

Association of hepatitis B virus mutations in basal core promoter and precore regions with severity of liver disease: an investigation of 793 Chinese patients with mild and severe chronic hepatitis B and acute-on-chronic liver failure

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Abstract

Objective To investigate the features of hepatitis B virus (HBV) basal core promoter/precore (BCP/PC) mutations and genotypes in a large number of mild/severe chronic hepatitis B (CHB-M/CHB-S), and acute-on-chronic liver failure (ACLF) patients and analyze the clinical implications of the virologic features.

Patients and methods Sera of 793 (325 CHB-M, 170 CHB-S, and 298 ACLF) patients admitted to or who had visited Beijing 302 Hospital from January 2005 to December 2008 were collected and successfully amplified for the HBV BCP/PC and a 1225-bp-long S/Pol (nt 54–1278) gene regions. Biochemical and serological parameters and HBV

DNA level were routinely performed. Viral DNA was extracted and subjected to a nested PCR. Genotypes/subgenotypes were determined based on complete genomic sequence or on analysis of the 1225-bp-long S/Pol-gene sequence. HBV genotyping was performed by direct PCR sequencing followed by molecular evolutionary analysis of the viral sequences. A *P* value of <0.05 (two-sided) was considered to be statistically significant.

Conclusions Our findings suggest that CHB patients infected with BCP/PC mutant viruses are more susceptible to severe hepatitis and ACLF than those with the BCP/PC wild-type virus and that ACLF patients with PC mutant viruses have an increased risk of death. As such, the HBV PC mutation is a potential predictive indicator of ACLF outcome.

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Keywords Hepatitis B virus · Basal core promoter mutation · Precore mutation · Genotype · Acute-on-chronic liver failure

Abbreviations

ACLF	Acute-on-chronic liver failure
ALF	Acute liver failure
ALT	Alanine aminotransferase
BCP	Basal core promoter
CHB-M	Mild chronic hepatitis B
CHB-S	Severe chronic hepatitis B
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
LC	Liver cirrhosis
PC	Precore
PTA	Prothrombin activity
TBIL	Total bilirubin
PCR	Polymerase chain reaction

Introduction

Hepatitis B virus (HBV) chronically infects about 350 million people worldwide and 93 million people in China, with high risk of evolution to liver cirrhosis (LC) and hepatocellular carcinoma (HCC) [1, 2]. Chronic HBV infection leads to a wide spectrum of clinical presentations, including the asymptomatic carrier state, mild and severe chronic hepatitis B (CHB-M, CHB-S), and acute-on-chronic liver failure (ACLF). In China, patients with hepatitis B-related ACLF account for more than 80% of total ACLF cases due to the high prevalence of chronic HBV infection. Without transplantation, such patients have a high mortality rate (60–80%), resulting in 22,600 deaths annually [3].

The pathogenesis of the severity of chronic HBV infection remains largely unclear. Both viral and host factors may play a role. HBV is a highly variable virus and is classified into at least eight genotypes (A–H) that may vary in geographical distribution, viral characteristics, and relationship to clinical outcomes [4]. HBV mutations in basal core promoter (BCP) and precore (PC) regions have attracted special attention because the BCP mutation may enhance HBV replication in vitro and the PC mutation abrogates translation of the HBe antigen (HBeAg) which is considered to be a tolerant protein to buffer immune attack of the infected hepatocytes [5–7]. Studies have been performed to clarify these virologic features in patients with acute liver failure (ALF) who developed fulminant hepatitis from acute HBV infection. The occurrence of the BCP double mutation A1762T/G1764A and the PC mutation G1896A has been documented to be higher in ALF patients than in those with acute hepatitis B [8–14]. A few other BCP/PC mutations have also been reported to be associated with increased HBV replication capacity and/or reduced HBeAg expression in vitro and, in some cases, also associated with ALF occurrence [15–18]. However, the findings have been inconsistent, and to date no obvious link has been identified between HBV BCP/PC mutations and ALF or fulminant hepatitis development [19–22]. Such clinical results may be partly due to inadequate sample sizes and the interference of viral genotypes.

There is still a paucity of data on the association of HBV BCP/PC mutations and genotypes with ACLF occurrence. Liu et al. [23] reported that these virologic features seemed not to be associated with the fulminant and subfulminant exacerbation of CHB, but their sample size was small. The severity of chronic HBV infection may exhibit a progressive process, i.e., from CHB-M to CHB-S and then to ACLF in many cases. In the study reported here, we investigated the features of HBV BCP/PC mutations and genotypes in a large number of CHB-M, CHB-S, and

ACLF patients and analyzed the clinical implications of the virologic features.

Materials and methods

Patients and samples

Sera of 793 patients who were admitted to or visited Beijing 302 Hospital from January 2005 to December 2008 were collected and successfully amplified for the HBV BCP/PC and a 1,225-bp-long S/Pol (nt 54–1278) gene regions. The patient cohort comprised 325 CHB-M, 170 CHB-S, and 298 ACLF patients. The patients came from various areas of China, but mainly from the north of the country. The diagnostic criteria were based on the Management Scheme of Diagnostic and Therapy Criteria of Viral Hepatitis [24] and Diagnostic and Treatment Guidelines for Liver Failure [25], issued by the Chinese Society of Infectious Diseases and Parasitology and the Chinese Society of Hepatology, respectively, and have been described in our previous studies [26, 27]. Briefly, all patients had persistent seropositivity of HBsAg for at least 6 months before enrollment. CHB-M patients met the following criteria: a history of chronic hepatitis based on a histopathological diagnosis and/or compatible laboratory data and ultrasonographic findings, with mild to moderate liver disease activities that did not reach the criteria of CHB-S. CHB-S patients had severe liver disease symptoms, including obvious clinical manifestations and significant alterations in their biochemical parameters, such as significant serum alanine aminotransferase (ALT) elevation. Based on the biochemical parameters, the diagnosis of CHB-S had to meet at least one of the following criteria: (1) serum albumin level ≤ 32 g/L; (2) serum total bilirubin (TBIL) >85.5 $\mu\text{mol/L}$; (3) plasma prothrombin activity (PTA) was 60–40%; (4) serum cholinesterase $<4,500$ IU/L. ACLF patients met the following criteria: recent development of increasing jaundice (TBIL >171.0 $\mu\text{mol/L}$ or rapid increase to >17.1 $\mu\text{mol/L/day}$) and decreasing PTA ($<40\%$), with a recent development of complications, such as hepatic encephalopathy (\geq grade 2), or an abrupt and obvious increase of ascites or spontaneous bacterial peritonitis or hepatorenal syndrome. The criteria for ACLF have been widely used in China and are similar (but not exactly identical) with the newly issued Asian Pacific Association for the Study of the Liver (APASL) criteria [28]. For all patients, there was no evidence of HCC or other metastatic liver disease; no evidence for concomitant hepatitis C/D virus (HCV/HDV) or human immunodeficiency virus (HIV) infection or autoimmune liver disease. The study was approved by the Ethics Committee of Beijing 302 Hospital.

Serological markers and quantitation of HBV DNA

The detection of biochemical and serological parameters and HBV DNA level were routinely performed in the Central Clinical Laboratory of Beijing 302 Hospital. The lower limit of HBV DNA detection is 500 copies/mL (equivalent to 100 IU/mL).

Detection of the BCP/PC mutations

Viral DNA was extracted and subjected to a nested PCR as described elsewhere [29]. The primers were 5'-GAC GTC CTT TGT YTA CGT CC-3' (sense, nt 1413–1432) and 5'-TCT GCG ACG CGG CGA TTG AG-3' (antisense, nt 2403–2422) for the first-round PCR and 5'-ACT TCG CTT CAC CTC TGC AC-3' (sense, nt 1583–1602) and 5'-ATC CAC ACT CCA AAA GAY ACC-3' (antisense, nt 2257–2277) for the second-round PCR. The first-round PCR consisted of equilibrating at 94°C for 3 min, followed by 10 cycles of 94°C for 35 s, 59°C for 35 s (decreasing by 2°C every other cycle), and 72°C for 70 s, and then by 30 cycles of 94°C for 35 s, 56°C for 35 s, and 72°C for 70 s. The second-round PCR consisted of 94°C for 3 min, followed by 35 cycles of 94°C for 25 s, 56°C for 25 s, and 72°C for 50 s. Sequencing was performed using an ABI 3730xl DNA Analyzers (Applied Biosystems, Foster City, CA). Analysis and assembly of sequencing data were performed with the Vector NTI Suite software package (Informax, Frederick, MD). Ten sites of interest were the focus of the analysis based on their clinical or potential clinical significance suggested in previous publications, namely, nt 1753, 1754, 1758, 1762, 1764, 1766, 1768, 1862, 1896, and 1899.

Typing of HBV genotypes/subgenotypes

The genotypes/subgenotypes were determined based on complete genomic sequence or on analysis of the 1225-bp-long S/Pol-gene sequence, which was amplified by an in-house nested PCR assay [29]. A total of 380 HBV complete genomic sequences from individual patients were used for HBV genotyping, including 115 sequences from CHB-M patients (GenBank accession no.: FJ386574–386689 except for FJ386590), 120 sequences from CHB-S patients (FJ562218–562340 except for FJ562263, 562306, and 562338), and 145 sequences from ACLF patients (EU939536–939681 except for EU939680). HBV genotyping was performed by direct PCR sequencing followed by molecular evolutionary analysis of the viral sequences using MEGA 4 software. Standard reference sequences were acquired from the online Hepatitis Virus Database (<http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi>).

Statistical analysis

Continuous variables were expressed as mean \pm standard deviation (SD) or median. Differences in continuous data were evaluated using Student's *t* test, analysis of variance (ANOVA), or nonparametric Wilcoxon signed-ranked test, where appropriate, and categorical data were analyzed using the chi-square test and Fisher's exact test. Multivariate analyses with logistic regression were used to determine independent factors. Statistical analysis was carried out in SPSS ver. 16.0 software (SPSS, Chicago, IL). A *P* value of <0.05 (two-sided) was considered to be statistically significant.

Results

Clinical background, HBV genotype, and BCP/PC mutation profiles of the patients

Table 1 summarizes the clinical background, HBV genotypes, and BCP/PC mutations in the three groups of patients. HBV genotypes C and B were detected in the samples of 648 and 145 patients, respectively. HBV/C2 and HBV/B2 were the dominant subgenotypes and found in 96% and 92% individual genotypes, respectively. The HBV genotyping based on the 1225-bp-long gene fragment had 100% (380/380) concordance for the classification of genotypes B and C and 98.7% concordance for the classification of subgenotypes B2 and C2 (B2 100%, C2 98.3%) in comparison with the genotyping based on complete HBV genomes. A slightly higher ratio of genotype B to C was found in ACLF patients than in CHB patients. Compared to CHB patients, ACLF patients had significantly higher mutation incidences at eight of the ten sites of interest, including T1753V (C/A/G), A1762T, G1764A, C1766T, and T1768A in the BCP region and G1862T, G1896A, and G1899A in the PC region. Notably, the frequencies of A1762T, G1764A, and G1896A hotspot mutations and the average substitution number at the ten sites of interest of the viral sequences increased in a step-wise manner in the three groups of patients, namely, CHB-M < CHB-S < ACLF patients. Correspondingly, the incidence of the BCP/PC wild-type sequence decreased in the same order. In addition, two interesting triple BCP mutations, T1753V/A1762T/G1764A and A1762T/G1764A/C1766T/T1768A-triplet (with any three substitutions at the four sites), were more frequently detected in ACLF patients than in CHB patients. The frequencies of former triple mutations were 15.7, 15.3, and 29.2% for CHB-M, CHB-S, and ACLF patients, respectively (*P* < 0.01). The frequencies of latter triple mutations were

Table 1 Clinical features, HBV genotype, and BCP/PC mutation profile of the 793 patients enrolled in the study

	CHB-M (<i>n</i> = 325)	CHB-S (<i>n</i> = 170)	ACLF (<i>n</i> = 298)	<i>P</i> value
Gender (M/F)	279/46	148/22	255/43	0.90
Age (years)	38.3 ± 11.9	39.1 ± 12.3	45.9 ± 11.8	<0.001
Total bilirubin (μmol/L)	16.4 ± 12.1	164.2 ± 170.24	418.5 ± 179.3	<0.001
Prothrombin activity (%)	89.3 ± 22.4	74.5 ± 20.1	24.1 ± 9.7	<0.001
Albumin (g/L)	44.3 ± 5.6	34.2 ± 4.1	29.0 ± 3.9	<0.001
Albumin/globulin	1.67 ± 0.36	1.37 ± 0.34	1.20 ± 0.50	<0.001
Cholinesterase (IU/L)	7110 ± 2419	3302 ± 1094	1840 ± 889	<0.001
ALT (IU/L)	70.2 ± 90.4	400.3 ± 559.1	516.5 ± 585.2	<0.001
HBV DNA (logcps/mL)	5.26 ± 1.65	5.99 ± 1.47	5.48 ± 1.60	<0.001
HBeAg negative/positive	134/191	100/70	199/99	<0.001
Anti-HBe negative/positive	232/93	69/101	160/138	<0.001
Genotype B	54 (16.6%)	28 (16.5%)	63 (21.1%)	0.271
Genotype C	271 (83.4%)	142 (83.5%)	235 (78.9%)	
Wild type	94 (28.9%)	18 (10.6%)	13 (4.4%)	
T1753C/A/G	51 (15.7%)	26 (15.3%)	90 (30.2%)	<0.001
T1754G	8 (2.5%)	5 (2.9%)	8 (2.7%)	0.950
T1758C	16 (4.9%)	10 (5.9%)	11 (3.7%)	0.535
A1762T ^a	172 (52.9%)	114 (67.0%)	235 (78.9%)	<0.001
G1764A ^a	177 (54.5%)	120 (70.6%)	256 (85.9%)	<0.001
C1766T	16 (4.9%)	11 (6.5%)	34 (11.4%)	0.008
T1768A	9 (2.8%)	2 (1.2%)	21 (7.0%)	0.003
G1862T	4 (1.2%)	2 (1.2%)	13 (4.4%)	0.019
G1896A ^a	105 (32.3%)	82 (48.2%)	155 (52.0%)	<0.001
G1899A	22 (6.8%)	8 (4.7%)	62 (20.8%)	<0.001
Substitution ^b	1.78 ± 1.45	2.24 ± 1.16	2.97 ± 1.31	<0.001

Where appropriate, values are given as the mean ± standard deviation

BCP/PC basal core promoter/precore, HBV hepatitis B virus, CHB-M mild chronic hepatitis B, CHB-S severe chronic hepatitis B, ACLF acute-on-chronic liver failure, M male, F female, HBeAg hepatitis B e antigen, ALT alanine aminotransferase

^a Of the sites of interest, the frequencies of A1762T, G1764A, and G1896A (hotspot BCP/PC mutations) and of average substitution number were significantly different among the three illness groups and showed a stepwise increase in the order of CHB-M < CHB-S < ACLF patients

^b Values represent average substitution numbers/sample at the ten sites of interest at HBV BCP/PC regions

3.4, 4.7, and 12.8% for CHB-M, CHB-S, and ACLF patients, respectively ($P < 0.01$). The quadruple mutation A1762T/G1764A/C1766T/T1768A was detected in four ACLF cases only.

To exclude the potential influence on the results brought by age differences among patient groups, patients aged from 25 to 38 and 39 to 52 years were analyzed as independent two subsets. As shown in Table 2, patients in different illness categories had a similar age within each subset; the frequencies of A1762T, G1764A, G1896A and the average substitution number remained significantly different among the three illness groups as did the stepwise increase in the order of CHB-M > CHB-S > ACLF patients.

In addition to the ten interested sites, significant difference in variant frequencies among the three illness categories was also found at A1846T (CHB-M 8.3%, CHB-S 36.5%, ACLF 37.5%, $P < 0.01$).

Individual profiles of BCP/PC mutations in patients infected with the genotypes B and C viruses

Because HBV genotype influences BCP/PC mutational rates, we analyzed the BCP and PC mutations in genotype B and C viruses individually. Table 3 summarizes the profiles of the BCP/PC mutations in patients infected with genotypes B or C virus, respectively. In patients infected with genotype B virus, a statistical difference in the occurrence of mutations among the three groups of patients was only observed at A1762T and G1764A, whereas in those infected with genotype C virus, there was a significant difference for four mutations among the groups, i.e., T1753V, G1862T, G1896A, and G1899A. The average substitution number/sample clearly increased in a stepwise manner in the order of CHB-S < CHB-M < ACLF patients for both genotypes B and C virus infection. The triple mutational pattern

Table 2 BCP/PC mutation incidence in different illness categories and different genotype virus infection

Age subset/BCP/PC mutation	Age 25–38 (<i>n</i> = 259)				Age 39–52 (<i>n</i> = 300)			
	CHB-M (<i>n</i> = 111)	CHB-S (<i>n</i> = 76)	ACLF (<i>n</i> = 72)	<i>P</i> value	CHB-M (<i>n</i> = 121)	CHB-S (<i>n</i> = 58)	ACLF (<i>n</i> = 121)	<i>P</i> value
Age (years)	31.61 ± 5.62	32.17 ± 4.03	33.12 ± 3.83	0.088	44.36 ± 3.75	44.66 ± 4.09	44.82 ± 4.05	0.664
BCP/PC (mutation) profile ^a								
Wild type	34 (30.6%)	6 (7.9%)	2 (2.8%)	<0.001	28 (23.1%)	4 (6.9%)	7 (5.8%)	<0.001
T1753C/A/G	19 (17.1%)	14 (18.4%)	22 (30.6%)	0.073	19 (15.7%)	8 (13.8%)	40 (33.1%)	0.001
T1754G	2 (1.8%)	4 (5.3%)	2 (2.8%)	0.399	5 (4.1%)	1 (1.7%)	3 (2.5%)	0.616
T1758C	4 (3.6%)	6 (7.9%)	5 (6.9%)	0.414	5 (4.1%)	2 (3.4%)	4 (3.3%)	0.939
A1762T	58 (52.3%)	52 (68.4%)	56 (77.8%)	0.001	68 (56.2%)	44 (75.9%)	96 (79.3%)	<0.001
G1764A	61 (55.0%)	57 (75.0%)	61 (84.7%)	<0.001	71 (58.7%)	45 (77.6%)	104 (86.0%)	<0.001
C1766T	7 (6.3%)	4 (5.3%)	6 (8.3%)	0.745	4 (3.3%)	4 (6.9%)	16 (13.2%)	0.017
T1768A	3 (2.7%)	2 (2.6%)	3 (4.2%)	0.824	3 (2.5%)	0	8 (6.6%)	0.059
G1862T	1 (0.9%)	1 (1.3%)	2 (2.8%)	0.592	2 (1.7%)	1 (1.7%)	7 (5.8%)	0.151
G1896A	36 (32.4%)	32 (42.1%)	37 (51.4%)	0.037	43 (35.5%)	28 (48.3%)	63 (52.1%)	0.029
G1899A	7 (6.3%)	4 (5.3%)	10 (13.9%)	0.103	9 (7.4%)	3 (5.2%)	28 (23.1%)	<0.001
Substitutions	1.78 ± 1.50	2.26 ± 1.08	2.78 ± 1.34	<0.001	1.91 ± 1.37	2.34 ± 1.13	3.03 ± 1.47	<0.001

^a Of the sites of interest, the frequencies of A1762T, G1764A, and G1896A (hotspot BCP/PC mutations) and of average substitution number were significantly different among the three illness groups and showed a stepwise increase in the order of CHB-M < CHB-S < ACLF patients

T1753V/A1762T/G1764A and any three substitutions of A1762T/G1764A/C1766T/T1768A were found to be significantly more frequent in ACLF patients than in CHB patients infected with genotype C virus.

HBV BCP/PC mutational patterns in relation to clinical features

To simplify the data analysis, we defined the A1762T/G1764A and G1764A/C1766A double mutations as basic BCP mutations and G1896A as a basic PC mutation, respectively. Accordingly, three basic patterns were analyzed, i.e., without both basic mutations (BCP–/PC–), with the basic BCP mutation only (BCP+/PC–), and with basic PC mutations regardless of basic BCP mutation concomitance (BCP±/PC+).

Table 4 summarizes the incidence of these three BCP/PC patterns in relation to the clinical laboratory parameters of the patients in the different illness categories. In CHB-M patients, TBIL and ALT levels escalated along with the emergence of the BCP–/PC–, BCP+/PC–, and BCP±/PC+ patterns. In contrast, CHB-S patients infected with the BCP–/PC– virus had a significantly higher TBIL level and a lower HBV DNA level than those infected with BCP/PC mutant viruses. In ACLF patients, no obvious influence of BCP/PC patterns was observed on TBIL, ALT, and HBV DNA levels. The PC mutant virus showed a strong positive influence on HBeAg seroconversion for all three groups of patients, whereas the BCP mutant showed

moderate positive influence on HBeAg seroconversion only in CHB-M patients.

The clinical and virologic characteristics in relation to mortality of ACLF patients

Of the 298 patients with ACLF, 184 patients had a fatal outcome and 90 patients survived >6 months after the onset of liver failure. Older age (≥40 years), higher TBIL level (≥408 μmol/L), lower PTA (≤24%), higher ALT level (≥311 IU/mL), and the HBV PC mutation were detected as independent risk factors of death (Table 5). The cutoff values were the median for biochemical parameters. A age of 40 years, 10⁵ copies/mL for HBV DNA, and 3 for the substitution number/sample were selected as cutoff values because they were the integral values closest to the median.

Discussion

The outcome of HBV infection depends on the interplay between the virus, the hepatocytes, and the host’s immune response. HBV BCP/PC mutations are considered to be likely involved in the driving factors of disease progression of HBV infection because BCP/PC mutations affect viral replication and/or HBeAg expression, which may in turn impact on immune responses to the virus. For example, it has been proposed that expression of HBeAg during perinatal infection, the major mode of HBV transmission in

Table 3 BCP/PC mutation incidence in different illness categories and different genotype virus infection

Clinical features, HBV genotype, and BCP/PC mutation profile	Genotype B (n = 145)					Genotype C (n = 648)				
	CHB-M (n = 54)	CHB-S (n = 28)	ACLF (n = 63)	Univariate P value	Multivariate P value	CHB-M (n = 271)	CHB-S (n = 142)	ACLF (n = 235)	Univariate P value	Multivariate P value
	Male	48 (88.9%)	23 (82.1%)	60 (95.2%)	0.134	0.688	231 (85.2%)	125 (88.0%)	195 (83.0%)	0.409
Age	33.6 ± 12.7	36.9 ± 14.0	47.3 ± 13.1	<0.001	<0.001	39.3 ± 11.5	39.5 ± 11.9	45.5 ± 11.6	<0.001	<0.001
HBV DNA (logcp/mL)	5.14 ± 1.5	6.15 ± 1.33	5.50 ± 1.69	0.024	0.143	5.28 ± 1.68	5.96 ± 1.50	5.61 ± 1.50	0.034	0.070
ALT	83.7 ± 124.5	863.9 ± 746.3	661.6 ± 717.5	<0.001	0.002	67.5 ± 82.0	820.4 ± 639.2	477.6 ± 539.6	<0.001	<0.001
BCP/PC (mutation) profile ^a										
Wild type	22 (40.7%)	2 (7.1%)	5 (7.9%)	<0.001	0.022	68 (25.1%)	13 (9.2%)	8 (3.4%)	<0.001	0.016
T1753V	2 (3.7%)	3 (10.7%)	6 (9.5%)	0.389	0.656	49 (18.1%)	23 (16.2%)	84 (35.7%)	0.001	0.031
T1754G	3 (5.6%)	3 (10.7%)	5 (7.9%)	0.698	0.648	5 (1.8%)	5 (3.5%)	4 (1.7%)	0.448	0.216
T1758C	0	1 (3.6%)	4 (6.3%)	0.172	0.181	16 (5.9%)	9 (6.3%)	7 (3.0%)	0.217	0.866
A1762T	15 (27.8%)	14 (50.0%)	40 (63.5%)	0.001	0.028	157 (57.9%)	100 (70.4%)	195 (83.0%)	<0.001	<0.001
G1764A	15 (27.8%)	14 (50.0%)	42 (66.7%)	<0.001	0.028	162 (59.8%)	106 (74.6%)	214 (91.1%)	<0.001	<0.001
C1766T	0	0	3 (4.7%)	0.136	0.976	16 (5.9%)	11 (7.7%)	31 (13.2%)	0.014	0.323
T1768A	1 (1.9%)	0	2 (3.1%)	0.611	0.977	8 (3.0%)	2 (1.4%)	19 (8.1%)	0.003	0.273
G1862T	1 (1.9%)	2 (7.2%)	3 (4.7%)	0.494	0.293	3 (1.1%)	0	10 (4.3%)	0.007	0.042
G1896A	23 (42.3%)	17 (60.7%)	34 (54.0%)	0.246	0.247	82 (30.3%)	65 (45.8%)	121 (51.5%)	<0.001	0.003
G1899A	4 (7.4%)	2 (7.2%)	11 (17.5%)	0.170	0.449	18 (6.6%)	6 (4.2%)	51 (21.7%)	<0.001	<0.001
Substitution ^b	1.28 ± 1.33	2.00 ± 1.12	2.43 ± 1.39	<0.001	0.070	1.90 ± 1.46	2.28 ± 1.17	3.12 ± 1.27	<0.001	0.085
Triplet A	3 (5.6%)	2 (7.1%)	5 (7.9%)	0.878	0.451	48 (17.7%)	24 (16.9)	82 (34.9%)	0.002	0.484
Triplet B	0	0	3 (4.8%)	0.136	–	11 (4.1%)	8 (5.6%)	35 (14.9%)	<0.001	0.004

^a Of the sites of interest, the frequencies of A1762T, G1764A, and G1896A (hotspot BCP/PC mutations) were significantly different among the three illness groups and showed a stepwise increase in the order of CHB-M < CHB-S < ACLF patients

^b Average substitution number/sample at the ten sites of interest of HBV BCP/PC regions. Triplet A, with T1753V/A1762T/G1764A; triplet B, with any three substitutions of A1762T/G1764/C1766T/T1768A. Triplet B may also be concomitant with T1753V

Table 4 Association of HBV BCP/PC mutations with clinical features

Clinical presentation	BCP-/PC-	BCP+/PC-	BCP±/PC+	<i>P</i> value	BCP-/PC-	BCP+/PC-	BCP±/PC+	<i>P</i> value
	Total bilirubin (μmol/L)				Prothrombin activity (%)			
CHB-M	13.9 ± 8.9	15.6 ± 10.8	20.0 ± 15.4	0.001	100.1 ± 28.8	89.6 ± 19.1	81.7 ± 17.4	<0.001
CHB-S	277.9 ± 282.1	139.2 ± 119.0	147.0 ± 142.5	0.005	76.2 ± 21.2	73.8 ± 20.6	74.4 ± 19.7	0.399
ACLF	385.9 ± 187.3	419.5 ± 184.2	422.7 ± 174.8	0.847	27.2 ± 8.4	23.6 ± 9.0	24.0 ± 10.4	0.953
	ALT (IU/L)				HBV DNA			
CHB-M	55.9 ± 60.7	65.8 ± 86.4	90.2 ± 115.4	0.016	5.43 ± 1.83	5.30 ± 1.55	5.03 ± 1.52	0.140
CHB-S	893.1 ± 571.4	771.2 ± 602.0	849.4 ± 720.7	0.314	5.25 ± 1.74	6.14 ± 1.42	6.11 ± 1.36	0.011
ACLF	518.3 ± 489.6	501.7 ± 523.8	527.7 ± 643.5	0.142	4.63 ± 2.13	5.51 ± 1.51	5.58 ± 1.55	0.100
	HBeAg negative (%)				Anti-HBe positive (%)			
CHB-M	20.0 (23/115)	40.0 (42/105)	65.7 (69/105)	<0.001	20.0 (23/115)	26.7 (28/105)	40.0 (42/105)	<0.001
CHB-S	41.7 (10/24)	43.8 (28/64)	75.6 (62/82)	<0.001	37.5 (9/24)	23.4 (15/64)	54.9 (45/82)	0.001
ACLF	64.0 (16/25)	50.8 (60/118)	79.4 (123/155)	<0.001	40.0 (10/25)	41.5 (49/118)	51.0 (79/155)	0.242

Basic BCP mutations: A1762T/G1764A and G1764A/C1766A double mutations. Basic PC mutation: G1896A. Three basic patterns: without both basic mutations (BCP-/PC-), with the basic BCP mutation only (BCP+/PC-), and with basic PC mutations regardless of basic BCP mutation concomitance (BCP±/PC+)

Table 5 Clinical and viral characteristics in relation to mortality of ACLF patients

Clinical and viral characteristics	Cutoffs	Survival (n = 90)	Non-survival (n = 184)	Univariate <i>P</i> value	Multivariate <i>P</i> value	Odds ratio
Age (year)	≥40	51 (56.7%)	138 (75.0%)	0.002	0.013	2.313 (1.201–4.454)
	<40	39 (43.3%)	46 (25.0%)			1
Total bilirubin (μmol/L)	≥408	36 (40.0%)	101 (54.9%)	0.021	0.006	2.672 (1.358–5.258)
	<408	54 (60.0%)	83 (45.1%)			1
Prothrombin activity	<24%	29 (32.2%)	108 (58.7%)	0.003	0.049	1.905 (3.623–1.004)
	≥24%	61 (67.8%)	76 (41.3%)			1
Albumin (g/L)	<29	43 (47.8%)	91 (49.5%)	0.794	0.652	
	≥29	47 (52.2%)	93 (50.5%)			
Cholinesterase (IU/L)	≥1690	42 (46.7%)	95 (51.6%)	0.440	0.093	
	<1690	48 (53.3%)	89 (48.4%)			
ALT (IU/L)	≥311	53 (58.9%)	85 (46.2%)	0.029	0.019	2.277 (4.464–1.161)
	<311	37 (41.1%)	99 (53.8%)			1
HBV DNA (copies/mL)	≥10 ⁵	49 (54.4%)	124 (67.4%)	0.037	0.962	
	<10 ⁵	41 (45.6%)	60 (32.6%)			
HBeAg	Negative	57 (63.3%)	129 (70.1%)	0.259	0.471	
	Positive	33 (36.7%)	55 (29.9%)			
HBV genotype	C	70 (77.8%)	144 (78.3%)	0.928	0.890	
	B	20 (22.2%)	40 (21.7%)			
PC mutation	Positive	37 (41.1%)	107 (58.2%)	0.008	0.043	2.088 (4.255–1.022)
	Negative	53 (58.9%)	77 (41.8%)			1
BCP mutation	Positive	78 (86.7%)	154 (83.7%)	0.595	0.294	
	Negative	12 (13.3%)	30 (16.3%)			
Substitution ^a	≥3	50 (55.6%)	133 (72.3%)	0.006	0.172	
	<3	40 (44.4%)	51 (27.7%)			

^a Average substitution number/sample of at the ten sites of interest of the HBV BCP/PC regions

Asia, induces immune tolerance. Another potential role of HBeAg in promoting persistent infection is to mimic CP so as to buffer the immune attack of the infected hepatocytes by the anti-HBc antibodies [5]. When the balance is disrupted by the emergence of HBV mutants with different phenotypes, the altered virus–cell relationship might trigger robust immune responses of the host in some instances, causing extensive hepatocyte necrosis [30]. Thus, the investigation of virologic features may shed light on the pathogenesis and identify predictors of ACLF development. However, the development of ACLF is related to multiple factors, all of which need to be studied extensively.

Clinical manifestations of ACLF are different from those of fulminant hepatitis on the basis of acute HBV infection. ACLF manifestation also differs from the acute exacerbation of CHB, which is defined as clinical symptoms along with an abrupt rise in serum ALT (>200 IU/L [31] or >500 IU/L [32]). ACLF patients are relatively rare compared to the large population of CHB patients. The Beijing 302 Hospital is one of the largest hospitals for infectious and liver diseases in China and is well known for its management of hepatitis B. Patients from various areas of China come to the hospital seeking treatment, and this has allowed us to collect a larger sample of ACLF patients.

The efficiency of PCR amplification of HBV BCP/PC regions is greatly reduced by the nick-gap structure encompassed in the regions. A sensitive nested PCR assay with a lower limit of detection of 2000 copies/mL was developed to overcome this obstacle. The technique allowed us to analyze samples of quite low viral load, as in our previous severe acute respiratory syndrome (SARS)–coronavirus studies [33, 34]. A significantly higher incidence of the BCP/PC mutations at eight of the ten sites of interest was found in ACLF patients than in CHB patients. In particular, the occurrence of three hotspot BCP/PC mutations and average substitution number in the ten sites of interest increased with the severity of illness in a step-wise fashion (Table 1), suggesting that the accumulation of the BCP/PC mutations could be one of the driving factors of illness severity. Taking into account that a longer duration of infection may increase the incidence of HBV BCP/PC mutation, we particularly compared different illness groups of comparable ages to minimize this factor. The results showed that the increasing trend of the three hotspot mutations (A1762T, G1764A, and G1896A) with disease severity remained unchanged in age-matched patients (Table 2). In addition to variants at the ten sites of interest, the frequency of A1846T was found to increase in CHB-S and ACLF patients compared to CHB-M patients, suggesting that this variant may also be associated with disease severity. A1846T has been reported to be more frequently detected in HBeAg-negative HBV infection than in HBeAg-positive HBV infection [35], but the

mechanism needs further clarification. More frequent BCP mutations and less frequent PC mutations were detected in genotype C virus than in genotype B virus which confirmed previous results [36, 37]. Interestingly, T1754G was an exceptional case which was more often seen in genotype B virus infection. The influence of HBV genotypes on the severity of HBV infection is still uncertain. As genotype B and C viruses have different BCP/PC mutational features, their influence on clinical outcome may be associated with the BCP/PC mutation. Nevertheless, the average substitution number per sample in BCP/PC regions clearly increased with the severity of the illness categories in both genotype B and C, suggesting that the accumulation of the BCP/PC mutations increased the risk of ACLF occurrence regardless of the HBV genotypes.

It has been reported that the co-existence of A1762T/G1764A with T1753V, C1766T, and T1768A may enhance viral replication *in vitro* and is associated with ALF and advanced liver disease [38–40]. We found that T1753V-, C1766T-, and/or T1768A-containing triple mutations were more frequent in ACLF patients than in CHB patients, suggesting highly replicative strains with T1753C, C1766T, and T1768A BCP mutations were also most likely to be associated with ACLF development. Among the 57 cases with triplet B patterns, 30 (52.6%) were A1762T/G1764A/C1766T, nine (15.8%) were A1762T/G1764A/T1768A, and 18 (31.6%) were G1764A/C1766T/T1768A. Noticeably, the last pattern has not been documented earlier and mainly occurred in ACLF patients. In the luciferase reporter system, the natural G1764A/C1766T/T1768A mutant sequences have been proven to have a 1.8-fold higher transcriptional regulation activity on average than the wild-type sequences generated by reverse site-directed mutagenesis (data not shown).

It has been suggested that the BCP mutations frequently emerge at the late HBeAg phase of infection, whereas the PC mutations usually emerge later, at the height of the anti-HBe immune response [15]. Therefore, we classified three BCP/PC mutation statuses as BCP–/PC–, BCP+/PC– and BCP±/PC+ and analyzed their implications. The results showed that CHB-M patients infected with BCP/PC mutants exhibited more enhanced inflammatory responses (significant increase in TBIL and ALT levels and a slight decrease of viral load) than those with the BCP/PC wild-type virus, whereas CHB-S and ACLF patients did not. In contrast, CHB-S patients infected with BCP/PC mutants had lower TBIL and HBV DNA levels than those with the wild-type virus. To date, few studies have been able to show an association between these clinical parameters and HBV BCP/PC mutations. BCP mutants have been shown to be positively correlated with increased ALT levels in chronic HBV carriers and CHB patients [41], and reduced viremia associated with BCP/PC mutants was explained by

the fact that enhanced HBV replication of the mutants would efficiently stimulate immune reactions, leading to the enhanced destruction of viral particles [13, 15]. The alternation of ALT and HBV DNA levels may be influenced by the use of antiviral treatment; furthermore, a blood test at a single time point may bias the evaluation of disease activity for some CHB patients with fluctuating ALT and HBV DNA levels, although these influences were relatively proportionate in large-size samples in this study. Further genotypic and phenotypic studies need to be performed to make a clearer outline.

The rates of HBeAg loss and anti-HBe positivity increased in a stepwise manner along with the emergence of BCP mutation and PC mutation in CHB-M patients. In CHB-S and ACLF patients, BCP mutation alone did not significantly increase HBeAg-negative/anti-HBe-positive rates, suggesting that BCP mutations may have diverse influence on HBeAg seroconversion in different illness categories. There have been reports of the BCP double mutations being associated with lower viral loads in HBeAg-positive individuals [42] and the PC mutation being correlated with high levels of HBV DNA in HBeAg-negative CHB patients [43]. However, these associations were not observed in our study (data not shown). Mutant viruses frequently coexist with wild-type viruses, and subpopulations comprising <20% of the total HBV population may be missed by direct sequencing techniques [44]. This may account for the detection of G1896A mutants in some HBeAg-positive patients.

Interestingly, we found that ACLF patients infected with PC mutants had a significantly higher mortality than those with PC wild-type HBV, indicating that the HBV PC mutation could serve as a predictive indicator for ACLF outcome. In summary, our findings suggest that CHB patients infected with BCP/PC mutant virus are more susceptible to severe hepatitis and ACLF than those with the BCP/PC wild-type virus and that ACLF patients with PC mutant virus have an increased risk of death. The results of this study further our knowledge of the virologic factors that are associated with the severity of chronic HBV infection.

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