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Quantitative dynamics of hepatitis B basal core promoter and precore mutants before and after HBeAg seroconversion

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Abstract

Background and Aims—Hepatitis B e antigen (HBeAg) seroconversion is an important clinical and virological “landmark” during the chronic hepatitis B virus (HBV) infection. Mutant viruses carrying the precore G1896A and/or the basal core promoter (BCP) A1762T/G1764A mutations are associated with HBeAg seroconversion. However, the exact role of these mutants in HBeAg seroconversion remains unclear, partly because the evolution of these mutant viruses before and after seroconversion has not been well studied.

Methods—Using our novel mutant quantification methods, the percentage of the mutant viruses was analyzed both cross-sectionally and longitudinally, before and after seroconversion.

Results—Cross-sectional analysis showed that the percentage of both precore and BCP mutants gradually increased with age in the HBeAg-positive population. Follow-up of 18 patients revealed that the mutant percentage may stay low and stable for many years prior to seroconversion, followed by a steady increase in the percentage of G1896A and/or A1762T/G1764A mutants, from <10% to 50–100%, within about three years of seroconversion. In all cases, increase of mutant percentage was preceded or accompanied by elevated serum alanine aminotransferase. After the seroconversion, the mutant percentage could remain high or decrease significantly, sometimes to below 20%.

Conclusions—Levels of G1896A and A1762T/G1764A mutants (of genotypes B and C) in the HBeAg-positive patients may predict the time of HBeAg seroconversion. The dominance of these

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

mutants in the HBeAg-positive phase is more likely the result of immune selection rather than the enhanced replication capability of the mutants. However, anti-HBe antibody may not be a major selection force for these mutants.

Keywords

HBV; G1896A; A1762T/G1764A; mutation; seroconversion; biomarker

Introduction

Chronic hepatitis B virus (HBV) infection significantly increases the risk of liver cirrhosis and hepatocellular carcinoma (HCC) and poses a major healthcare burden in the world. An estimated 400 million people worldwide are chronically infected by HBV and more than 500,000 people die of HBV-associated cirrhosis and HCC each year. Between 1.5 and 2 million U.S. residents have chronic HBV infection, and more than one billion dollars are spent each year on hepatitis B-related hospitalizations [1, 2].

The course of chronic hepatitis B can be generally divided into five stages: immune tolerance, immune active, inactive carrier, reactivation and immune clearance [3–5]. The immune tolerance phase is characterized by high levels of hepatitis B e antigen (HBeAg) and viral DNA in the circulation without liver inflammation or elevation of serum alanine aminotransferase (ALT). This phase could last for years or decades, followed by the immune active phase characterized by intermittent ALT elevations in addition to high levels of HBeAg and viral DNA. At the end of immune active phase, HBeAg becomes negative accompanied by the appearance of anti-HBe antibody, a process known as HBeAg seroconversion. After the seroconversion, most people enter the inactive carrier phase as defined by low or undetectable levels of viral DNA from serum (generally < 2000 IU or 10⁴ copies/ml), normalized ALT levels, and/or resolution of active necro-inflammation on liver biopsy. Thus, although some individuals in the inactive carrier state may later have reactivation and have HBeAg-negative (HBe–) hepatitis, HBeAg negativity is in general associated with reduced disease activity, while HBeAg positivity is associated with active viral replication, infectivity, and increased risk of disease progression. For these reasons, HBeAg seroconversion has attracted much attention as an important “landmark” of disease stage as well as a treatment end-point [6].

The precore G1896A mutation and/or the A1762T/G1764A double mutation in the basal core promoter (BCP) region have been studied extensively using the qualitative DNA sequencing technology. These mutants appeared to be associated with HBeAg seroconversion because they were more prevalent in HBeAg-negative patients than in the HBeAg-positive (HBe+) patients [7]. The precore G1896A mutation creates a premature stop codon that abolishes the synthesis of HBeAg, thus the dominance of this mutant could easily account for HBeAg seronegativity [8–10]. The BCP A1762T/G1764A double mutation can reduce the production of HBeAg by around 50% and thus may also contribute to HBeAg seroconversion [11–13]. However, the exact relationship between these mutant viruses and HBeAg seroconversion remains unknown. Although longitudinal studies were performed [14, 15], the findings were still limited to the correlation of mutants with seroconversion because the qualitative detection method (sequencing) used in the studies would not reveal the true dynamics of these HBV mutants. Since the precore G1896A and BCP A1762T/G1764A mutants make no or reduced level of HBeAg, they could escape the anti-HBe immunity and be selected passively due to a survival advantage [8]. On the other hand, these mutants may have replication advantage over the wild type (WT) viruses [11, 12, 16], and thus become dominant actively. These two hypotheses predict different patterns of mutant evolution which can only be determined by a quantitative analysis of the mutants.

Quantitative detection of HBV mutants was attempted [17, 18] but difficult due to the extensive nucleotide polymorphisms of the viral genome. We have recently overcome this technical difficulty and established reliable quantitative assays for the detection of the BCP A1762T/G1764A double mutation and the precore mutations (G1896A and G1896A/G1899A). These assays are highly sensitive, able to detect 0.001% mutant in the total viral population [19, 20]. These novel assays enabled us to determine mutant quantities in different patient populations as well as to trace the mutants longitudinally, before and after HBeAg seroconversion. Our data revealed a strong temporal correlation between the increase of mutant levels and HBeAg seroconversion. Therefore, the time of HBeAg seroconversion could be predicted by quantification of the HBV mutants; this might be useful in directing clinical management of chronic hepatitis B.

Patients and Methods

Patients

Samples tested were from the serum repository of the Liver Cancer Prevention Center at the Fox Chase Cancer Center, where 3754 HBV-infected individuals have been screened since 1982 in ethnic Asian communities in the Philadelphia area. Subjects who were identified as hepatitis B surface antigen (HBsAg) positive were offered further testing, free of charge, every 6 months for early detection of HCC. Serum samples from the initial screening and subsequent testing of those who remained in follow-up have been archived. The population has been described in more detail in the previous publications [21–23]. All subjects gave signed informed consent for participation in future studies of HBV and HCC. Sample collection, processing and storage were described previously [23]. HBeAg and anti-HBe were tested by EIA using commercial kits (Abbott Laboratories) [22]. For the cross-sectional study, 170 HBe+ and 30 HBe– samples were selected from the cohort based on the age (Table 1). Since this study is focused more on the mutant evolution before seroconversion, more samples and age groups were used for the HBe+ population. The HBe– samples were mainly used for population comparison purpose. In addition, serum samples collected sequentially from 18 individuals were used for longitudinal analysis. Each individual had 5–19 samples spanning 4–14 years of time. All the patients were treatment naïve and remained untreated during the total duration of this study.

DNA isolation from the patient serum samples and HBV genotyping

HBV viral DNA was isolated from 200 µl patient serum samples using the QIAamp DNA Blood Mini kit (Qiagen) and eluted into 50 µl Buffer EB according to manufacturers' instructions. HBV genotyping was performed according to Yeh *et al* 2004 [24].

HBV Mutant Quantification Assays and Mutant Designation

Detailed assay development and evaluation were described elsewhere [19, 20]. Briefly, a WT-selective PCR blocker was used in the first round PCR to suppress the amplification of the WT viral DNA but not the mutant DNA. Mutants amplified in the first round PCR were then quantified in a second round SimpleProbe real time PCR. Of all the potential mutations in the BCP region, only the A1762T/G1764A double mutation is designated here as the “BCP mutation”. The precore mutation assay could detect G1896A, G1899A and G1896A/G1899A mutations. Only the G1896A and G1896A/G1899A mutations are designated as the “precore mutations”. The G1899A mutation alone (in the absence of G1896A mutation) has not been reported to affect HBeAg production, thus it was not counted as the “precore mutation” in this study. HBV total viral load assay was virtually the same as the precore mutation assay except the PCR blocker was omitted [19]. A plasmid carrying both A1762T/G1764A and G1896A mutations was used as the concentration standard to ensure consistency in quantification in different assays.

Statistical Analysis

JMP software (version 8.0.2.2; SAS institute Inc) was used for statistical analysis. All the viral titer measurements underwent logarithmic transformation for statistic analysis, and a titer of zero was assigned a $\log_{10}$ value of 1. Wilcoxon-Mann-Whitney test was used for nonparametric comparison of means; $P < 0.05$ was considered significant. Significance of correlations was performed using Spearman's rho test. The scatter plot was used to show the range and distribution of values together with the Means Diamonds and 95% Confidence Intervals.

Results

Quantitative analysis of BCP and precore mutants in the HBe+ and HBe- populations

We first analyzed the mutant percentage in HBe+ and HBe- populations. Mutant percentage, the percentage of total viral load represented by the mutant virus, was calculated based on the measurement of the mutant titers and the total viral load. The total viral load was about 4 $\log_{10}$ copies/ml higher in the HBe+ population (mean 8.05 $\log_{10}$ copies/ml) than in the HBe- population (mean 3.38 $\log_{10}$ copies/ml, $P < 0.0001$); this is consistent with the published data [25, 26].

The percentage of the precore mutants was significantly higher in the HBe- (27.1%) than in the HBe+ population (10.8%; $P = 0.0236$). The precore mutant percentage also increased with age in both the HBe+ population (Spearman's rho = 0.1906, $P = 0.0128$) and the HBe- population (Spearman's rho = 0.4471, $P = 0.0132$) (Fig. 1B). The BCP mutant percentage showed a more obvious increase over the age groups in the HBe+ population (Spearman's rho = 0.3775, $P < 0.0001$; Fig. 1C). In the HBe- population, however, the increase in BCP mutant percentage was not statistically significant (0.1051, $P = 0.5806$). Thus, the average percentage of both BCP and precore mutants increased over time in the HBe+ population.

In the Age group 1 (1–9 years old), 24/28 (86%) HBe+ patients already had detectable precore mutants. One patient (a 7 year old) had 41% precore mutant; all the others had mutant percentage ranging from 0.001% to 5% (Table 2). The BCP mutants were more diverse, with only 9/28 (32%) had detectable mutant, but surprisingly 3 of them already had mutant as the dominant species (Table 2). Never the less, the mutant detection rate increased steadily with age, reaching 75% for the Age group 6. In comparison, the positive detection rate for the precore mutant stayed around 90% in all age groups (Table 3).

Seroconversion preceded by significant increase of precore and/or BCP mutants

To gain more insight into the evolution of the HBV mutants in a given individual, we followed the percentage of the mutants retrospectively over the course of 4–14 years in 18 patients. Thirteen of them experienced seroconversion, and all of them showed various degrees of increase in the percentage of precore and/or BCP mutants around the time of seroconversion. Out of the 13 seroconverters, 4 showed increase of precore mutant only, 3 had BCP mutant only, and 6 had an increase of both precore and BCP mutants. Nine cases are shown in Fig. 2. In the case #1 (Fig. 2A), HBeAg seroconversion was preceded by a steady increase of precore mutant percentage, from 0.8% to 78%, in about 4 years. Similarly in the case #2 (Fig. 2B), seroconversion was preceded by a marked increase of precore mutant percentage, from 0.04% to 82%, in less than 3 years. When HBeAg first became negative and anti-HBe became positive for this patient, 100% viruses had the precore mutation. Fig. 2D is a representative case in which seroconversion was accompanied by a significant increase of the BCP mutant only. In the cases where the percentage of both precore and BCP mutants increased significantly before the seroconversion, the two mutants may evolve separately (not in the same genome), such as the one shown in Fig. 2E, or

concurrently (Fig. 2F), suggesting that both mutants were in the same genome. Interestingly, one case showed a reversion back to HBeAg-positive status after the initial seroconversion, followed by a second seroconversion (Fig. 2I). In both incidences, seroconversion was accompanied by a marked increase of BCP mutant percentage. The increase of mutant percentage was more or less uni-directional except in one case (Fig. 2E) that the BCP mutant percentage had a significant drop before the seroconversion. In this case, however, the percentage of the precore mutant was already high, which made the increase of BCP mutant “redundant”.

We further asked the question whether a significant increase of mutant percentage would occur without seroconversion. To this end, we analyzed the mutant percentage in 5 individuals who remained HBeAg-positive during the entire 4.5–14 years of follow up. The percentage of the mutants was below 10% at all times in these individuals, except in three cases (Fig. 3B, 3C and 3D), where a significant increase of precore and/or BCP mutant percentage was observed in the last few available samples. It was possible that seroconversion occurred afterwards in these patients. This data provided further support of our hypothesis that a significant percentage of mutant (>10 to 20%) in the HBeAg-positive phase signals that the seroconversion is underway.

The mutant stable phase

In the pre-seroconversion samples, before the mutant percentage increased significantly, the mutant percentage was low (usually in the range of 0.1% to 1%) and stable for many years. For example, the precore mutant was stable at a level around 0.5% for three years before the marked increase (Fig. 2B). In Fig. 3A, 3B, 3D and 3E, the mutant levels were not significantly changed over a period of 6 to 9 years. Thus, there appeared to be a “mutant stable phase” during mutant evolution.

Mutant percentage increase preceded or accompanied by ALT elevation

Previous population (cross-sectional) studies pointed out association of ALT elevation with BCP or precore mutants, and suggested that these mutants may be more virulent than the WT virus [27–29]. Our cross-sectional analysis is consistent with the published results. Out of 170 HBe+ individuals, 7 had ALT levels above 150 U/L, and they were all distributed in the older age groups (data not shown). Spearman correlation analysis showed that the level of ALT, like the percentage of the mutants, increased with age (0.3316, $P < 0.0001$) in the HBe+ population. However, ALT levels only significantly correlated with the percentage of the BCP mutant (0.2884, $P = 0.0003$), but not with precore mutant percentage. In the longitudinal analysis, ALT elevation could occur before or during mutant percentage increase. However, a high percentage of mutants was not always accompanied by an ALT elevation (Fig. 2 and Fig. 3).

Mutant percentage varies after seroconversion

After the seroconversion, the percentage of precore or BCP mutant could remain high (Fig. 2C) or fluctuate dramatically; it sometimes could drop to as low as 20% (Fig. 2H) or even less than 1% (Fig. 2G). In Fig. 2E, both BCP and precore mutants reached 100% around the time of seroconversion, but the precore mutants decreased to ~70% and the BCP mutants decreased to about 20%; mutants below 20% would be undetectable by DNA sequencing. A summary of the three different mutant evolution phases around the time of seroconversion is shown in Figure 4A.

Prediction of HBeAg seroconversion by mutant percentage levels

The above data indicate that an increase of percentage of precore and/or BCP mutants could predict a near term HBeAg seroconversion. To assess the correlation quantitatively between the mutant percentage and the time required to reach seroconversion, we performed a spearman correlation analysis. The mid-time between the last HBeAg-positive sample and the first HBeAg-negative sample was taken as the assumed time point of seroconversion (the dashed lines in Fig. 2), and this was used to calculate how much time was needed to reach seroconversion. The data are listed in Fig. 4B and 4C. There was a significant correlation between the mutant percentage and the time to seroconversion. The Spearman's rho was -0.5771 ($p = 0.0077$) for the precore mutant, and -0.4863 ($p = 0.0031$) for the BCP mutant. The linear regression on the graph indicated that the higher the mutant percentage, the less time was needed to reach seroconversion. The precore mutant, once appeared, took less time to have seroconversion than the BCP mutant.

Discussion

In this study, the quantities of the HBV precore and BCP mutants were analyzed both cross-sectionally and longitudinally to gain better understanding of how these mutants evolve over time. The longitudinal follow-up revealed that mutant evolution followed three distinct phases around the time of seroconversion: the stable and the escalation phases before seroconversion and the variable phase after seroconversion (Fig. 4A). This pattern of mutant evolution has several important implications.

First, our study provided strong evidence that there is a significant temporal correlation between the increase of mutant percentage and HBeAg seroconversion. All the 13 cases showed a more or less unidirectional increase of the mutants prior to seroconversion, and none of the 5 non-converters showed any significant levels of mutants. Therefore, a significant level of precore or BCP mutant may be predictive of an approaching seroconversion. In fact, the time required to reach seroconversion may be predicted based on the level of the mutants (Fig. 4B and 4C). We understand that 18 sets of samples may not be statistically strong enough to draw any clear-cut conclusions. On the other hand, these serial samples covering many years of time are not readily available. We are therefore tempted to present our hypothesis for future testing.

Second, a stable phase of mutant evolution was identified before the mutant escalation phase. This stable phase implies that the G1896A or A1762T/G1764A mutation *per se* may not confer replication advantage over the wild type virus as indicated by the earlier *in vitro* studies [11, 12, 16]. Thus, our data does not support the hypothesis that these mutants became dominant prior to seroconversion because they have “replication advantage”. In fact, the replication advantage was not supported by other *in vitro* measurement of replication [30, 31]. Furthermore, there is in most cases a decrease in total viral load after the seroconversion, and it will be difficult to explain this viral titer decrease when the dominant mutant viruses have enhanced replication capability. The decrease of the mutant percentage after the seroconversion, found in this study and another [18], also argues against this hypothesis.

However, this study and others indicated that these mutants are indeed frequently associated with ALT elevation. This phenomenon was used to argue that these mutants may be more virulent than the wild type virus, and was possibly associated with their “enhanced” replication [8, 32]. However, these mutants could exist in significant amount (detectable by sequencing) in inactive carriers who have no apparent liver injuries [33–36]. It is important to notice that in our study the percentage increase of mutants was often preceded by ALT elevation, a hall mark of the immune active stage. It therefore appears more likely that due

to the change in the host immune system in the late HBeAg-positive phase, the precore and/or BCP mutants were selected passively shortly before seroconversion due to their survival advantage. This hypothesis may explain why the mutants could coincide with older age and ALT elevations. It also explains why the precore mutants were selected more quickly than the BCP mutants (Fig. 4B and 4C) because the precore mutants do not produce HBeAg at all and would have more survival advantage compared with the BCP mutants. Because the BCP mutants do not “trigger” seroconversion as effectively as the precore mutants, patients with high levels of BCP mutants can be “accumulated” in the older HBe+ age groups (Fig. 1C).

The survival advantage hypothesis implies that the anti-HBe immunity may be the selection force as both mutants have diminished HBeAg production thus could escape the anti-HBe immunity. However, even in the presence of anti-HBe immunity, as evidenced by the anti-HBe antibody, the dominance of both precore and BCP mutants could diminish significantly after seroconversion. The reason for this decline is not clear, but it indicates that the HBe antibody may not be the primary selection force of these mutants. The cellular immunity against HBeAg or the related core antigen (due to overlapping amino acid sequence) might play a more important role in the selection of these mutants. Further study is needed to clarify the role of cellular immunity in HBeAg seroconversion. It is also unclear why the patients remained HBeAg negative even when the mutant percentage decreased to below 20% (that is, the wild type, presumably HBeAg-producing viruses are dominant). One possibility is that after the seroconversion, the anti-HBe immunity is in upper hand, and thus even HBeAg could be produced, the antigen may be masked by the excessive antibodies. Or, there may be other mutations in these “wild type” viruses that prevent them from making HBeAg. Lastly, we are interested to see whether, after seroconversion, the ones with persistently high levels of mutants will be more likely to have HBe-negative hepatitis, while the ones with low levels of mutants may correlate with the inactive state. This is supported by our limited data that the percentage of the precore mutant (not BCP mutant) positively correlated with ALT elevation in the HBe- negative cross-sectional samples (data not shown). However, conclusive evidence regarding the role of these mutants in HBe-negative hepatitis will have to be obtained using a specific cohort designed for this purpose. Lastly, due to the limitation of our cohort (Asian population), the viral genotype was found to be either B or C. Thus our conclusions regarding the evolution of the mutant HBVs are limited to these genotypes.

In conclusion, our study represents the first systematic and quantitative analysis of the dynamics of precore and BCP mutants. Our data revealed a strong temporal correlation between seroconversion and a significant increase in the percentage of precore and/or BCP mutants. Thus, a quantitative measurement of these mutants can help to predict when HBeAg seroconversion may occur. This may have significant impact on the clinical management of chronic hepatitis B. For example, an HBe+ patient with high levels of viral DNA and elevated ALT but no significant level of precore/BCP mutant should probably better be on an antiviral therapy. However, if the patient also presents with a significant level of precore/BCP mutant, should the treatment be initiated, or postponed because the chance of an inactive disease state is 70–80% after seroconversion? Further, was the “treatment-induced” HBeAg seroconversion truly induced by treatment or the patient was about to undergo a “spontaneous” seroconversion anyway? Quantification of these mutants prior to treatment may help clarify this question. Additional clinical studies are warranted to substantiate the utility of the quantitative information of the precore and BCP mutants in patient management.

Abbreviations

HBV	hepatitis B virus
HCC	hepatocellular carcinoma
ALT	alanine aminotransferase
BCP	basal core promoter
HBe+	HBeAg positive
HBe–	HBeAg negative

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References

1. Sorrell MF, Belongia EA, Costa J, et al. National Institutes of Health consensus development conference statement: management of hepatitis B. *Hepatology*. 2009; 49:S4–S12. [PubMed: 19399804]
2. Kim WR. Epidemiology of hepatitis B in the United States. *Hepatology*. 2009; 49:S28–S34. [PubMed: 19399791]
3. Fattovich G. Natural history and prognosis of hepatitis B. *Semin Liver Dis*. 2003; 23:47–58. [PubMed: 12616450]
4. McMahon BJ. The natural history of chronic hepatitis B virus infection. *Hepatology*. 2009; 49:S45–S55. [PubMed: 19399792]
5. Shi YH, Shi CH. Molecular characteristics and stages of chronic hepatitis B virus infection. *World J Gastroenterol*. 2009; 15:3099–3105. [PubMed: 19575488]
6. Liaw YF. HBeAg seroconversion as an important end point in the treatment of chronic hepatitis B. *Hepatol Int*. 2009; 3:425–433.
7. Chu CJ, Keeffe EB, Han SH, et al. Prevalence of HBV precore/core promoter variants in the United States. *Hepatology*. 2003; 38:619–628. [PubMed: 12939588]
8. Carman WF, Jacyna MR, Hadziyannis S, et al. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet*. 1989; 2:588–591. [PubMed: 2570285]
9. Akahane Y, Yamanaka T, Suzuki H, et al. Chronic active hepatitis with hepatitis B virus DNA and antibody against e antigen in the serum. Disturbed synthesis and secretion of e antigen from hepatocytes due to a point mutation in the precore region. *Gastroenterology*. 1990; 99:1113–1119. [PubMed: 2394332]
10. Brunetto MR, Giarin MM, Oliveri F, et al. Wild-type and e antigen-minus hepatitis B viruses and course of chronic hepatitis. *Proc Natl Acad Sci U S A*. 1991; 88:4186–4190. [PubMed: 2034663]
11. Buckwold VE, Xu Z, Chen M, et al. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. *J Virol*. 1996; 70:5845–5851. [PubMed: 8709203]
12. Scaglioni PP, Melegari M, Wands JR. Biologic properties of hepatitis B viral genomes with mutations in the precore promoter and precore open reading frame. *Virology*. 1997; 233:374–381. [PubMed: 9217060]
13. Tong S, Kim KH, Chante C, et al. Hepatitis B Virus e Antigen Variants. *Int J Med Sci*. 2005; 2:2–7. [PubMed: 15968333]
14. Chen CH, Lee CM, Hung CH, et al. Clinical significance and evolution of core promoter and precore mutations in HBeAg-positive patients with HBV genotype B and C: a longitudinal study. *Liver Int*. 2007; 27:806–815. [PubMed: 17617124]

15. Jang JW, Lee YC, Kim MS, et al. A 13-year longitudinal study of the impact of double mutations in the core promoter region of hepatitis B virus on HBeAg seroconversion and disease progression in patients with genotype C chronic active hepatitis. *J Viral Hepat.* 2007; 14:169–175. [PubMed: 17305882]
16. Moriyama K, Okamoto H, Tsuda F, et al. Reduced precore transcription and enhanced core-pregenome transcription of hepatitis B virus DNA after replacement of the precore-core promoter with sequences associated with e antigen-seronegative persistent infections. *Virology.* 1996; 226:269–280. [PubMed: 8955047]
17. Brunetto MR, Randone A, Ranki M, et al. Quantitative analysis of wild-type and HBeAg minus hepatitis B viruses by a sequence-dependent primer extension assay. *J Med Virol.* 1994; 43:310–315. [PubMed: 7931193]
18. Pang A, Yuen MF, Yuan HJ, et al. Real-time quantification of hepatitis B virus core-promoter and pre-core mutants during hepatitis E antigen seroconversion. *J Hepatol.* 2004; 40:1008–1017. [PubMed: 15158343]
19. Nie H, Evans AA, London WT, et al. Quantification of complex precore mutations of hepatitis B virus by SimpleProbe real time PCR and dual melting analysis. *J Clin Virol.* 2011; 51:234–240. [PubMed: 21665330]
20. Nie H, Evans AA, London WT, et al. Ultrasensitive Quantification of Hepatitis B Virus A1762T/G1764A Mutant by a SimpleProbe PCR Using a Wild-Type-Selective PCR Blocker and a Primer-Blocker-Probe Partial-Overlap Approach. *J Clin Microbiol.* 2011; 49:2440–2448. [PubMed: 21562108]
21. London, WT.; Atleson, J.; Eto, T., et al. Early detection of hepatocellular carcinoma among Asians living in the United States. Houston: Portfolio Publishing Company; 1991.
22. Evans AA, Fine M, London WT. Spontaneous seroconversion in hepatitis B e antigen-positive chronic hepatitis B: implications for interferon therapy. *J Infect Dis.* 1997; 176:845–850. [PubMed: 9333140]
23. Evans AA, O'Connell AP, Pugh JC, et al. Geographic variation in viral load among hepatitis B carriers with differing risks of hepatocellular carcinoma. *Cancer Epidemiol Biomarkers Prev.* 1998; 7:559–565. [PubMed: 9681522]
24. Yeh SH, Tsai CY, Kao JH, et al. Quantification and genotyping of hepatitis B virus in a single reaction by real-time PCR and melting curve analysis. *J Hepatol.* 2004; 41:659–666. [PubMed: 15464248]
25. Chu CJ, Hussain M, Lok AS. Quantitative serum HBV DNA levels during different stages of chronic hepatitis B infection. *Hepatology.* 2002; 36:1408–1415. [PubMed: 12447866]
26. Volz T, Lutgehetmann M, Wachtler P, et al. Impaired intrahepatic hepatitis B virus productivity contributes to low viremia in most HBeAg-negative patients. *Gastroenterology.* 2007; 133:843–852. [PubMed: 17854594]
27. Fang ZL, Sabin CA, Dong BQ, et al. The association of HBV core promoter double mutations (A1762T and G1764A) with viral load differs between HBeAg positive and anti-HBe positive individuals: a longitudinal analysis. *J Hepatol.* 2009; 50:273–280. [PubMed: 19070921]
28. Omata M, Ehata T, Yokosuka O, et al. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. *N Engl J Med.* 1991; 324:1699–1704. [PubMed: 2034246]
29. Ehata T, Omata M, Chuang WL, et al. Mutations in core nucleotide sequence of hepatitis B virus correlate with fulminant and severe hepatitis. *J Clin Invest.* 1993; 91:1206–1213. [PubMed: 8450049]
30. Gunther S, Piwon N, Will H. Wild-type levels of pregenomic RNA and replication but reduced pre-C RNA and e-antigen synthesis of hepatitis B virus with C(1653) -->T, A(1762) --> T and G(1764) --> A mutations in the core promoter. *J Gen Virol.* 1998; 79((Pt 2)):375–380. [PubMed: 9472623]
31. Jammeh S, Tavner F, Watson R, et al. Effect of basal core promoter and pre-core mutations on hepatitis B virus replication. *J Gen Virol.* 2008; 89:901–909. [PubMed: 18343830]
32. Sato S, Suzuki K, Akahane Y, et al. Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. *Ann Intern Med.* 1995; 122:241–248. [PubMed: 7825758]

33. Okamoto H, Tsuda F, Akahane Y, et al. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. *J Virol.* 1994; 68:8102–8110. [PubMed: 7966600]
34. Lok AS, Akarca U, Greene S. Mutations in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal. *Proc Natl Acad Sci U S A.* 1994; 91:4077–4081. [PubMed: 8171038]
35. Akarca US, Greene S, Lok AS. Detection of precore hepatitis B virus mutants in asymptomatic HBsAg-positive family members. *Hepatology.* 1994; 19:1366–1370. [PubMed: 8188166]
36. Laskus T, Rakela J, Nowicki MJ, et al. Hepatitis B virus core promoter sequence analysis in fulminant and chronic hepatitis B. *Gastroenterology.* 1995; 109:1618–1623. [PubMed: 7557146]

Fig. 1. The total viral loads and mutant percentages in HBeAg-positive and -negative populations
Total HBV DNA titers transformed by log10 were plotted against different age groups (see Table 1) that are either HBeAg-positive (e+) or HBeAg-negative (e-) (A). The distribution and summary of precore mutant percentage (B) and BCP mutant percentage (C) are also shown. The means diamond indicates the mean (the line cross the diamond) and the 95% confidence interval (the vertical span of the diamond). The short horizontal lines flanking the means are overlap marks computed as $0.3536 \times$ confidence interval above and below the mean.

Fig. 2. Longitudinal analysis of precore and BCP mutants in 9 individuals who underwent seroconversion

Shown in each case, from top to bottom, are gender and HBV genotype, ALT status (ALT levels >40 U/L are shown by an upward triangle, while ALT ≤ 40 U/L are shown by a downward triangle), Log₁₀ total viral load (open square line graph), and mutant percentage (open circle for the precore mutant and solid circle for the BCP mutant). The data is plotted against the patient's age at which the sample was taken. The vertical dashed lines are in between the last HBeAg-positive sample and the first HBeAg-negative sample, and indicate the presumed time of seroconversion. The last case (I) had a second seroconversion. The HBeAg-positive samples before the second seroconversion are indicated by asterisks.

Fig. 3. Longitudinal analysis of precore and BCP mutants in 5 individuals who did not undergo seroconversion

Shown in each case, from top to bottom, are gender and HBV genotype, ALT status (ALT levels >40 U/L are shown by an upward triangle, while ALT ≤ 40 U/L are shown by a downward triangle), Log₁₀ total viral load (open square line graph), and mutant percentage (open circle for the precore mutant and solid circle for the BCP mutant). The data is plotted against the patient's age at which the sample was taken.

A **B** **C**

Fig. 4. Temporal correlation between an increase of mutant percentage and seroconversion
A, Evolutionary patterns of HBV precore and BCP mutants before and after HBeAg seroconversion. **B** and **C**, Correlation between the mutant percentage levels and the time of seroconversion. The data points were extracted from the longitudinal study of 13 cases in which seroconversion occurred. Linear regressions are also shown.

Table 1

Serum samples in different age groups

Age Group	Age	Number of individuals	
		HBeAg+	HBeAg–
1	1–9	28	
2	10–19	28	10
3	20–29	27	
4	30–39	29	10
5	40–49	30	
6	50–	28	10
Total		170	30

Table 2

Mutant and total viral titers in 28 young HBe+ patients

	Age	Gender	Total viral load (copies/ml)	Precore mutant (copies/ml)	Precore mutant (%)	BCP mutant (copies/ml)	BCP mutant (%)	ALT (U/L)	HBV Genotype
1	1	F	5.70E+08	3.02E+04	0.005	0	0	46	C
2	2	M	5.25E+06	3.74E+03	0.071	4.39E+03	0.084	18	C
3	3	M	6.45E+08	6.53E+05	0.101	0	0	13	B
4	3	F	2.19E+08	0	0	0	0	33	C
5	4	M	8.55E+07	1.61E+05	0.188	8.53E+07	99.749	49	C
6	4	M	3.51E+09	9.80E+07	2.797	1.15E+07	0.329	10	C
7	4	M	2.64E+07	7.84E+02	0.003	2.71E+07	100.000	8	C
8	5	F	2.32E+09	8.35E+07	3.599	0	0	24	C
9	5	M	3.05E+07	1.34E+06	4.404	0	0	91	C
10	5	M	1.29E+09	0	0	0	0	28	C
11	6	F	3.21E+08	6.37E+06	1.986	8.20E+04	0.026	8	C
12	6	F	1.93E+08	6.94E+05	0.360	0	0	34	B
13	6	M	3.29E+08	2.70E+05	0.082	0	0	22	C
14	7	M	9.95E+08	4.07E+08	40.905	0	0	23	C
15	7	M	1.01E+09	0	0	0	0	21	C
16	7	M	2.77E+07	0	0	0	0	9	B
17	8	F	1.79E+09	1.00E+07	0.560	0	0	8	C
18	8	F	2.88E+06	2.24E+03	0.001	2.90E+06	100.000	18	C
19	8	M	2.85E+09	9.21E+06	0.323	0.00E+00	0	47	C
20	8	M	2.64E+07	1.72E+04	0.065	9.36E+03	0.036	26	C
21	8	M	9.35E+07	1.65E+05	0.177	0	0	24	C
22	8	M	5.30E+08	6.26E+05	0.118	0	0	20	C
23	8	F	3.22E+08	1.89E+05	0.059	3.29E+06	1.022	19	C
24	9	M	8.65E+08	1.28E+07	1.479	3.75E+04	0.004	9	C
25	9	F	4.39E+08	8.47E+06	1.932	0	0	20	C
26	9	F	1.59E+07	7.60E+05	4.795	0	0	42	C

	Age	Gender	Total viral load (copies/ml)	Precore mutant (copies/ml)	Precore mutant (%)	BCP mutant (copies/ml)	BCP mutant (%)	ALT (U/L)	HBV Genotype
27	9	M	3.02E+08	2.90E+05	0.096	0	0	17	C
28	9	F	6.05E+08	1.20E+07	1.983	0	0	13	B

Table 3

Positive mutant detection rate in different age groups

	HBeAg positive					HBeAg-negative		
Age group	1	2	3	4	5	6	2	4
Precore mutants	85.7	92.9	96.3	82.8	100	78.6	0	60
BCP mutant	32.1	39.3	55.6	69.0	76.7	75.0	0	40
								20

Positive detection cut off was 0.001%. Most cases in the HBeAg-negative age group 2 had very low total viral load (see Fig. 1A) which affected mutant detection.