Original Article
Association of hepatitis B virus basal core promoter T1762/A1764 and precore A1896 mutations with serum hepatitis B virus DNA load, hepatitis B e antigen status, serum α-fetoprotein and hepatitis B virus-related liver disease progression

Wei Chen[1,2], Jie Chen[2], Hongjiang Wei[2], Donglin Cao[2], Yuhua Li[1]

[1]Department of Hematology, Zhujiang Hospital, Southern Medical University, Guangzhou, China; [2]Department of Laboratory Medicine, Guangdong No. 2 Provincial People's Hospital, Guangzhou, China

Abstract: The frequency of the basal core promoter (BCP) T1762/A1764 and precore (PC) A1896 mutations of Hepatitis B virus (HBV) and their association with clinical parameters in patients with chronic hepatitis B (CHB), cirrhosis and hepatocellular carcinoma (HCC) were assessed. Serum samples from 237 hepatitis B surface antigen-positive patients were collected. BCP T1762/A1764 and PC A1896 mutation were assessed by sequencing the precore region and the mismatch amplification mutation assay (MAMA-PCR). Serum HBV DNA was quantified by qPCR and hepatitis B e antigen (HBeAg), hepatitis B e antibody (HBeAb), α-fetoprotein (AFP) and clinical data were obtained from the hospital database. The BCP T1762/A1764 mutation was associated with HBV-related liver disease progression, reduced HBV replication, increased HBeAg seroconversion and seroclearance, and correlated positively with higher serum AFP (correlation coefficient 0.136, P<0.01) and HCC development (correlation coefficient 0.346, P<0.001). The PC A1896 mutation was not associated with HCC development (correlation coefficient 0.068, P>0.05), but was associated with HBeAg seroconversion (correlation coefficient 0.212, P<0.01) and higher serum AFP (correlation coefficient 0.109, P<0.05). This study suggested that the BCP T1762/A1764 mutant of HBV is closely associated with the development HBV-related cirrhosis and HCC, as it may prevent HBV replication, abolish HBeAg secretion and increase serum AFP. The PC A1896 mutation of HBV may play an important role in HBeAg seroconversion and was associated with higher serum AFP, but was not related to the development of HCC.

Keywords: BCP T1762/A1764 mutant, PC A1896 mutant, HCC, cirrhosis, CHB, HBeAg, serum HBV DNA level, AFP

Introduction

Hepatitis B virus (HBV) infection is a major health problem worldwide. In China, it is estimated that over 96 million patients have chronic hepatitis B (CHB) infection, which is responsible for 300,000 deaths due to liver cancer every year. Most cases of HBV infection are asymptomatic at the early stage; however, 15-40% of patients with CHB will progress to cirrhosis and hepatocellular carcinoma (HCC) [1]. Most patients with HCC have very serious liver damage at diagnosis and present at a late stage of disease, which has a 5-year survival rate less than 20% [2]. Although the exact mechanisms by which CHB progresses to HCC

is still unknown, a high serum HBV DNA level, HBV mutations and high level of serum cytokines among other factors have been associated with the occurrence and prognosis of hepatitis B-related HCC [3].

HBV is the smallest human enveloped DNA virus and contains partially double-stranded circular DNA harboring four overlapping reading frames: S (encoding the viral surface proteins, HBS), P (encoding viral polymerase), X (encoding the regulatory X protein, HBX) [4], and pre C (encoding the antigens "e" and "c") [5]. Since the viral reverse transcriptase has high error rate, the HBV genome evolves quickly over time, with an estimated nucleotide substitution rate

of 1.4-3.2×10^{-5}/sites per year [5]. Various genetic mutations have been considered as basic factors that influence the biological features of HBV [6]. It has been demonstrated that HBV accumulates mutations during replication that alter viral replication and HBV antigen epitopes, and are related to an exaggerated host immune response and resistance to antiviral agents. Therefore, HBV mutations are involved in persistent infection and severe damage to hepatocytes.

Previous studies confirmed that the basal core promoter (BCP) T1762/A1764 dual mutation and precore (PC) A1896 mutation of HBV are associated with an increased risk of HBV-related liver disease [7-9]. The BCP T1762/A1764 mutations increase viral replication, allowing rapid spread of the virus throughout the liver, and the PC A1896 mutation abrogates HBeAg synthesis, leading to a vigorous immune response [10, 11]. However, the relationships between the BCP T1762/A1764 and PC A1896 mutations and clinical serum parameters, such as serum HBV DNA level, HBeAg status and serum α-fetoprotein (AFP), remain to be elucidated. Therefore, we analyzed the frequency of the BCP T1762/A1764 and PC A1896 mutations in three groups of patients with HBV-related liver disease, CHB (n = 127), cirrhosis (n = 65) and HCC (n = 45), and investigated the correlations between these mutations and the clinical parameters serum HBV DNA load, HBeAg status and the liver cancer biomarker AFP to further explore the role of these HBV mutations in the development of HCC.

Material and methods

Patients and serum samples

The study population consisted of 127 cases of CHB, 65 cases of cirrhosis and 45 cases of HCC; all patients were hepatitis surface antigen (HBsAg)-positive at least for 6 months, and HBeAg/AntiHBe status at least for 3 months. Patients with HCV, alcoholic, cryptogenic, NAFLD/NASH liver diseases were excluded in this study. Considering that antiviral agents such as hepatitis B inferno and nucleot(s) ides might be affect HBV viral load and virus mutation, no antiviral agents treatment is one of inclusion criteria in sample selection. Diagnosis of CHB and cirrhosis were in compliance with the criteria of the Guidelines for Prevention and

Treatment of Chronic Hepatitis B (CMA, 2010 Edition). In addition to meeting the criteria of the Standard Specification for Diagnosis and Treatment of Primary Liver Cancer (Chinese National Health Planning Commission, 2011 Edition), the diagnosis of HCC was also confirmed by biopsy of the lesions in the liver. Procoagulant blood was drawn from enrolled patients and centrifuged at 3,000 rpm for 5 min to extract serum. Serum samples were analyzed immediately or cryopreserved at -80°C until analysis. The medical records of the patients were reviewed using the Easylink-Lis database of our hospital to obtain data on HBsAg, anti-HBs Ag, HBeAg, anti-HBeAg, and AFP. The baseline characteristics of the enrolled patients with HBV-related liver disease are summarized in **Table 1**. This study was approved by the Ethical Committee of the Faculty of Guangdong No. 2 Provincial People's Hospital. Informed consent was obtained according to the regulations of the committee and related documents have been stored at our hospital.

Detection of HBV genomic DNA BCP T1762/A1764 and PC A1896 mutations by sequencing

Serum virus DNA was extracted using the TIANamp virus DNA/RNA kit (DP315, Tiangen Biotech Co. Ltd, Beijing, China) according to the manufacturer's instruction. The HBV X gene fragment containing the BCP 1762/1764 and PC 1896 sites was amplified by nested PCR. Each PCR reaction mixture contained 5 μL of viral DNA (outer PCR) or 0.5 μl of outer PCR product (inner PCR) and 10 pM each of the forward and reverse primers. The sequences of the inner and outer PCR primers were as follows: outer forward primer: 5' TCG CTT CAC CTC TGC AC 3'; inner forward primer: 5' AAT GTC GAC CGA CCT T 3'; forward/inner reverse primer: 5' AAA AAA GTC AGA AGG CAA AAA 3'. The inner PCR products were sequenced by Life Technologies (Invitrogen Co. Ltd, Shanghai, China) to analyze mutation of the BCP 1762/1764 and PC 1896 sites.

Analysis of BCP T1762/A1764 and PC A1896 mutation rates using mismatch amplification mutation assay PCR (MAMA-PCR)

The mutation rates of the BCP T1762/A1764 and PC A1896 sites were analyzed by mismatch amplification mutation fluorescent PCR

Table 1. Baseline characteristics of the patients with hepatitis B surface antigen-positive CHB, cirrhosis and HCC

	CHB	Cirrhosis	HCC	*P*-value
Number of patients	127	65	45	
Male	72% (92)	83% (54)	89% (40)	
Female	28% (35)	17% (11)	11% (5)	
Age (years)	35 ± 9.8	47.2 ± 10.3	51.9 ± 13.3	CHB vs. HCC (cir) <0.05
α-Fetoprotein (ng/ml)	17.2 ± 57.3	14.7 ± 32.7	46368.7 ± 113963.1	HCC vs. CHB (cir) <0.001
HBV load ($\log_{10}$)	5.87 ± 1.846	4.24 ± 2.93	3.97 ± 1.89	

Note: Values are mean ± SD.

using a diagnostic kit for the 1762/1764 dot mutation in the BCP region of HBV-DNA (Biot Gene Co. Ltd., Wuhan, China) and diagnostic kit for the 1896 dot mutation in the pre-core region of HBV-DNA (Biot Gene Co. Ltd.). Two pairs of PC (or BCP) region-specific primers were included in each kit, one pair of primers (A) are designed to amplify total HBV DNA PC (or BCP)-specific fragments, another pair (B), the 1896 (or 1762/1764) mutant fragment. The mutation rate of the PC1896 (or BCP T1762/A1764) site was determined by assessing the Ct difference value (ΔCt) of these two products. According to the manufacturer's instructions, 100 µl of serum (refrigerated for less than 72 h at 4-8°C) and 100 µl of 15% PEG6000 were mixed well, centrifuged at 13,000 rpm for 5 min, the supernatant was discarded and 50 µl of EDTA (pH 8.0), Tris-HCl (pH 8.0) and NP-40 were added and mixed well, heated to 100°C for 10 min, and centrifuged at 13000 rpm for 5 min. Each 50 µl reaction volume mixture contained 5 µl of each sample, wild type control, negative control or mutant control, and 45 µl of PCR buffer A (including primer pair A for total PC products, probe and PCR mixture A) or 45 µl PCR buffer B (including primer pair B for mutant products, probe and PCR mixture B); the mixtures were separately amplified on two different PCR systems (A and B). MAMA-PCR was performed on an ABI 7500 Real-Time PCR system (ABI, Singapore) at 50°C for 2 min, 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 sec and 60°C for 1 min. Fluorescent signals were collected and analyzed using ABI 7500 Real-Time PCR software. Mutation rates were assessed as following: ΔCt was 0-2 and mutation rate was 100-50%, ΔCt was 2-5 and mutation rate was 50-5%, ΔCt was 5-7 and mutation rate was 5-1%, ΔCt was over 7 and mutation rate was 0.

Quantification of serum hepatitis B virus load

Absolute quantification of serum HBV DNA load was performed by qPCR using a Hepatitis B Viral DNA Quantitative Fluorescence Diagnostic kit (PCR-Fluorescence Probing; Sansure Biotech Inc, Changsha, Hunan, China) according to the manufacturer's instructions. For each sample, 5 µl of fresh serum was mixed with 5 µl of lysis buffer in a (200 µl) 96 well plate, incubated at room temperature for 10 min to release viral DNA, then 40 µl of PCR Mastermix (containing HBV DNA forward and reverse primers, fluorescent probe, dNTPs, Mg^{2+}, *Taq* polymerase, UNGrase and buffer) was added. Negative control, positive control, blank and five standard samples (0, $4×10^4$ IU/ml, $4×10^5$ IU/ml, $4×10^6$ IU/ml and $4×10^7$ IU/ml) were amplified in the same plate in parallel. Each sample was repeated in triplicate. The qPCR was performed on an ABI 7500 Real-Time PCR system at 50°C for 2 min for the UNGase reaction, then 94°C for 5 min for *Taq* polymerase hot start and 45 cycles of 94°C for 15 sec (template denaturation) and 57°C for 30 sec (annealing, extension and fluorescent signal detection).

Statistical analysis

Data is expressed as percentages, or mean and standard deviation values. Comparisons between groups were performed using the Chi-square test or Fisher's exact test for categorical variables and one-way ANOVA (non-parametric) or the Student's *t*-test for quantitative variables. *P*-values below 0.05 for a two-tailed test were considered statistically significant. All statistical analyses were performed using Graphpad Prism 5 software for windows 10.0 (Graphpad software Inc.).
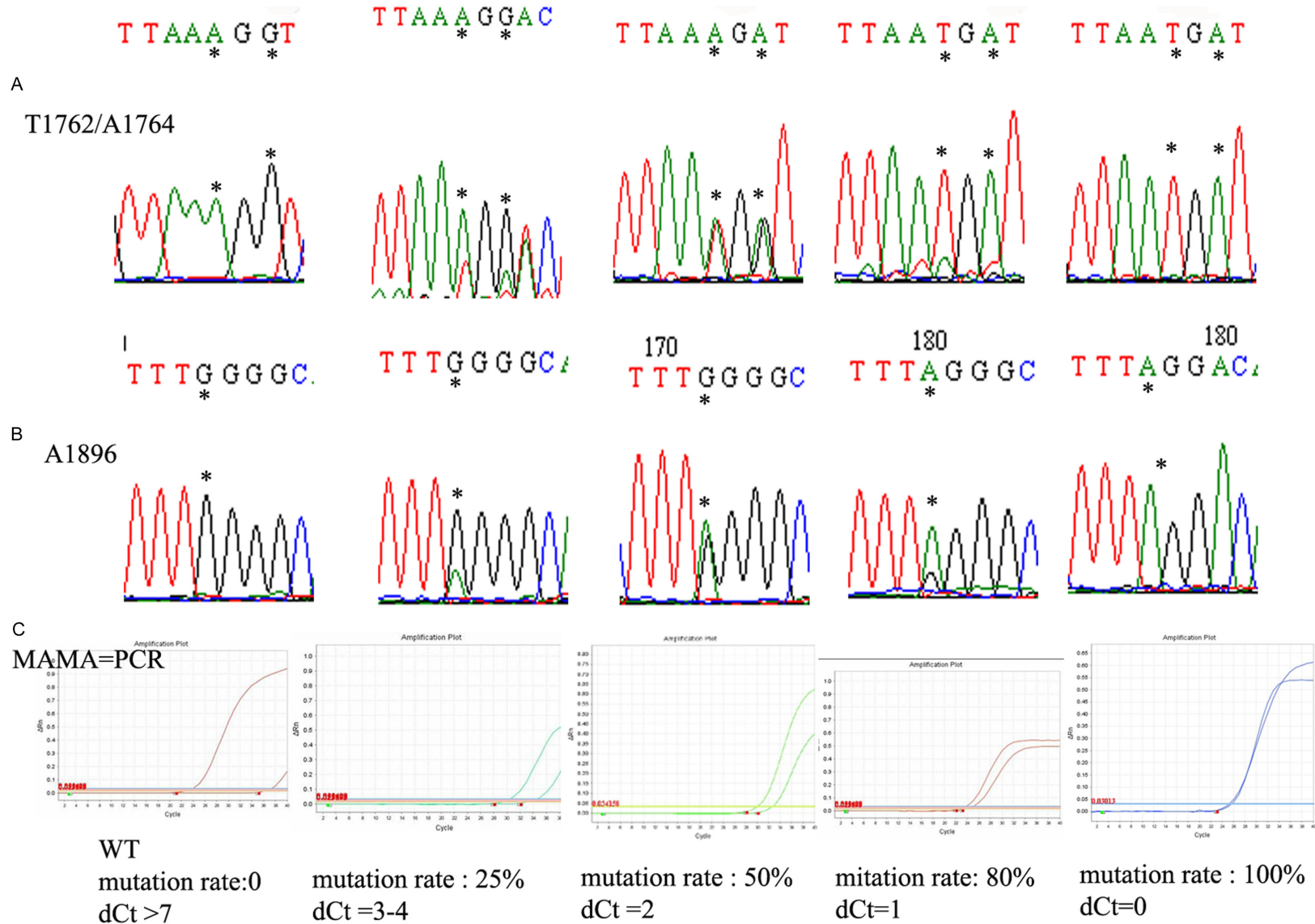
Figure 1. Representative mutation rate sequence peak graphs and MAMA-PCR amplification graphs of the BCP T1762/A1764 and PC A1896 mutations. A. T1762/A1764 sequence peak graph for 0, 25, 50, 80, and 100% mutation. B. A1896 sequence peak graph for 0, 25, 50, 80, and 100% mutation. C. MAMA-PCR amplification curves and ΔCt values for 0, 25, 50, 80, and 100% mutation. The * symbol indicates the mutation sites.
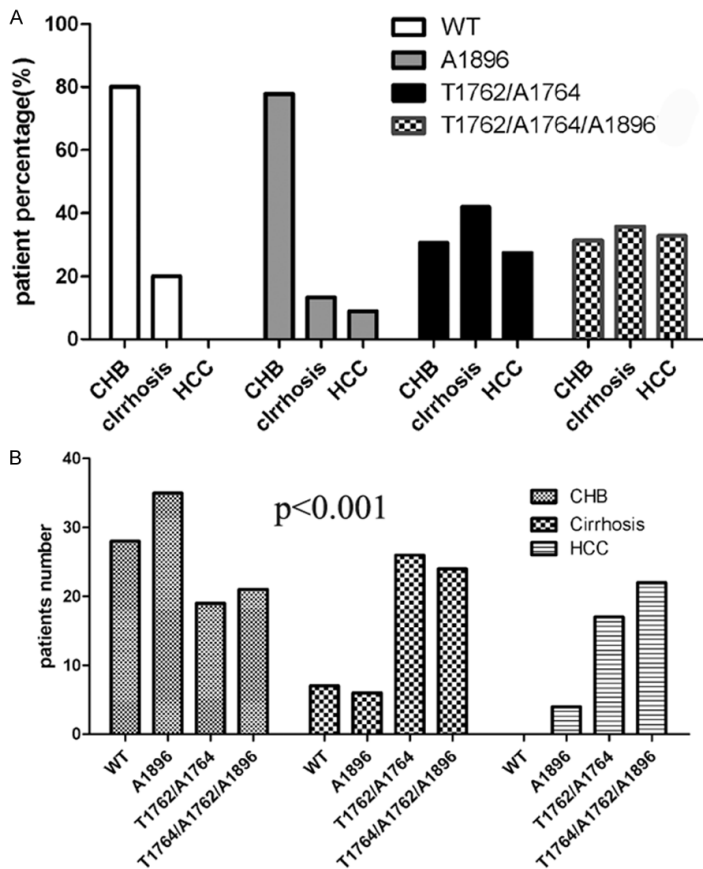
Figure 2. Distribution of the HBV BCP A1762/A1764 and PC A1896 mutations in the groups of patients with CHB, cirrhosis and HCC. A. Percentages of patients in the CHB, cirrhosis and HCC groups with the WT, A1896, T1762/A1764, and T1762/A1764/A1896 genotypes. B. Distribution of the BCP T1762/A1764 and PC A1896 mutations in the groups of patients with CHB, cirrhosis and HCC ($P<0.01$, Chi-square test).

Results

Clinical data

The baseline clinical data of the patients with HBV-related CHB, cirrhosis or HCC are shown in **Table 1**. Among the 237 patients, 127 had CHB, 65 had cirrhosis, and 45 had HCC. Overall, there were more male patients than female patients, with 72%, 83% and 89% males in the CHB, cirrhosis and HCC groups, respectively. The mean ages of the CHB, cirrhosis and HCC groups were 35 ± 9.8, 47.2 ± 10.3, and 51.9 ± 13.3 years-old. The patients with HCC had a significantly lower serum HBV load than the patients with CHB (HBV load $\log_{10}$: 3.97 vs. 5.87). The patients with HCC had significantly higher average serum AFP than the patients with cirrhosis and patients with CHD (46368.7 vs. 14.7 and 17.2 ng/ml).

Basal core promoter (BCP) T1762/ A1764 and precore (PC) A1896 mutation analysis

HBV DNA sequence and MAMA-PCR analysis were performed to analyze the BCP T1762/A1764 and PC A1896 mutations of HBV and calculate their mutation rates. Of the 237 patients, 35 had both wild-type BCP 1762/1764 and wild-type PC 1896 (WT), 84 had the BCP T1762/A1764 mutant and PC 1896 WT (T1762/A1764) mutant, 54 had WT BCP 1762/1764 and PC A1896 mutant (A1896), and 64 had both the BCP T1762/A1764 and PC A1896 (T1762/A1764/ A1896) mutations. The mutation rates were assessed using the ΔCt values of the total PC (BCP) specific product (A product) and the mutant product (B product). The five representative mutation rate graphs (0%, 20-25%, 50%, 80%, and 100%) and respective sequence peak graphs are shown in **Figure 1**.

Association of the BCP T1762/ A1764 and precore PC A1896 mutations with HBV-related disease progression

In order to evaluate the association between the BCP T1762/A1764 and PC A1896 mutations of HBV and HBV-related disease progression, the patients were categorized into four groups according to the BCP 1762/1764 and PC 1896 genotypes, as follows: WT group (wild-type BCP 1762/1764 and PC 1896), A1896 group (PC A1896 mutant, wild-type BCP 1762/1764), T1762/A1764 group (BCP T1762/A1764 mutant and wild-type PC 1896), and T1762/A1764/A1896 group (T1762/A1764 mutant plus A1896 mutant). As shown in **Figure 2A**, 80% of patients in the WT group had CHB, the remaining 20% had cirrhosis; no patients in this group had HCC. In the A1896 group, although 8.9% of patients had HCC, the majority of patients in this group had CHB (77.8%). However, disease progression was more evident in the T1762/A1764 and T1762/A1764/A1896 groups than that in the WT and A1896 groups,
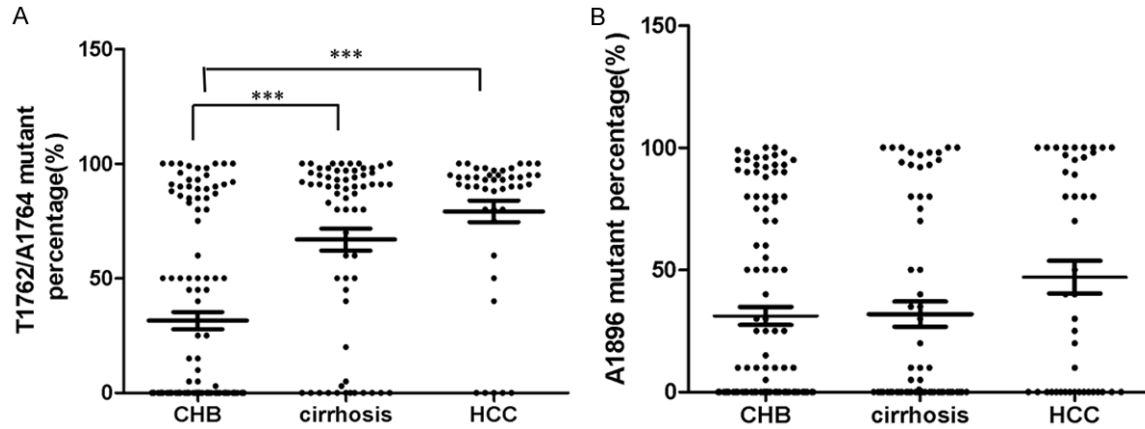
Figure 3. HBV BCP T1762/A1764 and PC A1896 mutation rates in the groups of patients with CHB, cirrhosis and HCC. A. BCP T1762/A1764 mutation rates for the groups of patients with CHB, cirrhosis and HCC. BCP T1762/A1764 mutation rates were significantly higher in the groups of patients with cirrhosis and HCC than the patients with CHB; P<0.001; one-way ANOVA [non-parametric]). B. A1896 mutation rates for the groups of patients with CHB, cirrhosis and HCC.

as the T1762/A1764 and T1762/A1764/A1896 groups contained higher proportions of patients with cirrhosis (41.9% and 35.8% vs. 20% and 13.3%) and HCC (27.4% and 32.8% vs. 0 and 8.9%) and lower proportions of patients with CHB (30.6% and 31.3% vs. 80% and 77.8%; **Figure 2A**).

We also analyzed the distribution of the BCP T1762/A1764 and PC A1896 mutations in patients with CHB, cirrhosis and HCC (**Figure 2B**). All four PC 1896 and BCP 1762/1764 sequence combinations were detected among the groups of patients with CHB, with 27.1%, 34.1%, 18.4%, and 20.4% of patients having the WT, A1896, T1762/A1764, T1762A1764/A1896 mutations, respectively. However, only 7 patients and 6 patients with cirrhosis and 0 patient and 4 patients with HCC had WT and mutant A1896. The T1762/A1764 and T1762/A1764/A1896 sequen-ces were the dominant genotypes in patients with cirrhosis and patients with HCC (41.3% and 38.1% of cirrhosis group, 40% and 51% of HCC group, respectively). The Chi-square test confirmed the distribution of the PC 1896 and BCP 1762/1764 mutations were significantly different between the groups of patients with CHB, cirrhosis and HCC (P<0.001).

We further analyzed the relationship between the BCP T1762/A1764 and PC A1896 mutation rates and HBV-related disease progression. As shown in **Figure 3**, the T1762/A1764 mutation

rate increased with disease progression, from 31.56% in patients with CHB, to 66.19% in patients with cirrhosis and 72.92% in patients with HCC; the differences between the CHB vs. cirrhosis and CHB vs. HCC groups were significant (P<0.001). A significant correlation between the T1762/A1764 mutant and HCC was observed (Pearson correlation 0.346, P< 0.001). Although the average A1896 mutation rate was slightly higher in patients with HCC than patients with CHB and patients with cirrhosis, these differences were not significant (**Figure 3B**; P>0.05). No correlation was detected between the A1896 mutation rate and HCC (Pearson correlation coefficient, 0.068, P> 0.05).

Association of the BCP T1762/A1764 and precore PC A1896 mutations with serum HBV-DNA load

Serum HBV DNA load is a critical clinic parameter in HBV-related liver disease. The mean serum HBV DNA load ($\log_{10}$) for patients with CHB was nearly 6, which was significantly higher than that of patients with cirrhosis and patients with HCC (5.88 ± 1.85 vs. 4.24 ± 2.40 and 3.97 ± 1.89, P<0.01; **Figure 4A**). Patients with cirrhosis and patients with HCC had similar mean HBV DNA loads (P>0.05). We also compared the serum HBV DNA loads of the WT, A1896, T1762/A1764, and A1896/T1762/A1764 groups. As shown in **Figure 4B**, the A1896 group had the highest mean serum HBV
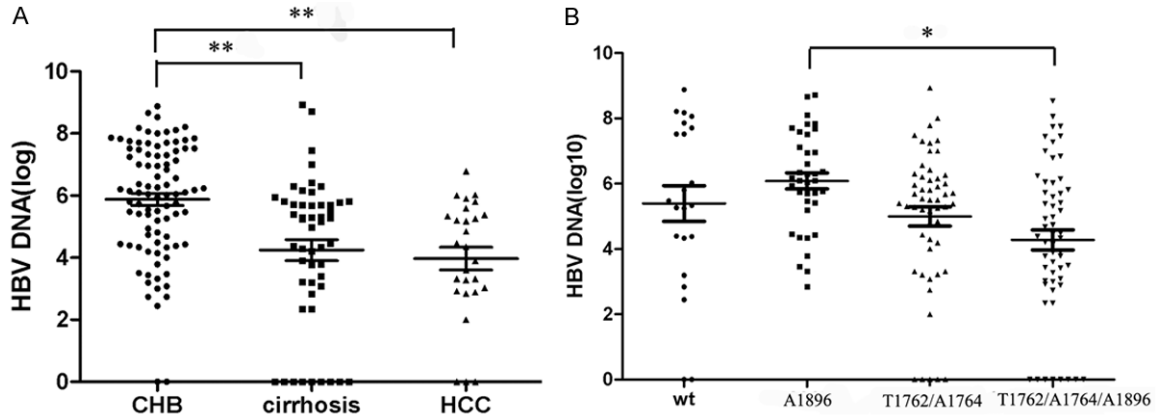
Figure 4. Serum HBV DNA loads for the groups of patients stratified by the type of HBV-related liver disease and the BCP T1762/A1764 and PC A1896 mutations. A. Serum HBV DNA loads were significantly higher in the patients with CHB than in the groups of patients with cirrhosis or HCC (P<0.01). B. Serum HBV DNA loads for all patients stratified by the BCP T1762/A1764 and PC A1896 mutations. No significant differences were observed between patients with the WT mutation and the three mutation groups; however, a significant difference existed between patients with the A1896 mutation and patients with the T1762/A1764 and A1896 mutations (P<0.05).
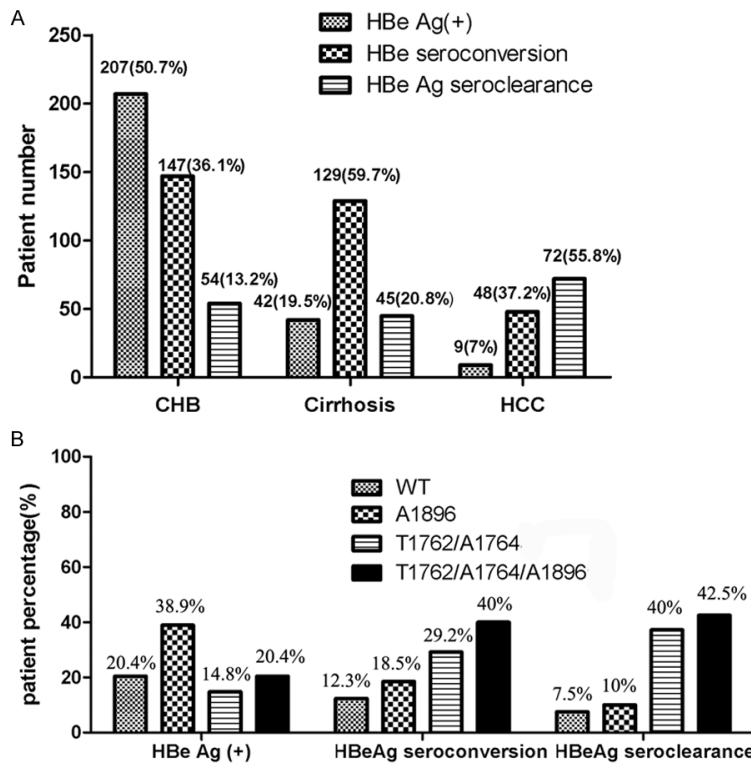
Figure 5. Relationships between HBeAg status and HBV-related liver disease progression and the HBV BCP T1762/A1764 and PC A1896 mutations. A. HBeAg status of the groups of patients with CHB, cirrhosis and HCC. B. Frequency of the T1762/A1764 and PC A1896 mutations in the patients stratified by HBeAg(+), HBeAg seroconversion and HBeAg seroclearance status.

group. However, only the difference between the A1896 group and T1762/A1764/A1896 group was significantly different (P<0.05).

Association of the BCP T1762/A1764 and precore PC A1896 mutations with serum HBeAg status

Among the patients with CHB, 50.7% were HBeAg-positive, 36.1% had HBeAg seroconversion (HBeAg(-)/anti-HBe(+)), and 13.2% had HBeAg seroclearance status (HBeAg(-)/anti-HBe(-)). However, the majority of patients with cirrhosis had anti-HBe positive status, and significantly fewer patients with cirrhosis were HBeAg positive compared to patients with CHB (19.5% vs. 50.7%; P<0.001). In the HCC group, more than half of patients had HBeAg seroclearance status and only approximately 7% were HBeAg-positive (**Figure 5A**). The Chi-square test confirmed that HBeAg status was significantly different between all groups (P<0.001).

DNA load, followed by the WT and T1762/A1764 groups, and T1762/A1764/A1896
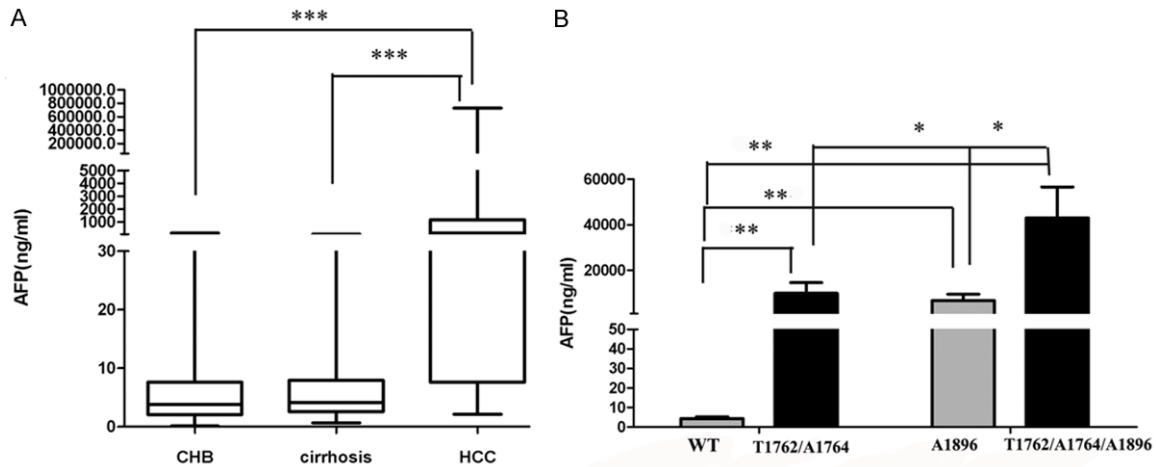
Figure 6. Relationship of serum AFP with HBV-related liver disease progression and the HBV T1762/A1764 and PC A1896 mutations. A. Serum AFP was significantly higher in patients with HCC than the groups of patients with CHB and cirrhosis (P<0.001). B. Serum AFP for patients with different combinations of the HBV T1762/A1764 and PC A1896 mutations. ***P<0.001, **P<0.01 and *P<0.05, one-way ANOVA [non-parametric]).

We also investigated the association between the BCP T1762/A1764 and PC A1896 mutations and HBeAg status in the 237 patients enrolled in this study. In HBeAg(+) patients, the A1896 mutant was dominant (38.9%), followed by the WT (20.4%), the T1762/A1764 mutations and the T1762/A1764/A1896 mutations (14.8% and 20.4%) (**Figure 5B**). In contrast to the HBe Ag(+) group, the T1762/A1764 and T1762/A1764/A1896 mutations were dominant among the HBeAg negative patients; the total frequencies of these two mutations in patients with HBeAg seroconversion and HBe seroclearance groups were 69.2% and 82.5%, respectively. The chi-square test indicated that there were significant differences in the frequencies of the BCP T1762/A1764 and PC A1896 mutations among the HBeAg-positive, HBeAg seroconversion status and HBeAg seroclearance status groups (P<0.01). Correlation analysis indicated that PC A1896 mu-tation was associated with HBeAg seroconversion status (0.212, P<0.01).

Association of the BCP T1762/A1764 and pre-core PC A1896 mutations with serum AFP

AFP is one specific biomarker for liver cancer. The average serum AFP level was significantly higher for patients with HCC than patients with cirrhosis and patients with CHB (**Figure 6A**; 46368.7 vs. 14.7 and 17.2 mg/ml; P<0.001). Correlation analysis showed that serum AFP was significantly and positively associated with

the development of HCC (correlation coefficient, 0.387; P<0.001).

We also observed correlations between the BCP T1762/A1764 and PC A1896 mutations and serum AFP (correlation coefficients, 0.136 and 0.109, respectively; P<0.05). Both BCP T1762/A1764 and PC A1896 were associated with significantly higher serum AFP (1762/1764WT/1896WT vs. 1762/1764MUT/1896-WT, P<0.001 and 1762/1764WT/1896WT vs. 1762/1764WT/1896MUT, P<0.01). Additionally, the BCP T1762/A1764 plus PC A1896 mutations were associated with significantly higher serum AFP compared to the BCP T1762/A1764 mutant or PC A1896 mutant alone (1762/1764MUT/1896MUT vs. 1762/1764-MUT/1896WT, P<0.05; 1762/1764MUT/18-96MUT vs. 1762/1764WT/1896MUT, P<0.05).

Association of the BCP T1762/A1764 and pre-core PC A1896 mutations with age

Age comparison among HBV patients (**Table 2**) revealed that mutations of PC A1896 and BCP T1762/A1764 occurred in adult, especially in middle-aged adult (19-60 years old). Patients aged between 19 and 36 had prominent distribution of PC A1896 mutation (66%). However, the occurrence of BCP T1762/A1764 mutation in 36-60 year-old patients was 64.1%. Similarly, within the T1762/A1764/A1896 mutation group, age 36-60 was the most prevalent age stage (60%).

Table 2. Prevalence of PC A1896 and BCP A1762/G1764 mutation among different age group

Mutation	Young	Adult		
	0-18	19-35	36-60	60-80
Wildtype	1	10	10	1
A1896	0	31	15	1
A1762/G1764	0	16	33	4
1762/1764/1896	0	14	34	10

Discussion

By combining gene sequencing and MAMA-PCR analysis, we quantified the mutation rates for the BCP T1762/A1764 and PC A1896 mutations of HBV in 237 patients with HBV-related diseases. To evaluate whether the BCP T1762/A1764 and PC A1896 mutations are related to disease progression from CHB to cirrhosis and HCC, an integrative approach was employed to assess the associations between these mutations and the clinical parameters of HBV-related liver disease.

It is well-recognized that disease progression varies widely in patients with hepatitis B-related liver disease, and is affected by several viral factors including HBV genotype, viral load and specific viral mutations [12, 13]. Our findings indicate the BCP 1762/1764 and PC 1896 mutations, as well as serum HBV viral load and HBeAg status, are significantly associated with disease progression from CHB to cirrhosis and HCC.

Previous studies demonstrated that the BCP T1762/A1764 mutation was independently associated with the development of HCC [8, 14, 15]. In this study, the BCP T1762/A1764 mutation, either alone or combination with the PC A1896 mutation, was much more frequent among patients with cirrhosis and patients with HCC than patients with CHB (**Figure 3A**). Furthermore, regression analysis proved the BCP T1762/A1764 mutation was strongly associated with disease progression to cirrhosis and HCC. These results are consistent with most of the 15 studies included in a recent meta-analysis [16], which investigated the association between the BCP T1762/A1764 double mutation and the risk of HCC. The overall pooled relative risk and 95% CI were 3.11 and 2.88-4.64 respectively. The BCP T1762/A1764 mutation may alter the binding ability of

the trans-regulating nuclear factors associated with liver carcinogenesis [17] and alter the amino acid sequence of the HBV X protein, which may lead to transactivation of oncogenes related to hepatocarcinogenesis [18].

The A1896 mutation of the PC region is another hotpot mutation commonly studied to assess the risk of developing HCC in HBV-related liver disease [19-21]. Our study indicated that 80% of patients with the PC A1896 mutant were in the CHB stage and no association was observed between the 1896 mutant and HCC, which is contradictory to the results of Liao et al. [22] but consistent with those of Yang et al. [16]. Our study indicated that BCP T1762/A1764 mutation played a critical role or were a principal driving force in the development of HCC and cirrhosis, and that the PC A1896 mutation alone does not promote the development of HCC but can exert a positive synergistic effect with the BCP T1762/A1764 mutation in the development of HCC.

Serum HBV DNA level is a critical clinical parameter that is assessed during the treatment HBV-related liver disease. In addition to causing persistent inflammation that promotes cancer initiation in hepatocytes [23], HBV inhibits the activation of c-Jun/AP-1 in HSCs, which can attenuate apoptosis and result in hepatic fibrosis. HBV also upregulates several endoplasmic reticulum stress genes associated with cell growth and fibrosis [24]. Several studies have suggested that serum HBV DNA load has prognostic value for HBV-related liver disease progression. HBeAg-negative patients with CHB and low HBV DNA are reported to have a low risk of disease progression [25], and a high viral load is associated with progression to cirrhosis [26, 27] or HCC [28-31]. However, our data indicates patients with CHB have significantly higher serum HBV DNA loads than patients with cirrhosis and patients with HCC. Serum HBV DNA load decreased by a mean of 2 log (10) as disease progression advanced from CHB to cirrhosis and HCC (**Figure 4A**). A recent study reported the HBV copy number was frequently lower in liver tumors than in non-tumor tissues [32]. We speculate that cirrhosis or HCC in the liver could make the hepatocyte microenvironment unfavorable for HBV replication. Previous clinical research suggested that compensated-stage cirrhosis patients with a detectable, but low, HBV viral load had a high risk of HCC, and antiviral treatment was

essential for these patients to prevent aggravation of liver damage [33].

The present study indicates mutation of both the PC 1896 and BCP 1762/1764 sites does not significantly affect the serum HBV load, though slight increases and decreases in serum HBV load were observed in patients with either the PC or BCP 1762/1764 mutations, respectively (**Figure 4B**). However, the number of patients with serum HBV DNA loads less than 10^2 IU/ml increased in the presence of the BCP T1762/A1764 mutation alone or in combination with the PC A1896 mutation, suggesting these variants prevent HBV replication and reduce viral load, while the presence of the A1896 mutation does not significantly affect viral replication.

HBeAg is derived from the precore/core protein, which are a strong immunogen and the major target of host immune clearance mechanisms [34]. HBeAg is commonly considered to be an indicator of viral replication and is used to assess a patient's degree of infectiousness, whereas the presence of anti-HBe antibodies often indicates a low level of viral replication [35]. We discovered HBeAg status significantly correlated with disease progression from CHB to cirrhosis and HCC in this cohort of HBs Ag-positive patients with HBV-related liver disease. HBeAg-positive, HBeAg seroconversion, and HBeAg seroconversion plus HBeAg seroclearance were the most frequent HBeAg statuses in patients with CHB, patients with cirrhosis, and patients with HCC, respectively (**Figure 5A**). Therefore, we conclude that HBeAg-negative status is significantly related to cirrhosis and the development of HCC.

In our study, PC A1896 was the dominant genomic HBV mutation in HBeAg-positive patients (38.4%), but was only present in 18.5% and 10% of patients with HBeAg seroconversion and patients with seroclearance, respectively (**Figure 6B**). Based on this data, our work is consistent with Suppiah et al. [36], who reported that the PC A1896 mutation was not associated with HBeAg-negative status. However, when assessed in combination with other previous studies [35, 37, 38], we conclude that the presence of the A1896 mutation is very closely associated with HBeAg seroconversion, as this mutation introduces a stop codon mutation in the precore region. We

hypothesize that this G-A substitution mutation at nucleotide 1896 of HBV viral genomic DNA leads to lower HBeAg expression in HBeAg-positive patients, and HBeAg seroconversion occurs when the G1896 wild-type genotype is completely replaced by the A1896 mutant. This would explain why the PC A1896 mutant was the dominant genomic mutation among HBeAg-positive patients and was present at significantly lower levels among patients with cirrhosis and patients with HCC. Patients with the BCP T1762/A1764 mutations, including T1762/A1764 and T1762/A1764 plus A1896, had HBeAg(-)/anti-HBe(+) and HBeAg seroclearance rates of 69.2 and 82.5%, respectively (**Figure 6B**), which suggests that BCP T1762/A1764 plays a critical role in the processes of HBeAg seroconversion and HBeAg seroclearance. Among all enrolled patients, 80.5% and 93% of patients with cirrhosis and HCC had HBeAg seroconversion and HBeAg seroclearance status, respectively; on the other hand, the BCP T1762/A1764 mutation was associated with cirrhosis and HCC and therefore may have potential as a biomarker to predict the development of cirrhosis and HCC.

AFP has been used as a tumor marker for the diagnosis of HCC since the 1970's [39, 40], and nearly 75% of patients with HCC have a serum AFP level higher than 10 μg/L [41]. As the results of this study suggested the BCP T1762/A1764 mutation has potential as a biomarker for the development of HCC, we investigated whether BCP T1762/A1764 was associated with the liver tumor-specific biomarker AFP. Both the BCP T1762/A1764 and PC A1896 mutations were associated with higher serum AFP, and a synergistic interaction between these two mutations appears to strengthen this association (**Figure 6B**).

The present study evidenced that the prominent prevalence of A1896 mutation was among 19-35-aged patients, which was consistent with previous studies [42, 43]. While highest prevalence of T1762/A1764 and T1762/A1764/A1896 mutations was distributed among patients of 36-60 years old. This finding suggested that A1896 mutation might be earlier than T1762/A1764 in CHB patients. The stop codon due to A1896 mutation leads to hepatitis B virus escape from recognition of patients' immune system, virus can still repli-

cate. Once T1762/A1764 mutation occurs, it means that the condition is worse.

In conclusion, this study indicates that the BCP T1762/A1764 mutant of HBV is closely associated with the development HBV-related cirrhosis and HCC, as it may prevent HBV replication, abolish HBeAg secretion and increase serum AFP. The PC A1896 mutation of HBV may play an important role in HBeAg seroconversion and was associated with higher serum AFP, but was not related to the development of HCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yuhua Li, Department of Hematology, Zhujiang Hospital, Southern Medical University, No. 253 Gongye Avenue, Haizhu, Guangzhou, China. Tel: +862061643188; Fax: +862061643188. E-mail: liyuhua2011gz@163. com; Dr. Donglin Cao, Department of Laboratory Medicine, Guangdong No. 2 Provincial People's Hospital, No. 466 Xingang Middle Road, Haizhu, Guangzhou, China. Tel: +862089168173; Fax: +86 2089168173; E-mail: caodlgz@sina.com

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