Title: Relationship between the development of precore and core promoter mutations and hepatitis B e antigen seroconversion in patients with chronic hepatitis B virus

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Relationship between the Development of Precore and Core Promoter Mutations and Hepatitis B e Antigen Seroconversion in Patients with Chronic Hepatitis B Virus

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Chinese patients with chronic hepatitis B virus (332 with and 44 without cirrhosis-related complications) were studied. Fifty percent of patients <30 years old had precore mutations. The prevalence of precore mutations among hepatitis B e antigen (HBeAg)-positive patients, although lower than that among anti-HBe–positive patients (44.2%), was already high (P < 0.031). Median HBV DNA level in anti-HBe–positive patients was $1.5 \times 10^8 - 1.55 \times 10^6$ copies/mL, irrespective of the presence or absence of precore mutations. There was no difference in the prevalence of precore mutations between patients with and without complications (P, not significant). However the prevalence of core promoter mutations was higher among patients with complications than among those without complications (90.5% vs. 69.3%, respectively; P < 0.003). In conclusion, precore mutations occurred in a large proportion of Chinese patients with chronic hepatitis B virus before HBeAg seroconversion. The development of complications was not related to precore mutations but was probably due to the persistence of significant viremia after HBeAg seroconversion.

It is believed that precore mutants emerge as a result of selection under immune pressure during the process of hepatitis B e antigen (HBeAg) seroconversion [1]. Precore mutants have been detected in 4%–10% of HBeAg-positive patients [2, 3]. Some studies suggest that it is associated with more severe hepatitis B virus (HBV) exacerbation and liver diseases [4, 5], but this has not been confirmed elsewhere [1, 6].

Core promoter mutants of HBV decrease the precore mRNA transcription while allowing the transcription of pregenomic mRNA. In in vitro studies, core promoter mutants decrease HBeAg production but enhance viral replication [7, 8]. However, in several in vivo studies, patients with core promoter mutants have lower HBV DNA levels, compared with patients without core promoter mutants [9, 10]. The core promoter mutations are reported to be related with hepatocellular carcinoma (HCC) [11, 12].

The primary objective of our study was to determine the relationship between the development of precore and core promoter mutants and HBeAg seroconversion, using an assay that is more sensitive than conventional sequencing for the detection of the mutants. The secondary aim was to define the clinical significance of these mutants.

Patients and Methods

In total, 376 Chinese patients with chronic HBV patients were recruited from the Hepatitis Clinic, University of Hong Kong, Queen Mary Hospital, Hong Kong, from January 1999 through December 2001. Three hundred thirty-two patients had no biochemical and clinical evidence of cirrhosis at presentation and subsequent follow-up. Among the remaining 44 patients, 20 presented with HCC and/or other cirrhosis-related complications, and 24 had developed complications by subsequent follow-up. Among these 44 patients, 24 had HCC only, 8 had HCC and cirrhosis-related complications, including ascites, encephalopathy, oesophageal varice, or spontaneous bacterial peritonitis, and 12 had cirrhosis-related complications without HCC. HCC and other cirrhosis-related complications are collectively referred to here as “complications.” All patients were positive for hepatitis B surface antigen (HBsAg) for at least 6 months. Serum was checked for HBeAg and antibody to HBeAg (anti-HBe), liver biochemistry, HBV DNA levels (measured by the Digene Hybrid Capture II assay, Digene [lower limit of detection, 0.14 x 10^6 copies/mL]), and precore and core promoter mutations at the time of presentation and at the time of the onset of complications for the 24 patients who developed complications during follow-up.

The precore and core promoter mutations of HBV were determined by use of an investigational research version of a line probe
assay (INNO-LiPA HBV Precore; Innogenetics). The line probe assay contains specific probes covering the following motifs: (1) core promoter nt 1762/1764 wild type (A/G), (2) core promoter nt 1762/1764 mutation (A/A), (3) core promoter nt 1762/1764 mutation (A/T), (4) core promoter nt 1762/1764 mutation (T/A), (5) precore codon 28 (nt 1896) wild type (TGG), and (6) precore codon 28 (nt 1896) mutation (TAG). The probes were applied on a nitrocellulose membrane. In brief, HBV DNA was amplified for the core promoter/precore region using 5’ biotinylated primers [13]. After reverse hybridization of the biotinylated polymerase chain reaction–fragments in the LiPA format, streptavidine–alkaline phosphatase incubation and color development was used to identify reactive probes. The presence of mixed population of wild-type and mutant viruses was verified by clonal sequencing in 5% of the tested serum to confirm the reactivity seen on the INNO-LiPA HBV Precore assay.

Statistical analysis. The Mann-Whitney U test was used for continuous variables, with skewed distribution and chi² test with Yates’ correction factor or Fisher’s exact test used for categorical variables. A general linear model was used to adjust confounding factors.

Results

Demographic data and prevalence of precore and core promoter mutations. The demographic data of the 332 patients without complications and 44 patients with complications are shown in table 1. Patients with complications had a significantly higher median age than did patients without complications (58 years [range, 32–80 years; interquartile range (IQR) expressed here as the difference between the 25th and 75th centiles], 15 years) vs. 41 years [range, 15–82 years; IQR, 16 years], respectively; \( P < .0001 \). Among the 332 patients without complications, 322 and 313 patients had the results for precore and core promoter regions, respectively, from the LiPA assay. Among the 44 patients with complications, 40 and 42 patients had the results for precore and core promoter regions, respectively. The prevalence of precore and core promoter mutations stratified according to HBsAg/anti-HBe status and alanine aminotransferase (ALT) levels are listed in table 2.

Relationship between the development of precore and core promoter mutations and HBsAg seroconversion. The prevalence of precore mutations in different age groups are as follows: aged 10–30 years, 50%; aged 31–40 years, 48.9%; aged 41–50 years, 50%; aged >50 years, 58.1%. There were no significant differences in the prevalence of precore mutations among the different age groups. Though anti-HBe–positive patients had a significantly higher prevalence of precore mutations (109/193 [56.5%]) than HBsAg-positive patients (57/129 [44.2%]; \( P = .031 \)), the prevalence of precore mutations in HBsAg-positive patients was already high.

Patients aged 10–30 years had a significantly lower prevalence of core promoter mutations (52.2%), compared with those aged 31–40 years (71.5%; \( P = .019 \)), 41–50 years (77.3%; \( P = .001 \)), and >50 years (69.7%; \( P = .001 \)). There were no significant differences in the prevalence of core promoter mutations among the latter 3 groups. There was no difference in the prevalence of core promoter mutations between HBsAg-positive patients (85/123 [69.1%]) and anti–HBe–positive patients (132/190 [69.5%]).

\( ALT \) and HBV DNA levels in precore mutations. There were no significant differences in the ALT and HBV DNA levels between patients with and without precore mutations (median ALT level, 59 U/L [range, 4–2660; IQR, 95] vs. 49 U/L [range, 13–1270; IQR, 77], respectively; \( P = .59 \)); median HBV DNA level, 1.58 \( \times \) 10^6 copies/mL [range, <1.0 \( \times \) 10^5–36,776 \( \times \) 10^6 copies/mL; IQR, 40.4 copies/mL] vs. 1.99 \( \times \) 10^6 copies/mL [range, <1.0 \( \times \) 10^5–36,080 \( \times \) 10^6; IQR, 26.19 \( \times \) 10^6, respectively; \( P = .53 \)].

For HBsAg-positive patients, there were no differences in the ALT and HBV DNA levels for patients with and without precore mutations (median ALT level, 59 U/L [range, 10–201 U/L; IQR, 20 U/L] vs. 42 U/L [range, 13–1200 U/L; IQR, 50.25 U/L], respectively; \( P = .29 \)); median HBV DNA level, 5.44 \( \times \) 10^6 copies/mL [range, <1.0 \( \times \) 10^6–36,480 \( \times \) 10^6 copies/mL; IQR, 257.17 \( \times \) 10^6 copies/mL] vs. 5.91 \( \times \) 10^6 copies/mL [range, <1.0 \( \times \) 10^6–1313.14 \( \times \) 10^6 copies/mL; IQR, 226.59 \( \times \) 10^6 copies/mL, respectively; \( P = .63 \)].

For anti-HBe–positive patients, there were again no differences in the ALT and HBV DNA levels for patients with and without precore mutations (median ALT level, 59 U/L [range, 4–2660 U/L; IQR, 81.5 U/L] vs. 60 U/L [range, 16–1270 U/L; IQR, 92 U/L], respectively; \( P = .45 \)); median HBV DNA level, 1.50 \( \times \) 10^6 copies/mL [range, <1.0 \( \times \) 10^6–36,776 \( \times \) 10^6 copies/mL; IQR, 18.38 \( \times \) 10^6 copies/mL] vs. 1.55 \( \times \) 10^6 copies/mL [range, <1.0 \( \times \) 10^6–36,080 \( \times \) 10^6 copies/mL; IQR, 8.93 \( \times \) 10^6 copies/mL, respectively; \( P = .75 \)].

\( ALT \) and HBV DNA levels in core promoter mutations. Patients with core promoter mutations tended to have high-

<table>
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<th>Table 1. Demographic data of patients with and without hepatocellular carcinoma and/or cirrhosis-related complications.</th>
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<td>Characteristic</td>
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<td>Age, years</td>
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<td>Follow-up, months</td>
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<td>ALT level, U/L</td>
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<td>HBV DNA level, ( \times ) 10^6 copies/mL</td>
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**NOTE.** Data are median (range); interquartile range [expressed here as the difference between the 25th and 75th centiles], except for sex ratio. ALT, alanine aminotransferase; HBsAg, hepatitis B e antigen; HBV, hepatitis B virus.
er ALT levels and lower HBV DNA levels, compared with those of patients without core promoter mutations (median ALT level, 59 U/L [range, 4–1270 U/L; IQR, 89.5 U/L] vs. 46 U/L [range, 7–2460 U/L; IQR, 65.5 U/L, respectively; \( P = .066 \]); median HBV DNA level, 1.7 \( \times \) 10^6 copies/mL [range, < 0.14 \( \times \) 10^6–36,776 \( \times \) 10^6 copies/mL; IQR, 18.05 \( \times \) 10^6 copies/mL] vs. 2.43 \( \times \) 10^6 copies/mL [range, < 0.14 \( \times \) 10^6–36,480 \( \times \) 10^6 copies/mL; IQR, 213.67 \( \times \) 10^6 copies/mL, respectively; \( P = .079 \)).

For HBeAg-positive patients, there was no difference in the ALT levels between patients with and without core promoter mutations (median ALT level, 43 U/L [range, 12–1220 U/L; IQR, 90 U/L] vs. 39.5 U/L [range, 12–2010 U/L; IQR, 129.5 U/L, respectively; \( P = .61 \)]. However, patients with core promoter mutations had significantly lower HBV DNA levels than did patients without core promoter mutations (median HBV DNA level, 1.42 \( \times \) 10^6 copies/mL [range, < 0.14 \( \times \) 10^6–36,472 \( \times \) 10^6 copies/mL; IQR, 124.26 \( \times \) 10^6 copies/mL] vs. 243 \( \times \) 10^6 copies/mL [range, < 0.14 \( \times \) 10^6–36,480 \( \times \) 10^6 copies/mL; IQR, 575.92 \( \times \) 10^6 copies/mL, respectively; \( P = .004 \)).

For anti-HBe–positive patients, patients with core promoter mutations had significantly higher ALT levels than did patients without core promoter mutations (median ALT level, 65.5 U/L [range, 4–1270 U/L; IQR, 85 U/L] vs. 48.5 U/L [range, 7–2460 U/L; IQR, 57.5 U/L, respectively; \( P = .032 \)]. However, the HBV DNA levels were comparable between anti-HBe–positive patients with and without core promoter mutations (median HBV DNA level, 1.85 \( \times \) 10^6 copies/mL [range, < 0.14 \( \times \) 10^6–36,776 \( \times \) 10^6 copies/mL; IQR, 11.29 \( \times \) 10^6 copies/mL] vs. 1.58 \( \times \) 10^6 copies/mL [range, < 0.14 \( \times \) 10^6–711.7 \( \times \) 10^6 copies/mL; IQR, 24.23 \( \times \) 10^6 copies/mL, respectively; \( P = .861 \)).

**Precore and core promoter mutations in patients with HCC and/or cirrhosis-related complications.** There was no difference in the prevalence of precore mutation between patients with (22/40 [55%]) and without complications (166/322 [51.6%]; \( P = .681 \)). Patients with complications had a significantly higher prevalence of core promoter mutations (38/42 [90.5%]) than patients without complications (217/313 [69.3%]; \( P = .003 \)). Because some studies reported that core promoter mutations were associated with the development of HCC [11, 12], we compared the prevalence of core promoter mutations between patients with HCC and patients without HCC. There was an increase in the prevalence of core promoter mutations in HCC patients (27/31 [87.1%]; \( P = .039 \)), compared with patients without HCC (217/313 [69.3%]; \( P = .039 \)), though this difference became insignificant after adjusting for age (\( P = .269 \)).

### Discussion

To our knowledge, this study was the first study to document the occurrence of precore mutations in a high proportion of patients from a very early age and before HBeAg seroconversion. Other studies have reported a low incidence of precore mutations (4%–10%) in HBeAg-positive patients [2, 3]. It has been suggested that precore mutants are selected through immune pressure during the process of HBeAg seroconversion. In this study, even though the prevalence of precore mutations was higher in anti-HBe–positive patients than in HBeAg-positive patients (\( P = .031 \)), a large proportion of HBeAg-positive patients (44.2%) were already harboring precore mutants. This discrepancy was most likely due to the methods used for the detection of the precore mutations. We used the LiPA test, which can detect the presence of 5%–10% of precore mutations in a mixed population of wild-type virus and precore mutants [14]. However, this test cannot quantify the exact proportion of wild-type virus and precore mutants. The method commonly used in many other studies is direct sequencing, with a sensitivity of detecting 30%–50% of precore mutations in a mixed wild-type and precore viral population. This probably led to a gross underestimation of the prevalence of the precore mutations in the HBeAg-positive patients.
in previous studies. The more sensitive LiPA assay used in the present study allowed a better approximation of the real prevalence of precore mutations in HBeAg-positive patients. Our findings suggest that precore mutations develop early during the phase of immune clearance of the hepatitis B virus, well before actual HBeAg seroconversion. This was supported by the fact that 50% of patients <30 years old already had precore mutations. On the other hand, even after HBeAg seroconversion, 43.5% (84/193) of our patients still had wild-type HBV alone (table 2), with a median HBV DNA level of $1.55 \times 10^6$ copies/mL, which is comparable to patients with precore mutations. The ALT and HBV DNA levels in our patients with and without precore mutations were comparable, regardless of the HBeAg or anti-HBe status. In addition, there was no difference in the prevalence of precore mutations in patients with (22/40 [55%]) and without complications (166/322 [51.6%]; $P = .681$). These findings suggest that precore mutations do not cause more-severe disease.

Thus, in Chinese patients with chronic HBV infections, there appears to be a spectrum of patients with wild-type virus alone, precore mutants alone, and a mixed population of wild-type virus and precore mutants both before and after HBeAg seroconversion. After HBeAg seroconversion, the median HBV DNA level of our patients, although lower, was still $>10^6$ copies/mL, irrespective of the presence or absence of precore mutations. The continuing development of cirrhosis and cirrhosis-related complications after HBeAg seroconversion in Chinese patients is probably related to this persistent viremia, but unrelated to the emergence of precore mutations. Some studies show that core promoter mutations are associated with the development of HCC [11, 12]. The present study suggests that core promoter mutations were indeed associated with the development of complications. However, the lack of correlation between core promoter mutations and HCC after age adjustment could reflect that some HCC may arise without cirrhosis, possibly in a genotype-dependent manner [15]. The role of HBV genotypes in hepatocarcinogenesis should be investigated in future studies.

In conclusion, to our knowledge this study was the first study to show that precore mutations occurred early in chronic HBV infection among the Chinese. The development of cirrhosis-related complications and HCC was unrelated to precore mutations, but was probably due to the persistence of significant viremia after HBeAg seroconversion. Core promoter mutations might also play a role.

References