

Longitudinal Study on Mutation Profiles of Core Promoter and Precore Regions of the Hepatitis B Virus Genome in Children

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ABSTRACT

Precore nucleotide 1896 and core promoter mutations may account for hepatitis B e antigen (HBeAg) seroconversion in chronic hepatitis B virus (HBV) infection, yet the mutational profiles of the core promoter are largely unknown in children. An age-matched, case-control study enrolled 110 chronic HBV-infected children, including 55 HBeAg seroconverters and 55 nonseroconverters. Precore and core promoter genes of HBV were sequenced and the serum viral genomes were genotyped from three serial serum samples of the seroconverters and from one serum sample of the nonseroconverters. Higher frequency of A1775G and G1799C mutation rates and lower frequency of A1752G mutation rate were found in the seroconverters. Precore 1896 mutation appeared more in seroconverters than in nonseroconverters (45.5% versus 10.9%; $p < 0.001$). 1762 + 1764 mutation rates were not different between the seroconverters (9.1%) and the nonseroconverters (5.5%). Genotype B was the

major type. Genotype C was associated with core promoter 1762 + 1764 mutations in the seroconverter group ($p = 0.023$). The conclusions of this study include the following: 1) mutations of core promoter at nucleotide position 1752, 1775, and 1799 have significant correlations with HBeAg seroconversion; 2) core promoter 1762 + 1764 mutations play a minimal role in HBeAg seroconversion; 3) precore 1896 mutant accounted for half of childhood HBeAg seroconversion; 4) genotype C is associated with 1762 + 1764 mutations during the process of HBeAg seroconversion. (*Pediatr Res* 56: 396–399, 2004)

Abbreviations

HBeAg, hepatitis B e antigen
HBV, hepatitis B virus
HBsAg, hepatitis B surface antigen

Hepatitis B e antigen (HBeAg), coded by the precore and core genes, is an important infectivity marker in hepatitis B virus (HBV) infection. HBeAg has been suggested to be a target of host cytotoxic T cell, and its disappearance may suggest an immune clearance of HBV (1). Seroconversion from HBeAg antigenemia to anti-HBe may occur spontaneously in the natural course of chronic HBV infection. This process usually suggests that HBV breaks through the immune tolerance and enters into a low replication phase (2). The factors that affect the rate of HBeAg seroconversion, including the age of the patients and maternal hepatitis B markers, were identified in our previous study (3). The G to A transition at nucleotide position 1896 (nucleotide position according to *EcoRI* site) was thought previously to be associated with HBeAg seroconversion. This mutation results in a stop codon

for the precore open reading frame (4) and was suggested to induce HBeAg loss and promote HBeAg seroconversion (5, 6). However, this mutation does not exist in every case of HBeAg seroconversion (7, 8).

Our previous long-term follow-up pediatric study revealed that the 1896 mutant and wild types could coexist before and after HBeAg seroconversion. Approximately half of the carrier children had no such mutant throughout the HBeAg seroconversion process (9). Some mechanisms other than 1896 precore mutant might account for HBeAg seroconversion.

Core promoter mutations, particularly an A to T mutation at 1762 and a G to A mutation at 1764 nucleotide position (1762 + 1764 mutations), have drawn much attention in the mechanism of HBeAg seroconversion in adults (10). Core promoter has been mapped to the nucleotide position 1744–1851 and regulates the transcription of both precore and core RNAs (11). Core promoter mutations may reduce precore transcription (12), thus leading to the loss of serum HBeAg and resulting in HBeAg seroconversion (13).

Little is known about these mutations in children (14). Previous studies were generally small in scale and lacked a

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control group; thus, we do not know the background information of these mutants (15). Age is a known important factor for HBeAg seroconversion (3). We thus performed a large-scale, case-control, and age-matched study to minimize this factor and focus on clarifying the role of core promoter mutations in children.

METHODS

Subjects. A total of 415 children with chronic HBV infection were followed-up prospectively every 6 mo at our outpatient clinic with their parents' consent. Among the 415 children, 169 had already undergone HBeAg seroconversion. HBeAg seroconversion was defined as the conversion from HBeAg seropositive to anti-HBe positive at two different time points. The timing of HBeAg seroconversion was arbitrarily assigned to be the midpoint between these two different time points. Among the aforementioned 169 subjects, 55 children (M:F = 34:21) were documented to undergo HBeAg seroconversion during follow-up and were followed up ≥ 2 y after HBeAg seroconversion. They were also free from other concurrent medical illness. They thus were enrolled as the subjects of this study. All of these 55 children were asymptomatic clinically. Their mean age at enrollment was 6.1 ± 4.6 y, 11.2 ± 4.1 y (mean \pm SD; range: 3.7–23.5 y) at HBeAg seroconversion, and 16.4 ± 5.8 y (5.6–32.0 y) at the last follow-up. For each case in this group, three blood samples were subjected to study: the first one was taken < 6 mo before HBeAg seroconversion, the second one was within 6 mo after HBeAg seroconversion, and the latest available one was an average 5.2 ± 3.5 y after HBeAg seroconversion. Maternal HBsAg was positive in 34 cases, negative in 20 cases, and unknown in 1 case.

Another 55 HBsAg carrier children with persistent HBeAg antigenemia formed the control group. The subjects of the control group were selected on the basis of their age and sex. Their mean age at enrollment was 5.5 ± 4.2 y, and the age at the latest check-up was 12.0 ± 3.7 y (2.6–21.6 y). The latter was matched with the seroconverters' age at seroconversion. The control group also has the same sex distribution as the study group. The presumed mode of acquisition was also matched (maternal HBsAg positive in 39 cases, negative in 14 cases, and unknown in 1 case). Their latest available serum samples were analyzed in this study. All 55 children were asymptomatic clinically, too.

All subjects were not treated with interferon or other antiviral agent during the study period. None of them had coexisting infection of hepatitis C or hepatitis D or HIV.

Serum hepatitis B markers and biochemical test. Hepatitis B surface antigen (HBsAg), anti-HBs, HBeAg, anti-HBe, and anti-HBc were tested by RIA kit (Abbott, Abbott Park, IL, U.S.A.). The liver function profile was determined by an autoanalyzer (Hitachi 736, Tokyo, Japan).

Nested PCR to amplify the core promoter region of HBV genome. DNA was extracted from 20 μ L of serum by the standard SDS-proteinase K/phenol/chloroform method. The extracted DNA was then subjected to PCR as the template. Nested PCR was performed using the primers and procedures

previously described (16). The sensitivity of our nested PCR was 10^{-5} pg of HBV DNA. Strict rules were followed to avoid PCR contamination (17).

Sequencing of PCR products. Cyclic sequencing was performed using ABI prism dye-labeled terminators and cycle sequencing ready reaction kit. The procedure was done mainly according to the manufacturer's instructions (Perkin Elmer, Foster City, CA, U.S.A.). Briefly, dye terminator, dNTP, Tris-HCl (pH 9.0), $MgCl_2$, thermal stable pyrophosphatase, AmpliTaq DNA polymerase, and M13 primer (0.8 pmol/L) mixed with 2 μ L of template (0.2 μ g/ μ L), and distilled water mixed to a total of 20 μ L. The mixture was then processed to the reaction in GeneAmp PCR system 2400 (Perkin Elmer), after which it was loaded and read by the sequencer. For preventing the artifacts of point mutations created by PCR, the sequencing reactions were done in both directions. All of the tests were duplicated to confirm the results. The entire study protocol was approved by the National Science Council, Taiwan.

Determination of HBV genotypes by nested PCR. All of the serum samples were checked for HBV genotypes. The genotype analysis strategy was to adapt the method of PCR using type-specific primers (18). When the results were not interpretable after double check, we applied the restriction fragment length polymorphism method developed by Lindh *et al.* (19). The nested PCR sensitivity was 10^{-5} pg of HBV DNA. All experiments were done at least in duplicate to confirm the results.

RESULTS

Clinical outcome. For the HBeAg seroconverter group, 47 of 55 had abnormal ALT changes (normal range of ALT < 40 U/L) in the process of HBeAg seroconversion. Conversely, 3 of 55 of the subjects in the nonseroconverter group had abnormal ALT. Peak ALT levels, which were the highest ALT levels during the follow-up, were different between the two groups (median peak ALT levels of seroconverter group *versus* nonseroconverter group: 77, range 1–253 U/L *versus* 9, range 2–150 U/L; $p < 0.0001$, Mann Whitney *U* test). When we compared the ALT of the postseroconversion serum sample (the third serum sample) of the seroconverter group with that of the nonseroconverter group, there was no difference (median ALT of the third serum sample of seroconverter group *versus* nonseroconverter group: 9, range 2–109 U/L *versus* 9, range 2–150 U/L; $p < 0.001$, Mann Whitney *U* test). Only two cases with ALT higher than $1.5 \times$ upper normal limit (> 60 U/L) were found in the third serum samples. Both bore no core gene mutations.

DNA sequence variations of the core promoter region and precore region. Nucleotide positions 1804–1845 were a relatively conserved region with no mutation found. Core promoter nucleotide positions 1752, 1775, and 1799 had significantly different mutation rates between the seroconverters and nonseroconverters after HBeAg seroconversion (Table 1).

When we considered the presence of G1896A mutation in any one of the three serum samples of the seroconverter group, we found this mutation appeared in 25 (45.5%) of 55 of the seroconverters during the follow-up course, whereas 6 of them

Table 1. Mutation at HBV core promoter and precore regions in HBeAg seroconverter and non-seroconverter groups

Nucleotide	Mutation	Nonseroconverters	Seroconverters		
			First	Second	Third
1752	A→G	36.4%	25.5%	7.3%*	1.8%*
1762/1764	A/G→T/A	5.5%	5.5%	7.3%	9.1%
1773	C→T	3.6%	10.9%	14.6%	12.7%
1775	A→G	3.6%	1.8%	9.1%	32.7%*
1799	G→C	29.1%	29.1%	65.5%*	81.8%*
1802-3	TT→CG	3.6%	7.3%	9.1%	9.1%
1846	A→T	7.3%	16.4%	18.2%	20.0%
1850	T→A	3.6%	9.1%	12.7%	18.2%
1896	G→A	9.1%	25.5%*	29.1%*	34.5%*

For each case in the HBeAg seroconverted group, three blood samples were subjected to study: the first one was taken before HBeAg seroconversion (<6 mo), the second was after HBeAg seroconversion (>6 mo), and the latest available one averaged 5.2 ± 3.5 y after HBeAg seroconversion. The first blood samples (before seroconversion) were compared with the nonseroconverted group, whereas the second and third blood samples (after seroconversion) were compared with the first blood samples.

* $p < 0.05$ (Fisher's exact test or χ^2 test with Yate's correction).

had this mutant reverted back to the wild type in the third serum sample. AG1762/1764TA was found in 6 (10.9%) of 55 of the seroconverters in any of the three serum samples, whereas 1 of them had this mutant reverted back to the wild type in the third serum sample.

Mutations of core promoter and precore region other than 1896 and 1762 + 1764 could be found in 19 (34.5%) of 55 of the seroconverter group. Only five (9%) in this group had neither precore G1896A mutation nor core promoter mutations in this study.

Only a minority of the nonseroconverters were with C1858 (3.6%; 2 of 55), whereas 32.7% (18 of 55) of the seroconverters were found to have C1858 ($p < 0.001$). Core promoter mutations of 1762 + 1764 did not correlate with C1858. All children with 1896 precore stop codon mutation were exclusively infected with T1858 HBV.

Genotype and precore and core promoter mutations. In the seroconverter group, 25 cases were ever associated with precore 1896 mutation; 21 were genotype B, and 4 were genotype C, whereas for the 30 cases without this mutation, 26 were genotype B and 4 were genotype C. The genotype distribution pattern is not different between those with and without precore 1896 mutation ($p = 0.78$, Fisher's exact test). Seven cases had core promoter 1762 + 1764 mutations (genotype B:genotype C = 4:3). Compared with the other 41 cases without such mutations in the seroconverters (genotype B:genotype C = 43:5), genotype C had a significantly higher association with core promoter 1762 + 1764 mutations ($p = 0.023$).

The nonseroconverter group consisted of only five cases with precore 1896 mutation and four were genotype B and 1 was genotype C, also not different from the data of those without precore 1896 mutation (4:1 versus 40:10; $p = 1$). Even fewer cases were associated with core promoter 1762 + 1764 mutations: two were genotype B and one was genotype C. It was also not significantly different from those without core promoter 1762 + 1764 mutation (2:1 versus 42:10; $p = 0.55$).

DISCUSSION

This study provides a good chance to focus on the viral mutation impacts in the event of HBeAg seroconversion. Pre-

core 1896 mutation was proposed to be one of the mechanisms to explain HBeAg seroconversion in the natural history of chronic HBV infection. However, there were still a proportion of patients who underwent HBeAg seroconversion without this mutation. In this study, we showed that 1896 mutations did appear more in the seroconverters. This precore mutation accounts for approximately half of the HBeAg seroconversions. Core promoter 1762 + 1764 mutations were reported to be associated with chronic hepatitis and may be a possible mechanism for HBeAg clearance in adults (20), because the mutations negate HBeAg expression and lead to HBeAg seroconversion. However, the data in this study showed that the mutation profile of 1762 + 1764 before seroconversion in the seroconverters was as low as in the nonseroconverters. This may suggest a minimal role of 1762 + 1764 mutations in childhood HBeAg seroconversion. However, we found that genotype C had an association with the core promoter mutation in this childhood study. This finding is similar to the adult study (21).

In this study, we demonstrated that the percentage of patients with core promoter mutations in A1752G, A1775G, and G1799C are statistically different before and after HBeAg seroconversion. The proportions of mutant virions in each child before and after HBeAg seroconversion are not known in this study. However, our previous study and a recent study had demonstrated that virions of precore and core promoter mutants gradually increased in the transition of HBeAg seroconversion (9, 22).

It is interesting that the A1752G mutation was found to appear more in the nonseroconverters and in the preseroconversion phase of the seroconverters. Nucleotide position 1752 is located in the Sp1 binding site of the core promoter (23). It may affect the transcription of precore RNA and reduce HBeAg production, thus relating to seroconversion. After HBeAg seroconversion, the mutants of A1752G were largely cleared away. A1775G and G1799C mutations were demonstrated in the sequence analysis of genotype B (24). As shown in the current study, genotype B is the most prevalent HBV genotype in Taiwan, either in children or in adults (25). Thus, the appearance of these mutations could be expected.

When we tally all of those with precore 1896 and/or core promoter mutations (Table 1), 91% of these chronic HBV-infected children had mutations in these regions during the seroconversion process. Only 9% of them underwent HBeAg seroconversion without any mutations in the precore and core promoter regions in this study. This finding suggests the important role of these regions in HBeAg seroconversion.

The nucleotide 1858 of the precore gene is closely related to the evolution of G-1896 to A-1896 mutation to form a stop codon. This is because U1858 (T1858 in DNA) can form a base pair with G- and A-1896. HBV genotypes A and F are well correlated with HBV C1858, whereas HBV genotypes B, D, and E are well correlated with HBV T1858 (19). In our study, T1858 was the major type and C1858 was the minor type in either seroconverters or nonseroconverters with different frequency. C1858 was found in 3.6% of the nonseroconverter group and 32.7% of the seroconverter group, respectively. The higher rate of C1858 in the seroconverter group is different from that reported in adults (26). That the precore 1896 mutation was found exclusively in those with T1858 in this study is compatible with the previous report. However, C1858 was not associated with the core promoter 1762 + 1764 mutation in this study.

For chronic HBV-infected children in Taiwan, we conclude that 1) mutations of core promoter nucleotide positions 1752, 1775, and 1799 associate with HBeAg seroconversion; 2) 1762 + 1764 mutations play a minimal role in HBeAg seroconversion in children; 3) more seroconverters had C1858 than the nonseroconverters, and C1858 tends to promote HBeAg seroconversion; and 4) precore 1896 mutant may account for 45% of childhood HBeAg seroconversion.

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