proceed to a rapid HBeAg seroconversion as Chen et al. have reported that G1896A might play an important role for naturally occurring HBeAg seroconversion in HBVgenotype Ba (Chen et al., 2007). However, the clinical relevance of the emergence of A1762T/G1764A mutations in HBeAg-positive patients has not been fully elucidated. Some previous studies found that patients with BCP or preC mutants were more likely to develop liver cirrhosis and HCC (Du et al., 2007; Chen and Yang, 2011; Kusakabe et al., 2011; Zheng et al., 2011). A follow-up study conducted by Yuen et al. disclosed that the high ALT level and presence of BCP/preC mutations at baseline were associated with a higher chance of achieving undetectable HBV DNA and higher rate of HBeAg seroconversion at week 52 under lamivudine treatment (Yuen et al., 2006). The follow up design will be needed to give a better understanding of the roles of these mutations in natural history of HBV infection and antiviral response.

The impact of A1762T/G1764A mutations on serum HBsAg levels has been rarely reported. In this study, we found that there were no statistical differences in serum HBsAg and HBV DNA levels between wild-type-only genotype B and C HBV, respectively. However, the significantly lower serum HBsAg level was found to be associated with A1762T/G1764A mutations rather than with HBV genotypes (Fig. 3). This finding was different from the finding that revealed genotype B HBV had a significantly higher serum HBsAg level than genotype C (Chen et al., 2008). Since A1762T/G1764A mutations were indeed highly prevalent in genotype C HBV strains regardless of the HBeAg status, the controversial results might reflect an overlook of the contribution of A1762T/G1764A mutations to HBsAg regulation in genotype C. Recently Moucari and Marcellin have reviewed the complex mechanisms underlying HBsAg expression and the clinical relevance of quantification of HBsAg, and suggested that serum HBsAg levels could be the result of the complex balance between the virus and the host's immune system as well as the product of the transcription of specific mRNAs rather than viral replication (Moucari and Marcellin, 2011). Our results added BCP A1762T/G1764A mutations as a possible influencing factor for regulating HBsAg expression, probably at a transcriptional level. Further investigation will be needed to reveal the fact.

In summary, A1762T/G1764A and G1896A mutations were prevalent in HBeAg-positive chronic hepatitis B patients with genotype B and C HBV infection in the studied population. Of note, most mutants coexisted with wild-type HBV as subpopulations, showing the overwhelming diversity of BCP and preC sequences in HBeAg-positive patients as a genetic pool for adaptation to various host immune pressures. In addition, both BCP and preC muta-

tions showed the decreased HBeAg expression *in vivo*. The emergence of A1762T/G1764A mutations was associated with genotype C HBV infection, an older age, an elevated ALT/AST level and a decreased HBsAg level in serum. However, the patient age and HBV genotype seemed to have no impact on occurrence of G1896A mutation before HBeAg seroconversion. In future, follow up studies focusing on the precise roles of T1762/A1764 and G1896A mutations in HBeAg-positive patients on the natural HBV infection and antiviral response would be useful in illustrating the viral pathogenesis and identifying valuable clinical applications.

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