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Universal Emergence of Precore Mutant Hepatitis B Virus along with Seroconversion to Anti-HBe Irrespective of Subsequent Activity of Chronic Hepatitis B

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Objectives: *It has been controversial whether or not the emergence of precore mutant HBV is related to the severe form of chronic hepatitis B (CH-B). To further clarify the role of the precore mutant HBV in the natural course of CH-B, we conducted a longitudinal analysis of precore-region sequences according to the biochemical severity along with seroconversion to anti-HBe in patients with CH-B.*

Methods: *The precore sequences of the ten sets of serial serum samples, obtained from 6 chronic hepatitis B patients with (group I) and from 4 patients without subsequent biochemical remission after seroconversion to anti-HBe (group II), were analyzed by direct sequencing of DNA amplified by PCR.*

Results: *The precore mutat HBV having a G-A mutation at the nucleotide 1896 was most commonly found (9/10). Wild-type precore HBV was detected in 4 of 6 (66.7%) in group I and 3 of 4 (75.0%) in group II during HBeAg-positive period ($p>0.05$), and during anti-HBe-positive period it was found in 2 of 6 (33.3%) in group I and 0 of 4 (0%) in group II ($p>0.05$). In contrast, precore mutant HBV was detected in 5 of 6 (83.3%) in group I and 2 of 4 (50.0%) in group II during HBeAg-positive period ($p>0.05$), and in all patients of both groups during anti-HBe-positive period.*

Conclusion: *The most common type of precore mutant HBV in Korea was the mutant with a G-A mutation at nucleotide 1896. The emergence of precore mutant HBV was a universal phenomenon during the natural history of CH-B; therefore, the precore mutant does not appear to have an pathogenic role in determining the severity of the CH-B.*

Key Words : *Precore mutant, Chronic hepatitis B, HBeAg, Hepatitis B virus*

INTRODUCTION

In chronic hepatitis B (CH-B), the disappearance of hepatitis B virus e antigen (HBeAg) and the subsequent seroconversion to antibody against HBeAg (anti-HBe) in serum are associated with the clearance of hepatitis B virus (HBV) DNA from serum and the resolution of histological activity[1]. However, in areas with high or intermediate HBV endemicity, HBV DNA and liver damage are persistently present in about 10% of anti-HBe-positive CH-B[2-5]. Virtually all of hepatitis B viruses found in these patients are precore mutant forms which show a translational stop codon in the precore region[6,15]. Therefore, the precore mutant HBV had been suggested as an important determinant of the severity and outcome of CH-B. However, the mutant HBV sequences were also detected in asymptomatic chronic carriers with anti-HBe in serum[16,17]. Thus, it is still a matter of controversy that the appearance or persistence of the precore mutant HBV is really related to the severity of CH-B.

To determine the timing of the emergence of the precore mutant HBV and its prevalence, and thus to further clarify the role of the precore mutant HBV in the natural course of CH-B, we conducted a longitudinal analysis of precore-region

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sequences according to the biochemical severity along with seroconversion to anti-HBe in patients with CH-B.

METHODS

1. Patients

We analyzed DNA sequences of HBV precore region in the serial sera collected from ten patients (9 males and 1 female, mean age 31.2 years) with CH-B who seroconverted spontaneously from HBeAg to anti-HBe during an observation period of 4 to 14 years (mean 8.3 years). The patients were divided into two groups according to the presence or absence of subsequent biochemical remission after the seroconversion; namely, group I consistd of 6 patients with subsequent normalization of serum ALT levels. Serologic markers for HBV(HBsAg and HBeAg/anti-HBe) were detected by commercially available radioimmunoassay kits (Ausria II and HBe Kit, respectively; Abbott laboratories, North Chicago, IL, USA).

2. Extraction of DNA from Serum

A 100-200 μl of each serum was centrifuged down after mixing with a third volume of 2.5 M NaCl and 20% polyethylene glycol. The pellet was redissolved in 300 μl of 5M guanidum thiocyanate and 0.5% Sarcosyl, and then the nucleic acid in solution was eluted with charged glassbeads (Glassmilk Bio101, La Jolla, CA, USA). The supernatant containing the eluted DNA was taken carefully, and a 5-10 μl of the eluant was used for polymerase chain reaction (PCR).

3. Amplification and Sequencing of the Precore Region of HBV DNA

A segment of HBV DNA that constitutes the entire precore region was amplified by a "nested" PCR method[18]. We prepared two pairs of synthetic oligonucleotide primers according to the HBV sequence reported by Kim et al[19]. Those were outer sense primer, 5'-AGGACTCTTGGACTCTCA-3' (1659-1678) and inner sense primer, 5'-GGGAGGAGATTAGGTTAA-3' (1744-1761); outer antisense primer, 5'-AGAATAGCTTGCCTGAGTGC-3' (2033-2052) and inner antisense primer, 5'-GGCAAAAAAGAGAGTAACTC-3' (1913-1932). A5-10 μl of specimen DNA was used for the first round of PCR amplification. The amplification reaction was performed for 20 cycles using an automatic thermocycler (GeneAmp 9600, Perkin-Elmer/Cetus, CA, USA). For the second round of PCR amplification, a 0.5 -1 μl of the first PCR product was amplified for 20 to 30 cycles. We carefully followed the recommendations of Kwok and Higuchi to avoid false-positive PCR[20].

The PCR products to be used for direct sequencing were purifed by spinning down in a microconcentrator (Centricon 30, Amicon, Beverly, MA, USA)[21]. Sequencing was performed by a modified version of the Sequenase protocol (United States Biochemical, Cleveland, OH, USA). Products of these reactions were electrophoresed on a 8% polyacrylamide/8M urea gel, dried and exposed to autoradiography (Fig. 1).

4. Statistical Analysis

The prevalence rates of the wild-type and precore mutant HBV before and after the

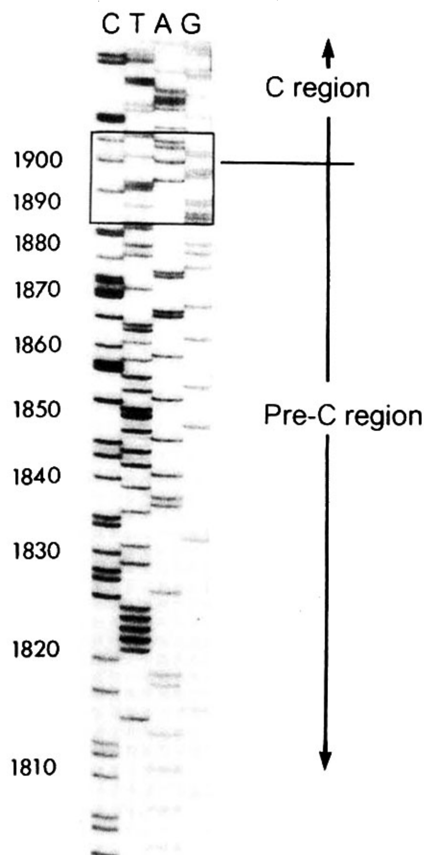


Fig. 1. Direct sequencing of amplified HBV DNA encompassing precore/core region.

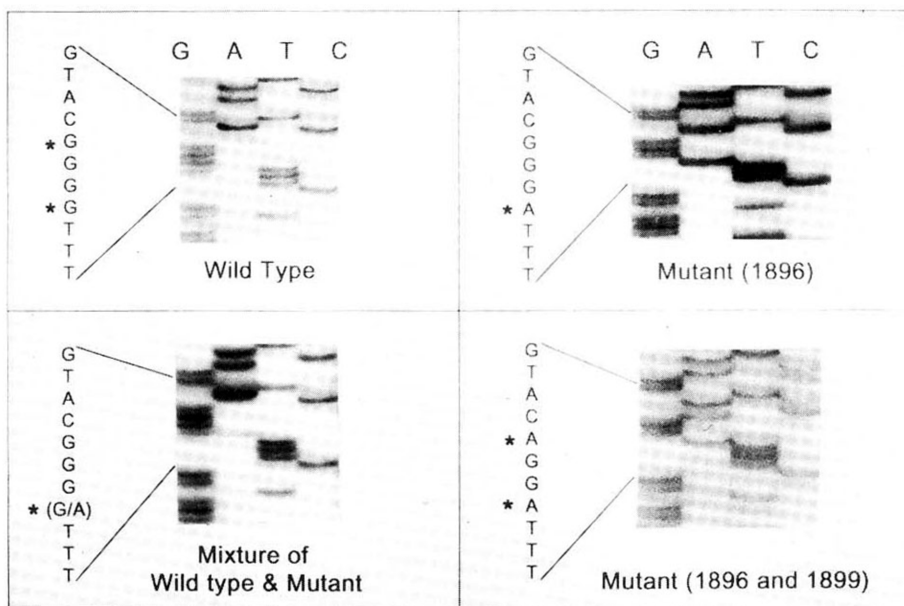


Fig. 2. Four autoradiograms showing direct sequencing of (1) wild-type HBV; precore mutants having (2) a mutation at nt 1896 and (3) two mutations at nt 1896 and 1899, and (4) a mixture of wild-type and a mutant HBV with a mutation at nt 1896, respectively.

seroconversion to anti-HBe in the two groups were compared with each otehr. Statistical differences were analyzed by Fishers exact test.

RESULTS

1. DNA Sequences of the Precore Mutant HBV

The most common (90%) precore mutant HBVs were those having a G-A mutation at the nucleotide 1896 (nucleotide 2 of codon 28) (9/10), and the mutant HBV with G-A mutations both at the nucleotide 1896 and 1899 was found only in one patient (10%) (Fig. 2).

2. Prevalence of Precore Mutant HBV

Of 10 patients, the precore mutant HBV was detected in 7 patients (70.0%) and in all patients (100%) during HBeAg- and anti-HBe-positive period, respectively (Table 1). In contrast, the prevalence rates of wild-type HBV were 70% and 20% during HBeAg- and anti-HBe HBe-positive period, respectively.

The prevalence rates of the mutant HBV according to the biochemical activity of CH-B before and after seroconversion to anti-HBe were depicted in Fig. 3. The precore mutant HBVs

Table 1. **Profile of Precore Mutant HBV in each Patient before and after the HBeAg/anti-HBe Seroconversion**

Patient	HBeAg(+)period		Anti-HBe(+)period	
	Wild	Mutant	Wild	Mutant
NL1	+	+	—	+
NL4	+	—	+	+
NL5	+	+	—	+
NL6	+	+	+	+
NL7	—	+	—	+
NL8	—	+	—	+
AB1	+	—	—	+
AB2	+	+	—	+
AB3	—	+	—	+
AB4	+	—	—	+

AB:patients with persistentyl elevated ALT levels after the seroconversion to anti-HBe
NL:patients with subsequent normalization of ALT levels after the seroconversion to anti-HBe

were consistently detected in all patients of each group, namely irrespective of subsequent activity of CH-B, after seroconversion to anti-HBe.

Two representative cases of group I (A, B) and one of group II (C), the courses of whom

were closely followed up for long period of time, are depicted in Fig. 4.

DISCUSSION

HBeAg is a protein encoded by the HBV core gene, which contains two start codons. When HBV DNA from HBeAg-negative patients with severe CH-B was sequenced, a point mutation was identified between the two start codons[6~15]. This mutation usually causes a stop codon which prevents the formation of the precore protein (the precursor for HBeAg) but does allow the nucleocapsid protein (HBcAg) to be produced by translation initiation at the second start codon. Such precore mutant HBVs are found chiefly in Asia and the Mediterranean region[6~18] and have been only rarely reported from Western Europe and North America[22~24]. A nucleotide transition at precore condon 28 that converts a tryptophan codon (TGG) into a stop codon (TAG) (a G-A mutation at the nucleotide 1896) is the most frequently encountered precore mutation in all areas of the world. An additional G-A mutation at 1899 was also frequently found in Mediterranean and Chinese patients[10,12].

We found in this study that besides the most common mutation of a G-Amutation at nucleotide 1896, a mutation at 1899 also occurred rarely in Korea. Therefore, the sites of point mutation at precore region and the prevalence of

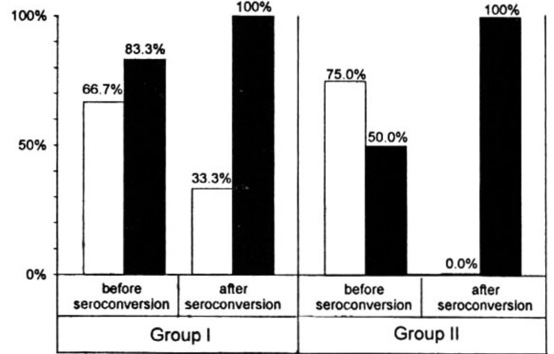
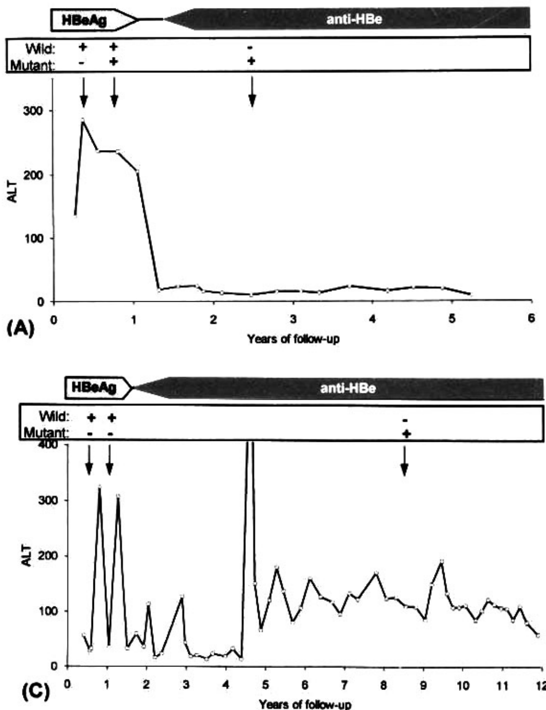


Fig. 3. The prevalence rates of wild-type(□) and precore mutant (■) HBV according to biochemical activity of chronic hepatitis B along with seroconversion to anti-HBe. Group I consisted of 6 patients with subsequent normali-zation of serum ALT levels, and group II included 4 patients with persistently elevated serum ALT levels.



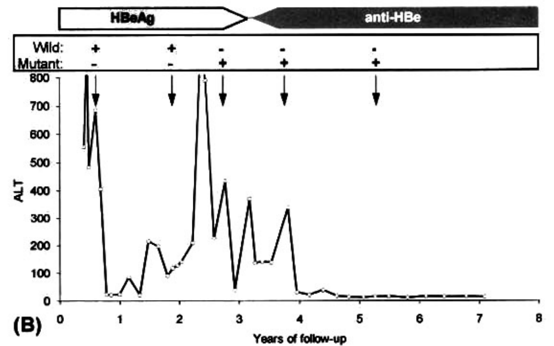


Fig. 4. Pattern of emergence of precore mutant HBV in three representative cases. Wild-type HBV was predominant in sera during HBeAg-positive period when ALT levels were fluctuating, and precore mutant HBV began to emerge just before the disappearance of HBeAg and was the main population after seroconversion to anti-HBe with (A, B) or without (C) subsequent normalization of ALT levels.

precore mutant HBV in Korea are very similar to those observed in Mediterranean countries and China[10,12], but quite different from those observed in the United States and Western Europe[22-24].

The causes of geographic difference in the prevalence rates of the precore mutant HBV still remain to be defined. It may be explained in part by the different distribution of genotypes[25], especially genotype A, in different part of the world. Le and his colleagues demonstrated that the distortion of a single base of G-C pair hampers viral replication and restricts the emergence of precore mutant HBV from this particular HBV genotype[26-28]. Therefore, the prevalence of precore mutant HBV may be inversely correlated with the prevalence of genotype A of HBV.

The present study which detected the precore mutant HBV in the sequential sera along with anti-HBe seroconversion has shown that the mutation develops naturally in all cases and that the dominant viral strain changed from wild to mutant type, irrespective of subsequent activity of CH-B. The findings of the present study, which demonstrated that precore mutant HBVs were found in all patients with normal ALT levels as well as in all of those with persistentyl elevated ALT levels, suggest that there is no causal relationship between the precore mutant HBV and activity of CH-B, implying that other factors should be involved in the pathogenesis and progression of chronic HBV infection.

Hepatitis B core antigen (HBcAg) is known as the main immune target for cytotoxic T cells[29] and the antigenic epitopes within the core protein that associate with the HLA antigens[30,31] have now been identified. Therefore, it is reasonable to presume that the variation in the antigenic epitopes within the core protein may have an etiologic role in determining the severity of CH-B. However, the results of the studies on the mutation of core region of HBV according to the severity of CH-B still remain to be seen.[32,33]

In conclusion, the emergence of precore mutant HBV along with seroconversion to anti-HBe is a universal phenomenon during natural history of CH-B; therefore, the precore mutant HBV does not appear to have a pathogenetic role in determining the severity and progression of the CH-B. It does not seem that a specific mutation at the specific region of HBV is involved in the pathogenesis of CH-B. It is more likely that the different severities of CH-B due to the HBVs having mutations at variable regions might be caused by a complex interaction between the mutation and the host immune response.

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