

•VIRAL HEPATITIS•

Distribution of HBV genotypes among HBV carriers in Benin: phylogenetic analysis and virological characteristics of HBV genotype E

Kei Fujiwara, Yasuhito Tanaka, Etsuro Orito, Tomoyoshi Ohno, Takanobu Kato, Kanji Sugihara, Izumi Hasegawa, Mayumi Sakurai, Kiyoaki Ito, Atsushi Ozasa, Yuko Sakamoto, Isao Arita, Ahmed El-Gohary, Agossou Benoit, Sophie I Ogoundele-Akplogan, Namiko Yoshihara, Ryuzo Ueda, Masashi Mizokami

Kei Fujiwara, Etsuro Orito, Tomoyoshi Ohno, Kanji Sugihara, Izumi Hasegawa, Mayumi Sakurai, Kiyoaki Ito, Atsushi Ozasa, Ryuzo Ueda, Department of Internal Medicine and Molecular Science, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Yasuhito Tanaka, Takanobu Kato, Masashi Mizokami, Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Yuko Sakamoto, Namiko Yoshihara, AIDS Research Center, National Institute of Infectious Disease, Tokyo, Japan

Isao Arita, Agency for Cooperation in International Health, Kumamoto, Japan

Ahmed El-Gohary, Clinical Pathology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt

Agossou Benoit, Central National Blood Transfusion Center, Cotonou, Benin

Sophie I Ogoundele-Akplogan, Hematology Lab-CNHUC, Cotonou, Benin

Correspondence to: Dr Masashi Mizokami, Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho, Nagoya 467-8601, Japan. mizokami@med.nagoya-cu.ac.jp

Telephone: +81-52-853-8292 Fax: +81-52-842-0021

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HBV/E was distributed throughout West Africa with very low genetic diversity (nucleotide homology 96.7-99.2%). Based on the sequences in the basic core promoter (BCP) to precore region of the nine HBV/E isolates compared to those of the other genotypes, a nucleotide substitution in the BCP, G1757A, was observed in HBV/E.

CONCLUSION: HBV/E is predominant in the Republic of Benin, and SC is estimated to occur in late teens in HBV/E. The specific nucleotide substitution G1757A in BCP, which might influence the virological characteristics, is observed in HBV/E.

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Key words: HBV genotype; West Africa; Basic core promoter

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Abstract

AIM: To determine the distribution of Hepatitis B virus (HBV) genotypes in Benin, and to clarify the virological characteristics of the dominant genotype.

METHODS: Among 500 blood donors in Benin, 21 HBsAg-positive donors were enrolled in the study. HBV genotypes were determined by enzyme immunoassay and restriction fragment length polymorphism. Complete genome sequences were determined by PCR and direct sequencing.

RESULTS: HBV genotype E (HBV/E) was detected in 20/21 (95.2%), and HBV/A in 1/21 (4.8%). From the age-specific prevalence of HBeAg to anti-HBe seroconversion (SC) in 19 HBV/E subjects, SC was estimated to occur frequently in late teens in HBV/E. The comparison of four complete HBV/E genomes from HBeAg-positive subjects in this study and five HBV/E sequences recruited from the database revealed that

INTRODUCTION

Hepatitis B virus (HBV) is a member of the family *Hepadnaviridae*. This virus is a small DNA virus with a partially double-stranded 3.2-kb genome. HBV has been classified into seven genotypes based on the sequence divergence over the entire genome exceeding 8%^[1,2]. The seven genotypes show a distinctive geographical distribution. HBV genotype A (HBV/A) is prevalent in Northwestern Europe, North America, and Africa^[3]. HBV/B and HBV/C are characteristics of Asia. HBV/D is predominant in the Mediterranean area, HBV/E in West Africa, and HBV/F in South America.

Recently, virological and clinical differences in genotypes have been reported^[4,5]. In addition, subtypes of HBV/B, HBV/Ba, 'a' indicating Asia, and HBV/Bj, 'j' indicating Japan, have been reported^[6]. The former has

recombination with HBV/C, but the latter does not, and clinical differences have been shown between them^[7]. Furthermore, HBV/A is phylogenetically classified into subtypes, namely HBV/Aa, 'a' indicating Asia/Africa, which is distributed in Asia and Africa, and HBV/Ae, 'e' indicating Europe, which is distributed in Europe^[8].

It is now recognized that mutations in basic core promoter (BCP) and precore region regulate hepatitis B e antigen (HBeAg) expression. It was shown in an *in vitro* study that the double mutations in BCP, A1762T and G1764A, downregulate precore mRNA and slightly increase the efficiency of pregenome mRNA and core mRNA^[9]. Additionally, the mechanism of HBeAg reduction through subtype-specific nucleotide substitutions just prior to the start of the precore open reading frame (ORF), known as "Kozak sequence" in the African subtype of HBV/A, has been shown in an *in vitro* study^[10].

In Africa, the distribution of HBV genotypes is HBV/A in the South, HBV/D in the North, and HBV/E in the West. For HBV/E, only four human HBV/E full genomes have been reported so far, and the virological and clinical characteristics have not yet been sufficiently clarified.

In this study, the distribution of HBV genotypes in the Republic of Benin was investigated for the first time, and the virological and clinical characteristics of the dominant genotype were analyzed.

MATERIALS AND METHODS

Patients

Five hundred blood donors, who visited the National Blood Transfusion Center in the Republic of Benin in 2002, were screened for hepatitis B surface antigen (HBsAg). Twenty-four were positive for HBsAg (4.8%). Sera from 21 donors were available in this study (17 males, 3 females, unknown in 1; mean age, 23.1±6.7 years). All the donors were negative for the antibody to HIV-1 (anti-HIV) and the antibody to human adult leukemia virus 1 (anti-HTLV-1). The antibody to hepatitis C virus (anti-HCV) was positive in one donor. Informed consent was obtained from each patient.

Serological testing

HBsAg, anti-HCV, and anti-HTLV-1 were tested by counting immunoassay (PAMIA, Sysmex, Kobe, Japan). HBeAg and the antibody to HBeAg (anti-HBe) were determined using a commercially available chemiluminescent enzyme immunoassay (EIA, Lumipulse f, Fujirebio Inc., Tokyo, Japan). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (T-Bil) were measured in all the samples.

Genotyping

The HBV genotypes were determined in the sera using an EIA with pre-S2 specific mAb^[12,13]. When the result of the EIA method was indeterminate, HBV genotypes were detected by restriction fragment length polymorphism, as previously described^[14].

PCR amplification and sequencing of HBV

The serum samples were stored at -80 °C until use. Total DNA was extracted from 100 µL of serum using microspin columns (QIAamp Blood kit, Qiagen KK, Tokyo, Japan). Purified DNA was resuspended in 80 µL of distilled water. PCR was carried out by the same protocol as described previously^[15]. Nucleotide sequences of the amplified products were determined directly by the dideoxy method, using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit with a fluorescent 3100 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

Real-time detection polymerase chain reaction (RTD-PCR)

serum HBV DNA was quantitatively detected by RTD-PCR based on Taqman chemistry as reported previously^[16]. Amplification was performed using primers corresponding to conserved sequences of the surface region. A 10-µL aliquot of DNA solution was used for RTD-PCR. A portion of the HBV surface region was amplified using primers: a forward primer HBSF2 (5'-CTTCATCCTGCTGCTATGCCT-3', nucleotide position (nt) 406-426) and a reverse primer HBSR2 (5'-AAAGCCCAGGATGGGAT-3', nt 646-627). A taqman probe was designed as HBSP2 (5'-ATGTTGCCCTT TGTCCCTCCTCTAATTCCAG-3', nt 461-488), with an additional G at the 3'-end of HBSP2 in the original method, the detection limit of this system was as low as five DNA copies/assay, and linear standard curve was obtained from 5 to 10⁶ DNA copies/assay.

Phylogenetic analysis

Complete sequences of 21 HBV isolates were aligned with the CLUSTAL W software program^[17], and the alignment was confirmed by visual inspection. Genetic distances were estimated by the six-parameter method, and phylogenetic trees were constructed by the neighbor-joining method using ODEN program of the National Institutes of Genetics (Mishima, Japan)^[18]. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1 000 times.

RESULTS

HBV genotypes

Distribution of HBV genotypes among 21 HBV carriers in the Republic of Benin was 20/21 (95.2%) for HBV/E, and 1/21 (4.8%) for HBV/A. HBV/B, C, D, F, and G were not found. HBV/E was the predominant genotype in the Republic of Benin.

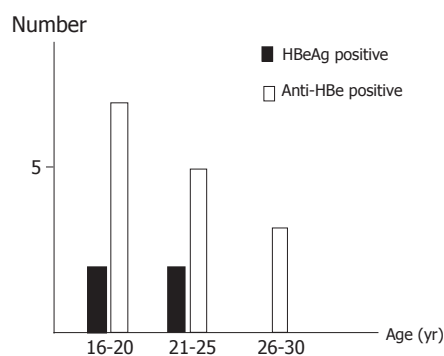
Clinical characteristics of asymptomatic HBV carriers in the Republic of Benin

HBeAg was positive for 4/21 (19.0%) subjects, and anti-HBe was positive for 17/21 (81.0%) subjects. The titers of four HBeAg-positive subjects were beyond the upper detection limit. In order to clarify the clinical characteristics of asymptomatic HBV carriers in this country, the clinical and laboratory data between HBeAg-positive patients

Table 1 Comparison of clinical characteristics between HBeAg-positive and anti-HBe-positive subjects

	HBeAg-positive (n = 4)	Anti-HBe-positive (n = 17)	P
Age (yr)	21.25±2.21	23.5±7.42 ¹	NS ²
Sex (M/F)	2/2	1/15 ¹	NS ³
Genotype (A/E)	0/4	1/16	NS ³
AST (IU/L)	25.8±8.3	18.0±5.4	NS ²
ALT (IU/L)	11.5±10.3	5.7±1.5	NS ²
T-Bil (mg/dL)	0.50±0.35	0.57±0.41	NS ²
HBV DNA (log (copies/mL))	7.76±0.29	<2.6	<0.0005 ²

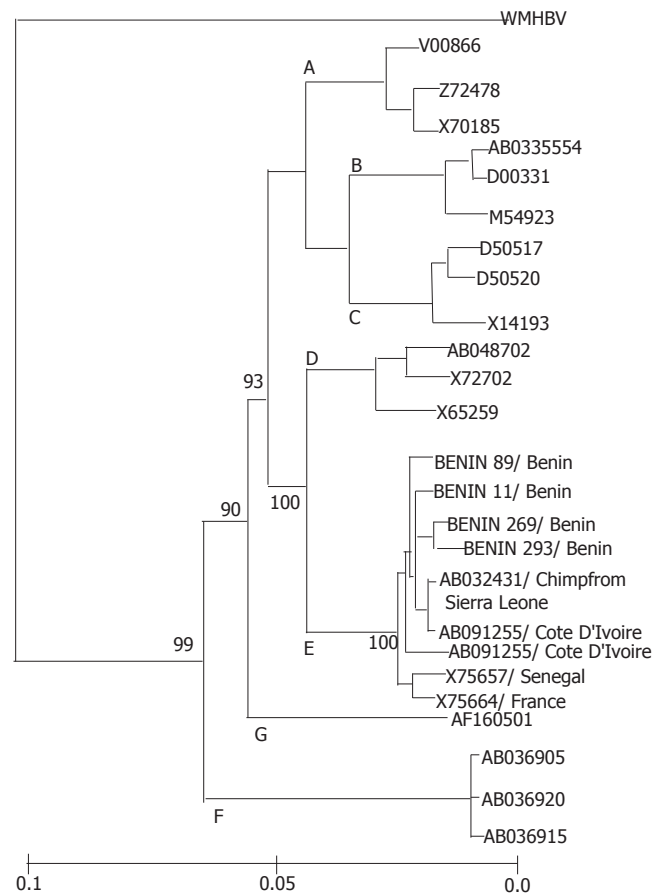
¹Sex and age unknown in one subject. ²P values were calculated by Welch's *t*-test. ³P values were calculated by Fisher's exact test. AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-Bil, total bilirubin.

**Figure 1** Age-specific prevalence of HBeAg/anti-HBe status in 19 HBV genotype E strains.

and anti-HBe-positive patients were compared (Table 1). The mean age of the HBeAg-positive group was not lower than that of the anti-HBe-positive group. As a matter of course, the difference in the mean HBV DNA titer between the two groups was statistically significant ($P<0.0005$). HBV DNA titers in anti-HBe-positive subjects were quite low. To examine the correlation between age and HBeAg/anti-HBe status in detail, the age-specific prevalence of the HBeAg/anti-HBe status in 19 HBV/E subjects was analyzed. Seroconversion (SC) during the late teens occurred in 7 of 19 subjects (36.8%, Figure 1). This was the reason why the comparison of age between the two groups was not statistically significant.

Phylogenetic analysis of four strains

HBV full genomes of four HBeAg-positive patients were determined to have a nucleotide length of 3 212 bp. Phylogenetic analysis of the complete genome sequences of these four strains compared to those of 22 HBV strains from the DDBJ/GenBank/EMBL database showed that four strains clustered with five other HBV/E strains (accession numbers: X75657, X75664^[2], and AB032431^[19]; AB091255, AB091256^[11]) (Figure 2). The nucleotide homology within the HBV/E cluster ranged from 96.7% to 99.2%. In addition, the phylogenetic analysis of four ORFs showed no recombination of HBV/E strains in the Republic of Benin (Figure 3). Genetic diversity among HBV/E strains was very low compared to the

**Figure 2** Phylogenetic tree constructed on complete nucleotide sequences of 26 HBV isolates. The nine HBV genotype E isolates were compared along with 16 HBV isolates representative of the other six genotypes (A–D, F and G), along with woolly monkey hepatitis virus (WMHBV) as an outgroup. The four HBV genotype E isolates determined in this study are indicated in boldface, and the other 22 HBV isolates are specified by accession numbers. The country of origin is indicated after the slash for each HBV isolate of genotype E.

other genotypes, excluding HBV/G. The nucleotide sequence data reported in this article will appear in the DDBJ/GenBank/EMBL nucleotide sequence databases with the accession numbers AB201287-90.

Characteristics of nucleotide substitutions in BCP and precore region

In order to find out the HBV/E-specific nucleotide substitution associated with HBe protein production, the BCP to the precore region of nine HBV/E strains and the strains of six genotypes were compared (Figure 4). The representative nucleotide sequences of HBV/A (Aa–Ae), B, C, D, F, and G were determined by aligning five sequences of each genotype, and the consensus nucleotide was deduced, if the identical nucleotide was detected in 60% or more of the sequences. In the BCP, the nucleotide substitution at A1757 was observed in all the strains of HBV/E. This substitution was not found in HBV/A–C, HBV/F, and HBV/G, but was found in more than half of HBV/D^[20]. BCP double mutation (T1762/A1764) was not found in the HBV/E strains; however, T1772C substitution was found in all the HBV/E strains as well

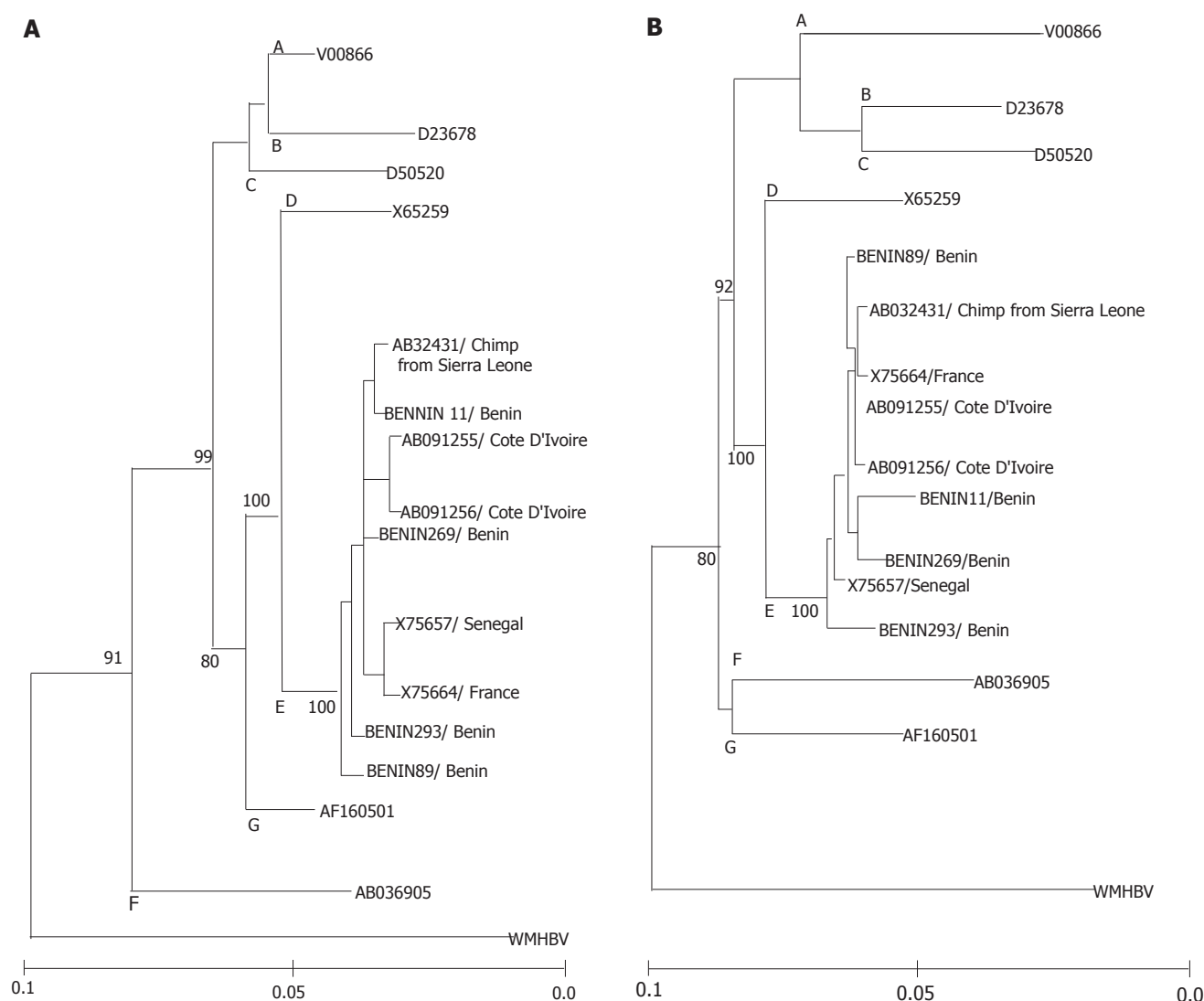


Figure 3 Phylogenetic trees representing the large S gene (A), and precore region plus core gene (B) along with the strains of the other six genotypes and WMHBV.

as in HBV/B, C and F. Other sequences in the BCP were conserved irrespective of genotypes, except for the above mutations. One HBV/E isolate had T1809 substitution prior to the start of precore ORF, which is recognized as the Kozak sequence frequently found in HBV/Aa. Precore stop mutation (A1896) was not found in the HBV/E.

DISCUSSION

In this study, the distribution of HBV genotypes among blood donors in the Republic of Benin was analyzed for the first time. In Africa, HBV/D is dominant in the Northern region^[21], with HBV/A found in South Africa. HBV/E is the major genotype in the Republic of Benin. This agrees with previous reports that HBV/E is prevalent mainly in West Africa^[2,11,22]. In addition, the distribution of HBV/E is restricted to West Africa, unlike other genotypes. Though slaves have migrated from West Africa to North America, HBV/E is not found in USA, suggesting that genotype E emerged from the mid to late

19th century^[22,23].

East Asia and Sub-Saharan Africa are the two most HBV endemic areas; however, clinical differences have been shown between the two areas. Vertical transmission is the main route in Asia, whereas horizontal transmission is the main route in Africa, and SC occurs much earlier in Africa than in Asia^[24,25]. It is important to examine the clinical characteristics of asymptomatic HBV carriers in Benin, which reflect the characteristics of HBV/E, because there are only a few reports concerning the clinical characteristics of HBV/E. As shown in Figure 1, SC is thought to occur at a young age in subjects with HBV/E in Benin. These data might explain one of the reasons why vertical transmission does not occur in West Africa. Recently, the difference in SC between HBV/B and HBV/C in Taiwan was reported^[5]. SC occurs a decade earlier in HBV/B patients^[26,27] and the mean SC age in HBV/B is approximately 30 years. In comparison with HBV/B, the mean SC age in HBV/E occurs even earlier. These data lead us to speculate that HBV/E has a better prognosis than other genotypes in terms of chronic liver disease.

		1759	1762	1764		1809	1812	1896
Genotype Ae		TAGGTTAAAGGCTTGTATTAGGAGGCTG				CAGCACCATGC		TTTGGGGCAT
Genotype Aa					T	T		
Genotype B					C			A
Genotype C			T	A	C			
Genotype D		A			T			
Genotype F					C			
Genotype G			T	A	T			A
Genotype E								
AB032431		A			C			
AB091255		A			C			
AB091256		A		A	C			
X75664		A			C			
X75667		A			C			
BENIN11		A			C			
BENIN89		A			C			
BENIN269		A			C			
BENIN293		A			C			

Figure 4 Nucleotide sequences constituting parts of the BCP to the precore of nine HBV genotype E isolates with the consensus sequences of the other six genotypes. The four Benin isolates determined in this study are shaded in gray.

Regarding hepatocellular carcinoma (HCC), a report from Gambia, where HBV/E is the most prevalent, revealed that chronic HBV infection is associated with HCC development at a younger age^[28]. Infection with HBV/B is associated with HCC development at a young age in Taiwan^[5]. In this sense, HBV/E in West Africa is clinically more similar to HBV/B than HBV/C, and virologically, HBV/E and HBV/B have a high frequency of a precore mutation, G1896A, in common^[11,29]. Further information about the clinical characteristics of HBV genotypes might reveal the reason why the age of SC and the age-related HCC incidence differ among genotypes.

So far, only five complete genomes of HBV/E (one from a chimpanzee) have been reported. By adding the four complete genomes reported here, phylogenetic analyses of the full genome and ORFs show much more clearly that HBV/E has a low genetic diversity, as previously reported^[11,22,23]. Although subtypes of HBV/B, which have recombination in the BCP to the core region^[7] and subtypes of HBV/A^[8,30] have been reported, the analysis of nine HBV/E genomes shows neither the possibility of subtypes nor the possibility of recombination.

As mentioned above, HBV/A is divided into two subtypes: HBV/Aa, 'Aa' indicates Asia and Africa, and HBV/Ae, 'Ae' indicates Europe^[8,30]. Subtype HBV/Aa has subtype-specific substitutions just before precore start codons, known as the "Kozak sequence". Substitutions reduce the HBeAg expression^[10,31]. Hence, genotype- and subtype-specific nucleotide substitutions in the BCP region are crucial, not only in viral replication but also in HBe SC. In this study, we identified the mutation in the BCP, A¹⁷⁵⁷, in HBV/E. Other genotypes have G¹⁷⁵⁷ except for HBV/D, in which A¹⁷⁵⁷ is observed in about 70%^[20]. BCP has been identified as a sequence that regulates both precore and pregenomic messages^[32]. The core promoter A1762T and G1764A double mutation is known to downregulate precore mRNA but does not seriously affect pregenome mRNA^[33]. The double mutation (1762/1764) occurs over time, as the viral load and HBeAg/anti-HBe status change, and the sequences

around the double mutations are conserved irrespective of the genotype so far. However, HBV/E seems to possess 1757A from the early stage of the infection, similar to Kozak sequence substitutions in genotype Aa. Thus, the different functions in relation to nuclear receptor binding between 1757G and 1757A was shown in an *in vitro* study, when the mechanism of suppression of HBV precore RNA transcription by core promoter double mutation was sought. The double mutation in 1762 and 1764 changes the nucleotide sequences of the nuclear-factor binding site of BCP. When the mutation occurs, the nuclear factor known as COUP TF I cannot bind to BCP. Instead, a different nuclear factor, HNF1, binds, and this alters the efficiency of precore and core mRNA transcription. The artificial mutation was created with the intention to create a sequence that neither COUP TF I nor HNF-1 can bind. This mutation is G1757A, and the mutation abolishes both the nuclear factors (COUP TFI and HNF1) binding to BCP in the *in vitro* experiment^[34]. This unique mutation and its alteration of nuclear factor binding capacity to the BCP region might influence the clinical characteristics of HBV/E. Further studies, including *in vitro* studies, are expected.

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