Clinical features of hepatitis B e-antigen positive chronic hepatitis B patients co-existing precore and/or basal core promoter mutations

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Aim: Characterize the clinical features of treatment-naïve hepatitis B e-antigen (HBeAg)-positive chronic hepatitis B (CHB) patients harboring either precore (PC) or basal core promoter (BCP; PC/BCP) mutations. **Methods:** Alanine aminotransferase, HBsAg, HBV-DNA, HBV genotypes, PC/BCP mutations and liver stiffness measurement were assessed for 186 treatment-naïve HBeAg-positive CHB patients. **Results:** PC/BCP mutations were found in 40.31% of the treatment-naïve HBeAg-positive CHB patients. Patients with PC/BCP mutations showed significantly higher alanine aminotransferase and liver stiffness measurement levels. The serum HBsAg and HBV DNA levels in patients with BCP mutations were significantly lower than those in patients with wild-type virus. PC/BCP mutations were more prevalent in patients in immune clearance phase than those in immune tolerance phase. **Conclusion:** The emergences of PC/BCP mutations possibly indicate that HBeAg positive patients are experiencing the phase of immune clearance.

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Keywords • basal core promoter • HBeAg-positive • hepatitis B virus • immune clearance • immune tolerance • precore

Hepatitis B is a major public health problem worldwide. Approximately 257 million persons worldwide are chronic hepatitis B (CHB) patients, particularly in low-and middle-income countries [1]. CHB is the leading cause of liver cirrhosis and primary liver cancer in China [2]. The clinical outcome differs among individuals with CHB [3]. The pathogenesis of CHB infection can be attributed to the complex interaction between the host's immune response and viral proteins.

Hepatitis B e-antigen (HBeAg) is a key viral protein which is translated from the precore (preC) transcript of HBV genome. HBeAg is capable of eliciting immune tolerance and involving in upregulating viral replication and facilitating the chronicity and persistence of infection in the host [4]. The presence of HBeAg is accepted as a classical indicator of HBV replication. The loss of HBeAg and the production of anti-HBe antibodies are considered a line of demarcation in the natural history of CHB.

HBV replicates via reverse transcription of pregenomic RNA, during which the proof-reading function is missing, making it prone to mutations [5]. Mutations in the HBV genome are frequent in CHB patients. The mechanisms of HBeAg seroconversion are not fully understood. The precore (PC) and basal core promoter (BCP) regions regulate HBV genomic replication and preC mRNA synthesis. HBeAg is translated by preC mRNA. Therefore, mutations in the preC and BCP regions may impact HBeAg seroconversion. In this study, we focused on mutations in the preC and BCP regions.

A predominant variant contains a G to A change at nucleotide 1896 in preC region (G1986A), which results a premature stop codon. This mutation prevents translation to a full-length preC protein and the production of HBeAg antigen is completely abolished. The most common BCP mutations result in a paired A to T at nt1762

and G to A at nt1764 (A1762T/G1764A) [6]. Consequently, this A1762T/G1764A mutation inhibits the preC mRNA synthesis and decreases the production of HBeAg [7]. Since the G1896A and A1762T/G1764A mutations compromise the HBeAg production, they are more expressed in HBeAg negative patients than in HBeAg positive patients with chronic HBV [8].

PreC and BCP mutations in patients with chronic HBV infection have been studied extensively. One report has described the frequency of PC/BCP mutations in CHB patients, especially HBeAg-negative patients [9]. Their association with the development of liver cirrhosis, primary liver cancer and liver failure has also been proposed [10,11]. Most of these studies selected HBeAg negative and/or HBeAg-positive patients with chronic HBV, including those who received antiviral therapy. Antiviral treatment may have an impact on the frequency of PC/BCP mutations. However, data regarding the frequency of PC/BCP mutations in treatment-naïve HBeAg-positive patients are still limited.

Recently, a study by Pan *et al.* [12] described the clinical features of CHB in treatment-naïve HBeAg positive Asian patients, which coexisting preC and/or BCP (PC/BCP) mutations. In contrast to traditional observations, PC/BCP mutations were found to be more than 60% in HBeAg-positive CHB patients. Current knowledge is still lacking with regard to the effect of PC/BCP mutations on host and viral factors in HBeAg positive treatment-naïve CHB patients, including serum ALT and HBsAg levels, HBV genotype, viral load and liver stiffness measurement (LSM).

Therefore, this study aimed to characterize the clinical features of HBeAg-positive treatment-naïve CHB patients harboring PC/BCP mutations. The correlations between PC/BCP mutations and ALT, serum HBsAg, HBV DNA level and LSM were achieved accordingly.

Patients

A total of 186 adult patients with chronic HBV infection were enrolled from April 2010 to June 2012 in the Center of Infectious Disease of West China Hospital of Sichuan University. All of the recruited chronic HBV infection patients were HBeAg positive and treatment naïve. Based on 2015 Asian Pacific Association for the Study of the Liver guideline on the management of hepatitis B, the enrolled patients were classified into patients in immune tolerance (IT) phase and patients in immune clearance (IC) phase [11].

Exclusion criteria were coinfection with HIV, HCV or HDV; alcohol consumption exceeding 40 g per day and decompensated liver cirrhosis or hepatocellular carcinoma. Patients receiving antiviral therapy were also excluded because of the possible impact on PC/BCP variants. Clinical information including age, gender and family history was collected. This study was approved by the ethics committee of West China hospital, Sichuan University.

Serologic markers of HBV & HBV DNA

Serum samples were tested for quantitative HBsAg titer using chemiluminescent microparticle immunoassay by ARCHITECT HBsAg Kit (Abbott Diagnostics, IL, USA). Antibody to HBsAg, HBeAg and anti-HBe were measured using a microparticle enzyme immunoassay (Abbott Diagnostics, IL, USA). Serum HBV DNA was quantified with quantitative fluorescence PCR (Shanghai Kehua Bio-engineering Co., Ltd, Shanghai, China). HBV DNA levels were expressed as log₁₀ copies/ml.

HBV genotyping & detection of BCP & PC region mutations

HBV genotypes were measured by fluorescent PCR in the KingMed Diagnostics Laboratory. The fluorescent probes were designed and synthesized based on the sequences of HBV genotypes. Detection of HBV PC and BCP mutations were performed in the Kingmed Diagnostics Laboratory using PCR-Reverse Dot Blot.

Liver stiffness measurement

LSM was performed using FibroScan as reported [13]. More than 10 valid measurements were obtained for each patient. The median value was recorded for LSM. Measurements with a success rate of less than 60% or an interquartile range of more than 30% were considered discarded. The unit of LSM is kilopascals (kPa).

Statistical analysis

Statistical analyses were performed with SPSS version 20.0 (SPSS, IL, USA). Normally distributed quantitative variables were expressed as the mean ± standard deviation. Non-normally distributed quantitative variables were expressed as median values (range, minimum–maximum). Qualitative variables were expressed as counts and per-

Table 1. Clinical characteristics of patients.

Patient group	Mean $\pm$ SD or n (%); n = 186
Age, years	33.26 $\pm$ 8.99
Gender, n (%):	
Male	133 (71.50)
Female	53 (28.50)
ALT, IU	32 (5–294)
TB, μ mol/l	13.77 $\pm$ 11.68
HBV genotype:	
B	123 (66.13)
C	60 (32.26)
B/C	3 (1.61)
HBV DNA, log ₁₀ copies/ml	7.30 $\pm$ 1.26
HBeAg, log ₁₀ IU/ml	4.12 $\pm$ 0.86
HBV variants, n (%)	
WT	111 (59.68)
PC	39 (20.97)
BCP	25 (13.44)
BCP/PC	11 (5.91)
HBV immune phase	
IT phase	127 (68.28)
IC phase	59 (31.72)
LSM, kPa	8.56 $\pm$ 6.82

BCP: Basal core promoter; IC: Immune clearance; IT: Immune tolerance; kPa: Kilopascal; LSM: Liver stiffness measurement; PC: Precore; SD: Standard deviation; WT: Wild type.

centages. HBeAg and HBV DNA were log-transformed. Normally distributed continuous variables were compared by Student's *t*-test. Non-normally distributed quantitative data were analyzed with the Mann–Whitney *U* test. Qualitative variables were analyzed by χ^2 test. *p*-values less than 0.05 were considered statistically significant.

Results

Patient characteristics

A total of 186 eligible patients were consisting of 127 patients in IT phase and 59 patients in IC phase. Basic characteristics are shown in Table 1. The PC/BCP mutations were detected in 40.31% of HBeAg-positive treatment-naïve patients with chronic HBV infection. The prevalence of HBV WT, PC mutation, BCP mutations and combined PC/BCP mutations was 59.68% (111/186), 20.97% (39/186), 13.44% (25/186) and 5.91% (11/186), respectively. There were 123 (123/186, 66.13%) patients infected with genotype B, 60 (60/186, 32.26%) patients infected with genotype C and 3 patients coinfecting with genotypes B and C (3/186, 1.61%).

Clinical characteristics of HBeAg-positive CHB patients with PC/BCP mutations versus those with wild-type virus

When mutations were analyzed in the study, patients were classified into one of the following four categories based on their mutation point(s): wild-type; PC mutation; BCP mutations and combined PC/BCP mutations. The median ALT values were 25 (5–219), 47 (10–294), 53 (9–215) and 68 (8–146) in patients with wild-type virus, PC mutation, BCP mutations and combined PC/BCP mutations, respectively (Table 2). The corresponding mean LSM values were 6.48 $\pm$ 4.04, 10.24 $\pm$ 10.25, 12.29 $\pm$ 5.21 and 15.74 $\pm$ 8.63 kPa (Table 2), respectively. Patients with any PC or BCP mutations had significantly higher ALT and LSM values (Figure 1A & D).

HBeAg-positive patients with PC mutations had similar median HBeAg values to those with wild-type HBV (4.16 vs 4.33 log₁₀ IU/ml, *p* = 0.26). Mean serum HBeAg values in patients with BCP mutation or combined PC/BCP mutations were significantly lower than in patients with wild-type virus (3.34 vs 4.33 log₁₀ IU/ml, *p* < 0.001; 3.74 vs 4.33 log₁₀ IU/ml, *p* = 0.002)(Figure 1B). Mean serum HBV DNA levels in patients with BCP mutations or combined PC/BCP mutations were also significantly lower than in patients with wild-type virus (6.43 vs 7.42 log₁₀ copies/ml, *p* < 0.001; 7.16 vs 7.42 log₁₀ copies/ml, *p* = 0.012)(Figure 1C).

Figure 1. Comparison of clinical characteristics in the HBeAg-positive chronic hepatitis B patients with nt1762/1764 wild-type, PC mutant, BCP mutant and PC/BCP mutant HBV infection. (A) ALT (IU/l) (B) HBsAg ($\log_{10}$ IU/ml) (C) HBV DNA ($\log_{10}$ copies/ml) (D) LSM (kPa). BCP: Basal core promoter; LSM: Liver stiffness measurement; PC: Precore; WT: Wild type.

The precore &/or basal core promoter mutations in different immune phases of CHB

The prevalence of PC and/or BCP mutations stratified by different immune phases of patients with chronic HBV infection is shown in Figure 2A. The prevalence of HBV WT, PC mutation, BCP mutations and combined BCP/PC mutations was 71.65% (91/127), 14.17% (18/127), 10.24% (13/127) and 3.94% (5/123), respectively, in patients of the IT phase. The corresponding prevalence was 33.90% (20/59), 35.59% (21/59), 20.34% (12/59) and 10.17% (6/59), respectively, for patients of the IC phase. PC and/or BCP mutations were more prevalent in HBeAg-positive CHB patient in the IC phase than in the IT phase (39/59 vs 36/129, $p < 0.001$).

PC &/or BCP mutations in patients with genotype B and C infection

The prevalence of PC and/or BCP mutations stratified by HBV genotype is shown in Figure 3A. The prevalence of HBV WT, PC mutation, BCP mutations and combined BCP/PC mutations was 65.86% (81/123), 25.20% (31/123), 6.50% (8/123) and 2.43% (3/123), respectively, in patients with genotype B. The corresponding prevalence was 50% (30/60), 10% (6/60), 28.33% (17/60) and 11.67% (7/60) in patients with genotype C, respectively. BCP mutations (including BCP mutations and PC/BCP mutations) were more prevalent in patients with genotype C than genotype B (24/60 vs 11/123, $p < 0.001$). For PC mutations (including PC mutations only and PC/BCP mutations), there was no statistically significant difference between the two genotypes (13/60 vs 34/123, $p = 0.385$). Among PC mutation patterns, genotype B was more prevalent, similar to the wild-type

Figure 2. The patterns of distribution of PC and BCP mutations in patients with IT phase versus IC phase.
BCP: Basal core promoter; PC: Precore; IC: Immune clearance phase; IT: Immune tolerance phase; WT: Wild type.

pattern (31/39 vs 81/111, $p = 0.143$). Genotype C was more prevalent in BCP and PC/BCP mutations patterns (17/25 vs 30/111, $p < 0.001$; 7/11 vs 30/111, $p < 0.001$) (Figure 3B).

Discussion

PC and BCP mutations have been investigated widely in patients with chronic HBV infection, especially in those with HBeAg disappears and seroconversion to anti-HBe [14]. Therefore, PC/BCP mutations are expected to be common in HBeAg-negative patients. However, data regarding the frequency of PC/BCP mutations in treatment-naïve HBeAg-positive patients is still limited. In this study, a total of 40.31% (74/186) of patients harbored HBV PC or BCP mutations. The prevalence of PC/BCP mutations in this study is lower than two previous studies in China [15,16]. Yan *et al.* [17] reported that PC/BCP mutations existed in 65.10% (125/192) of treatment-naïve HBeAg positive Chinese patients. Pan *et al.* [12] found PC/BCP mutations in 60.45% of HBeAg-positive treatment-naïve Asian patients. In line with Yan *et al.* [17], we found that BCP mutations were more prevalent in patients with genotype C than genotype B. The predominant HBV genotype was B in the present study, while

Figure 3. The patterns of distribution of PC and BCP mutations in patients with genotype B versus genotype C. BCP: Basal core promoter; PC: Precore; WT: Wild type.

the predominant HBV genotype was C in the study by Yan *et al.* [17]. In this study, patients who received antiviral therapy were excluded because the treatment may influence the host immune pressure on HBV and increase the prevalence of PC/BCP mutations. HBV genotype, treatment-naïve experience and ethnicity may account for the varied prevalence of PC/BCP mutations in HBeAg-positive cases.

It was surprising that the rate of PC mutation was higher than that of BCP mutations in our population with positive HBeAg. Previous studies demonstrated that HBV genotype B predicted the presence of PC mutation, while genotype C predicted the presence of BCP mutations [18,19]. In HBeAg-positive patients, we also observed that BCP mutations were associated with genotype C infection. The present results are consistent with previous studies. However, the predominant HBV genotype was B in this study. This may be the reason for a slightly higher proportion of the overall prevalence of PC mutation.

Table 2. Clinical characteristics of hepatitis B e-antigen-positive chronic hepatitis B patients with wild-type versus precore/basal core promoter mutations.

Characteristics	Wild-type (n = 111)	PC mutation (n = 39)	BCP mutations (n = 25)	Combined PC/BCP mutations (n = 11)
Age, years	32.23 ± 7.89	33.28 ± 10.09	34.84 ± 10.53	39.91 ± 9.69
Gender, n (%):				
– Male	79 (71.17)	28 (71.79)	20 (80.00)	6 (54.55)
ALT, IU/l	25 (5–219)	47 (10–294)	53 (9–215)	68 (8–146)
TB, μ mol/l	13.56 ± 10.74	15.24 ± 17.04	13.47 ± 6.38	11.42 ± 6.36
HBV genotype:				
– B	81 (72.97)	31 (79.49)	8 (32.00)	3 (27.27)
– C	30 (27.03)	6 (15.38)	17 (68.00)	7 (63.64)
– B/C	0	2 (5.13)	0	1 (9.09)
HBV DNA, log ₁₀ copies/ml	7.42 ± 1.27	7.53 ± 0.91	6.43 ± 1.52	7.16 ± 0.51
HBsAg, log ₁₀ IU/ml	4.33 ± 0.82	4.16 ± 0.78	3.34 ± 0.82	3.74 ± 0.52
LSM, kPa	6.48 ± 4.04	10.24 ± 10.25	12.29 ± 5.21	15.74 ± 8.63

BCP: Basal core promoter; LSM: Liver stiffness measurement; PC: Precore.

The principal finding of this study was that HBeAg-positive patients with BCP mutations were more likely to have lower serum HBV DNA levels compared with those patients with wild-type HBV. The previous study demonstrated that the HBV strain with BCP mutations supported a higher level of pregenomic RNA transcription and HBV DNA replication, compared with the wild-type strain *in vitro*.

As to the impact of BCP mutations on viral replication, variants in the core promoter regions can enhance genome replication capacity and reduce HBeAg expression *in vitro* through regulation by different liver-enriched transcription factors involved in viral transcription [20,21]. However, there is a contradiction of enhanced *in vitro* replication and reduced *in vivo* viremia. Parekh *et al.* [22] amplified full-length HBV genomes by PCR from sera of HBeAg positive individuals, cloned into vectors, transfected and detected HBV replication. They found that clones derived from highly viremic individuals showed low replication capacity *in vitro*, while some clones derived from low-viremia individuals had much higher replication capacity *in vitro*. It indicated that under the influence of the interaction between the virus and the host immune system, the BCP mutations affect viral replication in ways that are not captured *in vitro* [23].

The relationship between liver fibrosis and PC/BCP mutations in HBeAg-positive patients is also accessed in this study. LSM by FibroScan is reliable for detecting advanced liver fibrosis and cirrhosis in patients with CHB [24,25]. Among HBeAg-positive patients, individuals with any PC or BCP mutations had significantly higher LSM compared with individuals with WT. Although elevated ALT has a slight impact on LSM, 81.7% of patients enrolled in this study had normal ALT. Therefore, the elevated LSM represented advanced liver fibrosis. It indicated that treatment-naïve HBeAg-positive individuals with any PC or BCP mutations might have advanced liver fibrosis.

In our study, PC/BCP mutations were more prevalent in patients with IC-phase than IT-phase infection. It suggest that among patients with HBeAg positivity, unlike those with wild-type HBV who are in the immune tolerance phase, patients with PC/BCP mutations are in the immune clearance phase and are experiencing greater immune pressure, leading to a lower level of viremia and HBsAg. Our results demonstrated that the emergence of PC/BCP mutations was associated with the strong immune clearance for the HBeAg-positive patients.

Nevertheless, we are aware that there are several limitations in this study. First, the sample size of only 186 patients was relatively small. Despite the small sample size, we detected valuable clinical applications in chronic HBV patients with PC/BCP mutations. Second, longitudinal investigation is needed to give a better comprehension of the roles of PC/BCP mutations in natural history of chronic HBV infection. Third, PC/BCP mutations are not detected by sequencing. Fortunately, PCR-reverse Dot Blot is a accurate, reproducible for detection of PC/BCP mutations.

Conclusion

Our study showed that PC/BCP mutations frequently occurred, even in treatment-naïve HBeAg-positive patients. HBV BCP mutations were associated with low levels of serum HBsAg and HBV DNA but with high levels of

ALT and LSM. The emergence of PC/BCP mutations indicates that HBeAg-positive patients are experiencing the phase of immune clearance.

Future perspective

Future large longitudinal studies are needed to investigate the long-term clinical outcomes when PC/BCP mutations are detected in HBeAg-positive patients as it may impact the natural history in such patients.

Executive summary

- Precore/basal core promoter (PC/BCP) mutations are frequently detected in hepatitis B e-antigen (HBeAg)-negative chronic hepatitis B (CHB) patients, but little is known about their prevalence and clinical features in HBeAg-positive CHB patients especially in China.
- The PC/BCP mutants are also frequent (40.31%) in treatment-naïve HBeAg-positive CHB patients.
- HBV BCP mutations are associated with low levels of both serum HBsAg and HBV DNA, but a higher alanine aminotransferase levels and liver stiffness measurement values in treatment-naïve HBeAg-positive CHB patients.
- The emergence of PC/BCP mutations indicates that HBeAg-positive patients are experiencing the phase of immune clearance.

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The authors declare that they have no competing interests.

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