A Case-Control Study for Differences Among Hepatitis B Virus Infections of Genotypes A (Subtypes Aa and Ae) and D

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There are two subtypes of hepatitis B virus genotype A (HBV/A) and they are provisionally designated Aa ("a" standing for Africa/Asia) and Ae ("e" for Europe). In a case-control study, 78 HBV/Aa, 78HBV/Ae, and 78HBV/D carriers from several countries were compared. The prevalence of HBe antigen (HBeAg) in serum was significantly lower in carriers of HBV/Aa than in carriers of HBV/Ae (31% vs. 49%; $P = .033$), with a difference more obvious in the carriers aged 30 years or younger (34% vs. 67%; $P = .029$). HBV DNA levels in the carriers of HBV/Aa (median, 3.46 log copies/mL; 95% CI, 2.93–3.95) were significantly lower than those of carriers of HBV/Ae (6.09 log copies/mL; 95% CI, 4.24–7.64) or of carriers of HBV/D (5.48 log copies/mL; 95% CI, 4.06–7.02), regardless of the HBeAg status ($P < .001$). The most specific and frequent substitutions in 54 HBV/Aa isolates were double substitutions for T1809 (100%) and T1812 (96%) immediately upstream of the precore initiation codon, which would interfere with the translation of HBeAg in HBV/Aa infections. They were not detected in 57 HBV/Ae or 61 HBV/D isolates examined. The double mutation in the core promoter (T1762/A1764) was more frequent in both HBV/Aa (50%) and HBV/Ae (44%) than in HBV/D isolates (25%; $P < .01$), whereas the precore mutation (A1896) occurred in HBV/D isolates only (48%; $P < .0001$). In conclusion, the clearance of HBeAg from serum may occur by different mechanisms in HBV/Aa, HBV/Ae, and HBV/D infections, which may influence clinical manifestations in the Western countries where both genotypes A and D are prevalent. (HEPATOLOGY 2004;40:747–755.)

Seven major genotypes of hepatitis B virus (HBV) have been classified by a sequence divergence in the entire genome in excess of 8%, and they are named by capital alphabet letters from A through G.[1–3] An eighth genotype is proposed under the designation H,[4] but its independence from genotype F has yet to be verified by strict phylogenetic analyses. HBV genotypes have distinct geographical distributions.[5,6] Thus, genotype A (HBV/A) is predominant in Europe and Africa; HBV/B and HBV/C are prevalent in East and South Asia; HBV/D is common in the Mediterranean area, the Middle East, and India; HBV/E is restricted to sub-Saharan Africa; and HBV/F is localized in the Central and South America. All seven genotypes occur in the United States with frequencies dependent on the ethnicity.[7] More recently, HBV/G was found in France, Germany, and the United States,[3,7,8] and always coinfects hosts with genotype A.[3,9]

There have been increasing lines of evidence to indicate influences of HBV genotypes on the outcome of liver disease and the response to antiviral therapies, especially between genotypes B and C, common in Asia.[10–13] Evidence for clinical differences between genotype A and D infections is scarce.[14,15]

Abbreviations: HBV, hepatitis B virus; nt, nucleotide; HBeAg, hepatitis B e antigen; HCC, hepatocellular carcinoma; anti-HBe, antibody to hepatitis B e antigen; PCR, polymerase chain reaction; ALT, alanine aminotransferase.

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HBV isolates even of the same genotype may differ both virologically and clinically. Two subtypes of genotype B in distinct geographical distributions, designated Ba ("a" standing for Asia) and Bj ("j" for Japan) provisionally,[16] and clinical differences between patients infected with HBV/Ba and HBV/Bj are coming to the fore.[17,18] In a phylogenetic analysis of the pre-S2/S region, Bowyer et al.[19] found that African HBV/A isolates cluster on a branch separate from the original genotype A from Europe and reported it under the designation subgroup A′. Their findings have been extended, by comparison of the entire nucleotide (nt) sequences of many HBV/A isolates, leading to the proposal of two subtypes or subgenotypes of genotype A.[20–22] The one subtype of genotype A originally reported and distributed widely in European countries and the United States was tentatively designated subtype Ae ("e" standing for Europe), whereas the other prevailing in sub-Saharan Africa,[21,23] corresponding to subgroup A′ of Bowyer et al.[19] and also found in Asia,[20] was provisionally named subtype Aa ("a" for Africa/Asia).

There have been some lines of evidence for virological and clinical differences between subtype Aa in Africa and subtype Ae in Europe and the United States. Infection with subtype Aa is associated with low serum levels of HBV DNA as well as low prevalence of hepatitis B e antigen (HBeAg) in serum and is implicated in the high incidence of HBV-induced hepatocellular carcinoma (HCC) in Africa.[24,25] In 1999, Baptista et al.[26] reported the presence of nucleotide substitutions within the Kozak sequence, immediately upstream of the initiation codon of the precore region that were shown subsequently to interfere with the translation of HBeAg precursor.[27] These mutations are found exclusively in subtype Aa isolates and probably contribute to the early loss of HBeAg from serum in the individuals infected with this subtype in Africa.[28,29]

For the purpose of determining clinical and virological differences between subtypes Aa and Ae infections, in comparison with genotype D infection prevalent along with genotype A in the Western countries, a case-control study was performed of 234 carriers from several countries who were infected with one of the two subtypes of genotype A (Aa and Ae) or genotype D (78 in each group). Sequence analyses for mutations affecting the synthesis of HBeAg on HBV DNA from these patients suggest distinct mechanisms for the loss of HBeAg from serum that could affect clinical outcomes of patients infected with one of the two subtypes of genotype A or genotype D.

Patients and Methods

Serum Samples. Sera were obtained from 234 patients infected with subtype Aa (78 patients) or Ae (78 patients) of genotype A or with genotype D (78 patients) from several countries who were controlled for sex, age, and severity of liver disease: nine were from Bangladesh, 84 from India, 29 from Japan, 7 from Nepal, 14 from South Africa, 10 from Tanzania, and 81 from the United States. None were positive for serological markers of infection with hepatitis C virus or human immunodeficiency virus type 1. HBV DNA sequences were examined for mutations affecting the synthesis of HBeAg, and the results were correlated with HBeAg and HBV DNA levels in serum, as well as subtypes of genotype A (Aa and Ae) and genotype D. The study protocol was approved by ethics committees of the institutions in accordance with the 1975 Declaration in Helsinki, and an informed consent was obtained from each patient.

Serological Assays for HBV Markers. HBeAg was detected by Chemiluminescent enzyme immunoassay (Ortho Clinical Diagnostics, Tokyo, Japan). The seven major HBV genotypes (A–G) were determined by enzyme-linked immunosorbent assay with monoclonal antibodies directed to distinct epitopes on the preS2-region products,[30] with use of commercial kits (HBV GENOTYPE EIA; Institute of Immunology Co., Ltd., Tokyo, Japan) used for genotypes other than G. The genotype G was determined by the combination of preS2 serotype for genotype D and serotype *adw* of HBsAg; it is characteristic of this genotype.[9]

Detection and Quantification of Serum HBV DNA. HBV DNA sequences spanning the S gene were determined by real-time detection polymerase chain reaction (PCR) according to the method of Abe et al.[31] with a forward primer (HBSF2: 5′-CTT CAT CCT GCT GCT ATG CCT-3′ [nt 406–426]), a reverse primer (HBSR2: 5′-AAA GCC CAG GAT GAT GGG AT-3′ [nt 646–627]), and Taq Man probe HBSP2 (5′-ATG TTG CCC GTT TGT CCT CTA ATT CCA G-3′ [nt 461–488]), with an additional G at the 3′-end of HBSP2 in the original method. The detection limit of this study was 100 copies/mL.

PCR-Restriction Fragment Length Polymorphism for Confirmation of Genotype A and Determination of Subtype Ae. Nucleic acids were extracted from 100 μL of serum using QIAamp DNA Blood Mini Kit (Qiagen Inc., Hilden, Germany). A novel method for confirmation of genotype A and specific determination of HBV/Ae consisted of two PCR cycles with heminested primers followed by restriction fragment length polymorphism with the restriction site specific for HBV/Ae.[32] The first-round PCR was performed with a sense primer (HB7F: 5′-GAG ACC ACC GTG AAC GCC CA -3′ [nt 1611–1630]) and an antisense primer (HB7R-2: 5′-CCT GAG TGC TGT ATG GTG AGG-3′ [nt 2072–2052])

in a 96-well cycler. The second-round PCR was performed twice separately with different sets of primers. Set I was used for confirmation of genotype A and was composed of a sense primer (HBxA: 5′-ATT GGT CTG CGC ACC A-3′ [nt 1793–1808]) deduced from the conserved regions of the X gene and an antisense primer (HB7R-2), and set II was comprised of a sense primer (HBxAe: 5′-ATT GGT CTG CGC ACC AGG AC-3′ [nt 1793–1812]) bearing sequences specific for HBV/Ae at the 3′-end and an antisense primer (HB7R-2). For coping with occasional atypical isolates, amplification products were tested by restriction fragment length polymorphism for the restriction site of *Bgl*II that is possessed by HBV/Ae but not HBV/Aa sequences. Amplicons by PCR with set II of 282 base pairs were digested with 10 U *Bgl*II (New England BioLabs, Beverly, MA). The digest was run by electrophoresis on 3% (wt/vol) agarose, was stained with ethidium bromide, and was observed in ultraviolet light. Amplification products on HBV/Ae isolates gave rise to two fragments of 221 and 61 base pairs, respectively, whereas those on HBV/Aa isolates did not.

Amplification and Sequencing of the Core Promoter as Well as the Precore Region Plus Core Gene. HBV DNA sequences bearing the core promoter and precore or core regions were amplified by PCR with heminested primers by the method described previously.[33] Thereafter, PCR products were sequenced directly with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer (Applied Biosystems).

Molecular Evolutionary Analyses of HBV. Reference sequences were retrieved from the DDBJ/EMBL/GenBank database along with their accession numbers for identification. Nucleotide sequences of HBV were aligned by the program CLUSTAL X, and the genetic distance was estimated with the six-parameter method[34] in the Hepatitis Virus Database.[35] Based on these values, a phylogenetic tree was constructed by the neighbor-joining method with the midpoint rooting option. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1,000 times.

A Case-Control Study for Clinical and Virological Differences Among HBV/Aa, HBV/Ae, and HBV/D Infections. Clinical and serological data were available for the 78 individuals persistently infected with HBV/Aa (9 from Bangladesh, 29 from India, 3 from Japan, 7 from Nepal, 6 from the United States, 14 from South Africa, and 10 from Tanzania). Serving as controls were 78 carriers of HBV/Ae and 78 carriers of HBV/D randomly recruited from India (none and 55 carriers, respectively), Japan (26 and none, respectively), and the United States (52 and 23); they were matched for mean age and gender with the 78 carriers of HBV/Aa. They also were matched for the severity of liver disease in each group, including 22 asymptomatic carriers with normal alanine aminotransferase (ALT) levels and 56 patients with liver disease. An asymptomatic carrier was defined as a HBsAg-positive individual with normal ALT levels over 1 year (examined at least four times at 3-month intervals), without the presence of portal hypertension. Chronic hepatitis was defined as persistent elevation of ALT levels ($>1.5 \times$ upper limit of normal [35 U/L]) over a 6-month period (at least three readings at 2-month intervals), accompanied by histological documentation of grading and staging scores.[36] Cirrhosis was determined mainly by ultrasonography (coarse liver architecture, nodular liver surface, and blunt liver edges) and evidence of hypersplenism (splenomegaly on ultrasonography), a platelet count $<100{,}000/mm^3$, or a combination thereof. Confirmation by fine-needle biopsy of the liver was performed as required. Available histological results among the studied chronic hepatitis patients indicated no significant differences among HBV/Aa, HBV/Ae, and HBV/D groups: 0.93 ± 0.53, 1.19 ± 0.84, 0.88 ± 0.78 in grades, respectively, and 1.38 ± 0.81, 1.22 ± 0.90, 1.48 ± 1.01 in stages. None had received antiviral treatment during the follow-up period.

Statistical Analysis. Statistical differences were evaluated by the Mann-Whitney *U* test, Fisher exact probability test, and chi-square test with Yates's correction, where appropriate, with use of STATA software, version 8.0 (StataCorp. LP, College Station, TX). Differences were considered significant for *P* values less than .05.

Results

Phylogenetic Relatedness of the Two Subtypes of HBV/A and HBV/D. For further characterization of HBV genotypes in the 234 carriers participating in a case-control study, 54 (69%) HBV/Aa, 57 (73%) HBV/Ae, and 61 (78%) HBV/D isolates could be amplified and were sequenced over the core promoter and precore or core regions spanning 398 base pairs. The remaining 62 isolates could not be sequenced over the target because of their low HBV DNA levels in serum. Together with 14 HBV/A and HBV/D sequences retrieved from the database, the 54 HBV/Aa and 57 HBV/Ae isolates sequenced in the present study were subjected to a phylogenetic analysis along with the six sequences representative of subtypes Ba and Bj of genotype B, as well as genotypes C, E, F, and G, respectively (Fig. 1). The 54 HBV/Aa isolates in the present study, including 4 from Bangladesh, 21 from India, 3 from Japan, 7 from Nepal, 9 from South Africa, 7 from Tanzania, and 3 from the United States clustered with the HBV/Aa isolates from India, Malawi, Philippines, and South Africa retrieved from the database; all

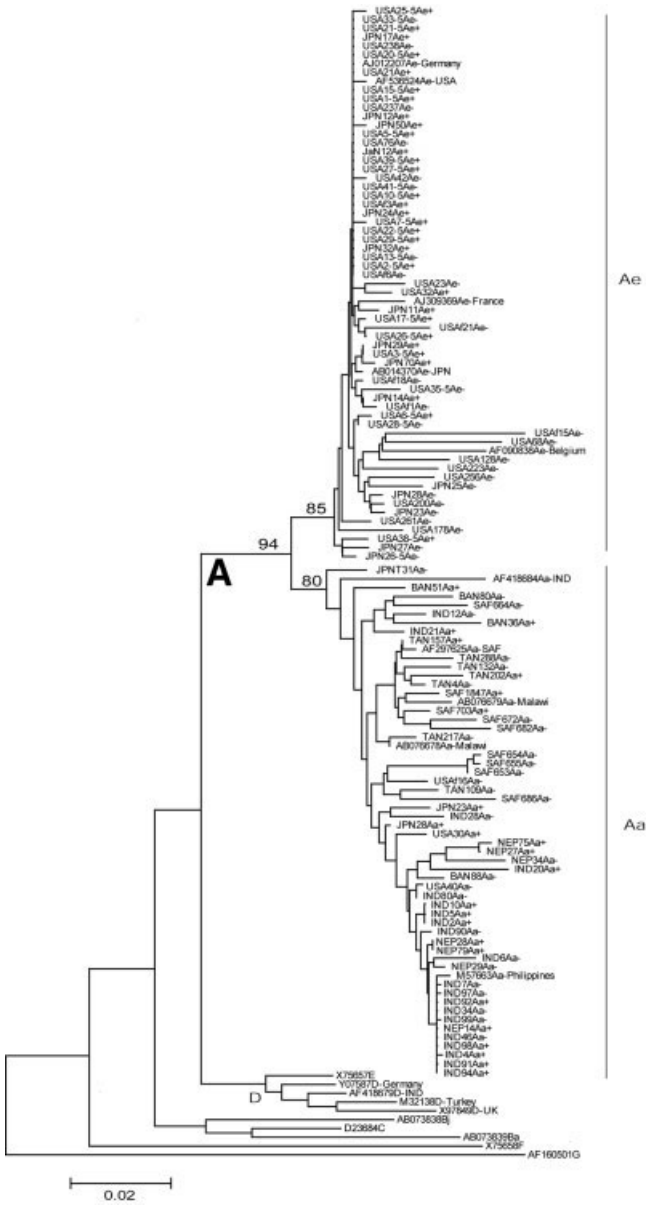

Fig. 1. A phylogenetic tree constructed on the X gene, precore region and core gene sequences. Together with 14 sequences of hepatitis B virus genotype A (HBV/A) and hepatitis B virus genotype D (HBV/D) isolates retrieved from the database, the 111 HBV/A sequences determined in the present study were subjected to phylogenetic analyses along with six HBV isolates representative of genotypes Ba, Bj, C, E, F, and G, respectively. Isolates sequenced in the present study are shown with the country of origin in abbreviation for Bangladesh (BAN), India (IND), Japan (JPN), Nepal (NEP), South Africa (SAF), Tanzania (TAN), and the United States (USA). Reference isolates from the database are identified with accession numbers. The numbers on nodes indicate bootstrap reliability (1,000×). Plus and minus signs at the end of isolates represent positive and negative hepatitis B e antigen in serum of carriers from whom HBV isolates were recovered.

three U.S. patients were Asians from the Philippines. Although their sequences diverged widely, no clusterings were indigenous to any countries of origin. In contrast, the 57 HBV/Ae isolates, including 14 from Japan and 43 from the United States, had less sequence divergence and clustered closely with the HBV/Ae isolates from the Western countries and Japan retrieved from the database. Thus, the mean genetic distance in the target sequence among HBV/Aa isolates was significantly greater than that among HBV/Ae isolates (0.0279 ± 0.0045 vs. 0.0098 ± 0.0017; $P < .01$), thereby suggesting that HBV/Aa would be of a much older phylogenetic origin and have infiltrated into the world more deeply than HBV/Ae. There were no differences in the HBeAg status between carriers of HBV/Aa and HBV/Ae from whom they were recovered (24/54 [44%] vs. 30/57 [53%]). The 61 HBV/D isolates, including 49 from India and 12 from the United States, clustered with the HBV/D isolates from the Western countries and India.

Serum HBeAg in Patients Infected With HBV/Aa, HBV/Ae, or HBV/D. Table 1 compares the HBeAg status and HBV DNA levels, as well as mutations in the core promoter and precore region, among 78 each carriers of HBV/Aa, HBV/Ae, or HBV/D in a case-control study. There were no significant differences in ALT levels among the patients, because they were equalized for the severity of liver disease. The prevalence of HBeAg was significantly lower in the carriers of HBV/Aa than in the carriers of HBV/Ae (24/78 [31%] vs. 38/78 [49%]; $P = .033$), although there were no significant differences between HBV/A (HBV/Aa or HBV/Ae) and HBV/D infections. Figure 2 depicts the prevalence of HBeAg in carriers of HBV/Aa, HBV/Ae, or HBV/D stratified by the age. The prevalence of HBeAg tended to decrease with age in all carriers, regardless of HBV genotypes. HBeAg was significantly less frequent in carriers of HBV/Aa than in carriers of HBV/Ae who were aged 30 years or younger (11/32 [34%] vs. 16/24 [67%]; $P = .029$).

HBV DNA Levels in Patients Infected With HBV/Aa, HBV/Ae, or HBV/D, with or Without Serum HBeAg. In the patients with HBeAg in serum, HBV DNA levels were significantly lower in those infected with HBV/Aa (median, 5.49 log copies/mL; 95% CI, 4.64–6.79]) than in those infected with HBV/Ae (median, 8.36 log copies/mL; 95% CI, 7.62–8.67) or HBV/D (8.57 log copies/mL; 95% CI, 7.73–8.91; $P < .0001$; Fig. 3). In the HBeAg-negative patients, HBV DNA levels were significantly lower in those carrying HBV/Aa (2.74 log copies/mL; 95% CI, 2.05–3.32) than in those carrying HBV/Ae (3.60 log copies/mL; 2.90–4.46; $P = .0031$) and those carrying HBV/D (3.63 log copies/mL; 95% CI, 3.12–4.13; $P = .034$; Fig. 3).

Mutations in the Core Promoter and Precore Region in Patients Infected With HBV/Aa, HBV/Ae, or HBV/D. An alignment of sequences covering the basic core promoter and the encapsidation signal (ϵ) in HBV of distinct genotypes or subtypes allowed the identification

Table 1. Demographic, Clinical and Virological Characteristics of Patients Infected with HBV of Genotype Aa, Ae, or D

Features	HBV Genotypes			Differences
	Aa (n = 78)	Ae (n = 78)	D (n = 78)	*P* Value
Men	65 (83%)	65 (83%)	65 (83%)	Matched
Age (yr)	37.9 ± 14.0	39.1 ± 12.6	37.6 ± 12.6	Matched
Clinical status				
Asymptomatic	22 (28%)	22 (28%)	22 (28%)	Matched
Liver disease	56 (72%)	56 (72%)	56 (72%)	Matched
Cirrhosis (HCC)	24 (5)	25 (2)	26 (8)	
ALT (IU/L)	36 (26–43)	36 (29–62)	36 (32–46)	NS
HBeAg	24 (31%)	38 (49%)	29 (37%)	.033†
HBV DNA (log copies/mL)	3.46*	6.09	5.48	<.001
95% CI	(2.93–3.95)	(4.24–7.64)	(4.06–7.02)	
Mutations in the core promoter				
T1762/A1764	27/54 (50%)	25/57 (44%)	15/61 (25%)*	<.01
Mutations in the ATG initiator codon in the precore region				
T1809	54/54 (100%)*	0/50 (0%)	0/61 (0%)	<.0001
T1812	52/54 (96%)*	0/57 (0%)	0/61 (0%)	<.0001
Mutations in the precore region				
T1858	0/54 (0%)	0/57 (0%)	61/61 (100%)*	<.0001
T1862	40/54 (74%)*	0/57 (0%)	0/61 (0%)	<.0001
H1888	42/54 (78%)*	0/57 (0%)	0/61 (0%)	<.0001
A1896	0/54 (0%)	0/57 (0%)	29/61 (48%)*	<.0001

Note. Median values and 95% confidence intervals in parentheses are shown for ALT and HBV DNA.
*Significantly different from the other genotypes.
†Significantly different between Aa and Ae.

of specific substitutions in HBV/Aa isolates, namely T1809, T1812, T1862, and H1888 (H representing non-G; Fig. 4A), which were not present in HBV/Ae (Fig. 4B) or HBV/D isolates (data not shown). T1809 and T1812 prevailed in HBV/Aa isolates (54/54 [100%] and 52/54 [96%], respectively), but were not detected in any HBV/Ae or HBV/D isolates. Triple nucleotide substitutions at positions 1809, 1811, and 1812 were detected in 12 HBV/Aa isolates recovered from carriers negative for serum HBeAg. Remarkably, the point mutations within ϵ, G to T at nt 1862 and G to A, C, or T at nt 1888, occurred frequently in HBV/Aa isolates (40/54 [74%] and 42/54 [78%], respectively) but were not seen in any HBV/Ae (0/57) or HBV/D isolates (0/61).

The precore stop mutation (A1896), accompanied by a C-to-T mutation at nt 1858 forming a base pair with it, was not found in HBV/Aa isolates, whereas another pre-

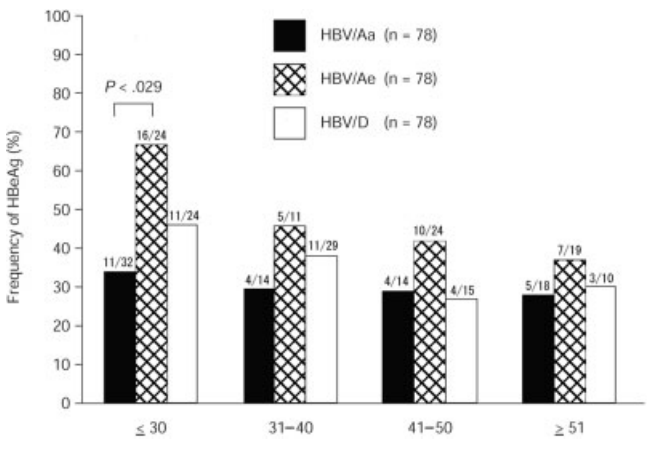

Fig. 2. Age-specific prevalence rates of hepatitis B e antigen (HbeAg) in carriers of hepatitis B virus genotype Aa (HBV/Aa), hepatitis B virus genotype Ae (HBV/Ae), or hepatitis B virus genotype D (HBV/D).

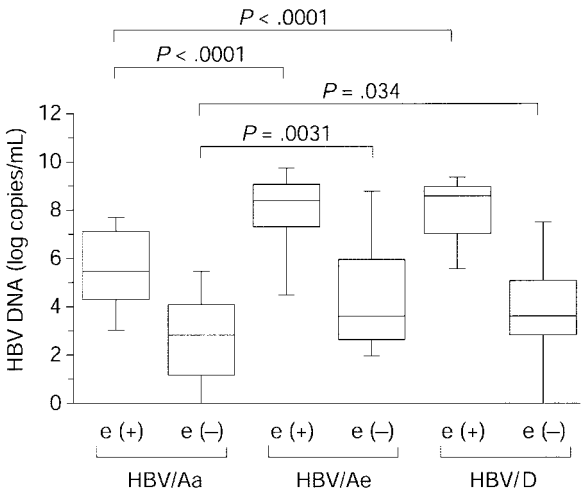

Fig. 3. Hepatitis B virus (HBV) DNA levels in carriers of hepatitis B virus genotype Aa (HBV/Aa), hepatitis B virus genotype Ae (HBV/Ae), and hepatitis B virus genotype D (HBV/D) with or without hepatitis B e antigen (HBeAg) in serum. HBV DNA was quantitated by real-time detection polymerase chain reaction, and the results are shown in log copies per milliliter. The box indicates 75 (upper edge), 50 (in between [median]), and 25 (lower edge) percentiles and bars indicate 90 and 10 percentiles, respectively.

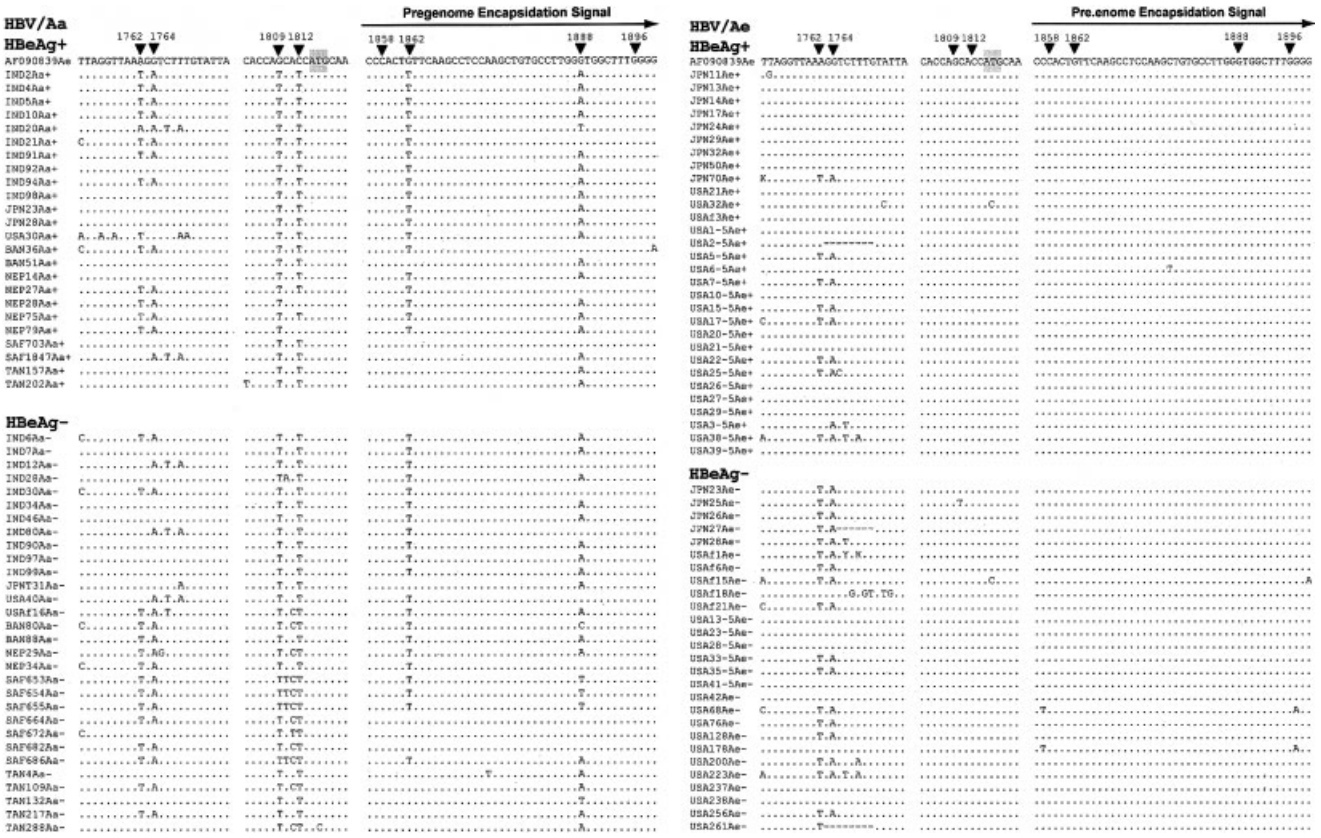

Fig. 4. Nucleotide sequences spanning the basic core promoter and pregenome encapsidation signal in hepatitis B virus (HBV) isolates of (A) subtype Aa and (B) subtype Ae. Positions of the double mutation (nucleotide [nt] 1762 and 1764), two substitutions frequent in subtype Aa (nt 1809 and 1812), and C1858 making a pair with G1896, as well as those of mutations at nt 1862 and nt 1888 specific for subtype Aa are indicated by inverted triangles. The ATG initiation codon of the precore region is shaded. The reference sequence is shown for the AF090839Ae of subtype Ae at the top. HBeAg, hepatitis B e antigen; +, positive; –, negative; BAN, Bangladesh; IND, India; JPN, Japan; NEP, Nepal; SAF, South Africa; TAN, Tanzania; USA, United States.

core stop mutation (A1897), accompanied by a C-to-T mutation at nt 1857, was found in two HBV/Ae isolates from HBeAg-negative carriers in the United States (Fig. 4B). The double mutation in the basic core promoter (T1762/A1764) was found significantly less often in carriers of HBV/D than in carriers of HBV/Aa (25% vs. 50%; $P = .0065$) or HBV/Ae (25% vs. 44%; $P = .033$), with remarkably more mutations in carriers of HBV/Aa (Table 1). Figure 5 compares the frequency of core promoter and precore mutations between carriers of HBV/Aa, HBV/Ae, or HBV/D with and without HBeAg in the serum. The basic core promoter double mutation was significantly more frequent in HBV/Ae carriers without than with HBeAg (63% vs. 27%; $P = .013$). In sharp contrast, the HBeAg status did not influence the frequency of these mutations in HBV/Aa carriers. In HBV/D carriers, both core promoter and precore stop mutations were significantly more common in those without than with HBeAg (36% vs. 11%; $P = .035$; and 67% vs. 25%; $P = .0019$, respectively).

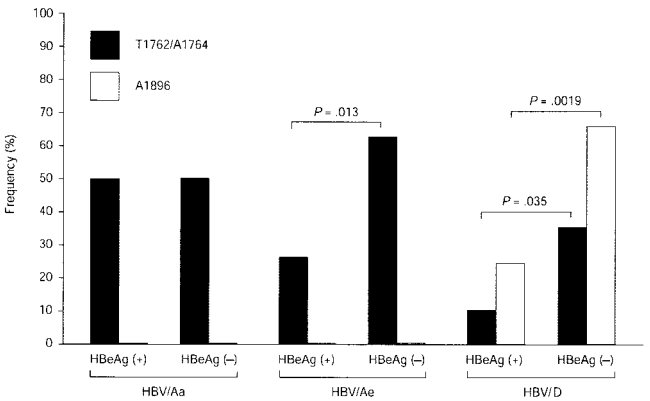

Fig. 5. The frequency of basic core promoter (BCP) mutation (T1762/A1764) and precore mutation (A1896) among carriers of hepatitis B virus genotype Aa (HBV/Aa), hepatitis B virus genotype Ae (HBV/Ae), and hepatitis B virus genotype D (HBV/D) with or without serum hepatitis B e antigen (HBeAg). The prevalence of BCP mutation in HBV/Ae isolates from carriers without HBeAg was significantly higher than that from those with HBeAg (63% vs. 27%; $P = .013$). The prevalence of BCP or precore mutation in HBV/D isolates from carriers without HBeAg was significantly higher than that from those with HBeAg (36% vs. 11%; $P = .035$; or 67% vs. 25%; $P = .0019$). +, positive; –, negative.

Discussion

In the present case-control study, the prevalence of HBeAg was found to be significantly lower in the carriers of HBV/Aa than in carriers of HBV/Ae. This is consistent with the observation that Africans infected with HBV frequently lose HBeAg from serum in infancy or early childhood,[28,29] in contrast to inhabitants of other countries where HBV is endemic, who lose HBeAg later in life.[37–39] Moreover, subtype Aa, first reported in 1997 as subgroup A′ by Bowyer et al.,[19] is the major genotype of HBV in southern Africa[19,21,22] and is common in young patients with HCC in South Africa (Kew MC et al., submitted for publication, 2004).[40] These findings suggest that HBV/Aa in Africa is distinct from HBV/Ae in Western countries[20–22] and is associated with HCC in young ages (Kew MC et al., submitted for publication, 2004).

HBV/Aa isolates have been reported to have distinctive sequence characteristics in the basic core promoter and precore or core regions that could affect HBeAg expression at different levels.[20,21] In this study, the double mutation in the core promoter (T1762/A1764) that has been shown to affect transcription[41] was found significantly more frequently in HBV/Ae isolates without than with HBeAg, suggesting that it would be associated with HBeAg clearance in HBV/Ae carriers (Fig. 5). However, although T1762/A1764 was highly prevalent in isolates from carriers of HBV/Aa, it did not associate with the HBeAg status. This is in agreement with the results of a previous study that also showed a high prevalence of these mutations in HCC patients compared with asymptomatic carriers (66% vs. 11%).[26] Furthermore, the classic precore stop codon mutation (A1896) was detected more often in HBV/D than in HBV/Aa or HBV/Ae isolates.

At the level of translation, double nucleotide substitutions at nt 1809 and 1812 immediately upstream of the precore ATG initiation codon, 5′-*T*CA*T*CATGC-3′ (precore start codon underlined and double substitutions in italics), and occasional triple nucleotide substitutions with an additional mutation at nt 1811 were described by Baptista et al.[26] and Ahn et al.[27] Using elegant expression studies, Ahn et al.[27] demonstrated that an adenosine or guanine, at the −3 position with reference to the ATG start codon, affected the translation efficiency of the HBeAg precursor by a leaky scanning mechanism,[42] providing an account on the early loss of HBeAg in carriers of HBV/Aa in Africa.[28,29] Notably, these 1809–1812 mutations are highly prevalent in HBV/Aa strains from South Africans with acute infection, during childhood, and in the HBeAg-positive phase of infection,[27] indicating these mutations as stable traits of HBV/Aa, rather than as adaptive changes under anti-HBe immune pressure. Indeed, simultaneous substitutions at nt 1809 and 1812 have not been documented in any HBV isolates other than HBV/Aa from the database. Inasmuch as carriers harbor HBV/Aa since infancy in Africa, the process for HBeAg loss may start from the outset for an early seroconversion.[28,29] In outstanding contrast, carriers of HBV/Ae or HBV/D contract infection with the wild-type with no mutations, causing loss or downregulation of HBeAg synthesis. Furhtermore, HBV DNA levels in the carriers of HBV/Aa, who would have a high rate of HBeAg clearance in young ages,[28,29] were significantly lower than those in the carriers of HBV/Ae or HBV/D, regardless of HBeAg in serum (Fig. 3). These data indicate a mechanism of HBeAg seroconversion different between carriers of HBV/A and HBV/D, or even between carriers with distinct subtypes of genotype A, Aa, and Ae. Such different characteristics among carriers of HBV/Aa, HBV/Ae, and HBV/D mimic those among subtypes Ba and Bj of genotype B and among HBV/C infections in Asian countries. In our previous case-control study, HBeAg and the double mutation in the core promoter were significantly more frequent in carriers of HBV/C than HBV/B.[10] Furthermore, the core promoter mutation was significantly more frequent in carriers of HBV/Ba than HBV/Bj, in sharp contrast to the precore stop mutation that was more frequent in carriers of HBV/Bj than in carriers of HBV/Ba or HBV/C.[18]

Of an additional virological relevance, nt 1862 was invariably G in all HBV/Ae and HBV/D isolates, whereas it was T in 40 of the 54 (74%) HBV/Aa isolates. Furthermore, nt 1888 was exclusively G in HBV/Ae isolates, but it was replaced by A in 36, C in 1, and T in 5, adding up to 42 of the 54 (78%) HBV/Aa isolates, in confirmation of earlier reports.[20,21,24,25] These two nucleotides are positioned in the 6-nt bulge and upper stem, respectively, and make essential elements in the pregenome encapsidation signal. The nt 1862 is G in the wild-type HBV and occupies the third position in the 6-nt bulge. The conversion of G1862 to any of the other three nucleotides can affect the replication of HBV[43] and can reduce the production of HBeAg at the post-translation level in expression studies *in vitro*.[44]

At the protein level, substitutions of nt 1809 and 1812 result in double amino acid replacements in the X protein, A146S and P147S. The core promoter substitutions at nt 1762 and 1764, which are prevalent in HBV/Aa, also result in double amino acid changes in the X protein, K130M and V131I. As the transactivation domain of X protein is mapped in the carboxyl-terminal portion,[45,46] these changes not only may affect the replication of HBV, but also may play a role in HBV-related hepatocarcinogenesis as previously proposed.[47] Because sub-Saharan Af-

ricans take the highest dietary aflatoxin B1, which induces typical G-to-C to T-to-A transversions at the third base in codon 249 of p53,[48] making it, together with HBV, the major causes of HCC mortality in this geographic area, further studies are necessary.

In the U.S. population, there was an association between ethnicity and HBV genotypes. Among 52 U.S. patients with HBV/Ae, most of them were white persons born in the United States and included one African American, three Hispanics, and two Asians born in the Philippines. However, five of six U.S. carriers with HBV/Aa were Asians from the Philippines. Among 23 patients with HBV/D, most were white, some of whom were born in Europe, and four were Asians born in India, Afghanistan, or Samoa. In agreement with the distribution of HBV genotypes in association with prevalent HBV genotypes in the birthplace,[7] the present study reinforced a possible association between ethnicity and subtypes (Aa, Ae) of HBV/A among U.S. carriers.

In conclusion, specific mutations in HBV/Aa isolates affect the translation of HBeAg, as well as the replication of HBV, and may modify clinical outcomes of HBV infections. A prospective study in carriers of HBV/Aa isolates, with and without simultaneous substitutions at nt 1809 and 1812, is required to evaluate the validity of this hypothesis. Because HBV/Aa strains are different both in virological characteristics and clinical manifestations from HBV/Ae and HBV/D strains, these two subtypes of HBV/A would need to be distinguished. It is hoped that the results of this study would encourage research in a number of epidemiological and clinical settings of Western countries, where genotypes A and D prevail, for evaluating clinical outcomes that would be different between carriers not only of these two genotypes, but also subtypes Aa and Ae of genotype A.

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References

1. Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol 1988;69: 2575–2583.
2. Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 1994;198:489–503.
3. Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 2000;81:67–74.
4. Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol 2002;83:2059–2073.
5. Magnius LO, Norder H. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. Intervirology 1995;38:24–34.
6. Miyakawa Y, Mizokami M. Classifying hepatitis B virus genotypes. Intervirology 2003;46:329–338.
7. Chu CJ, Keeffe EB, Han SH, Perrillo RP, Min AD, Soldevila-Pico C, et al. Hepatitis B virus genotypes in the United States: results of a nationwide study. Gastroenterology 2003;125:444–451.
8. Vieth S, Manegold C, Drosten C, Nippraschk T, Gunther S. Sequence and phylogenetic analysis of hepatitis B virus genotype G isolated in Germany. Virus Genes 2002;24:153–156.
9. Kato H, Orito E, Gish RG, Bzowej N, Newsom M, Sugauchi F, et al. Hepatitis B e antigen in sera from individuals infected with hepatitis B virus of genotype G. HEPATOLOGY 2002;35:922–929.
10. Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, et al. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. HEPATOLOGY 2001;33: 218–223.
11. Chu CJ, Hussain M, Lok AS. Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C. Gastroenterology 2002;122:1756–1762.
12. Kao JH, Chen PJ, Lai MY, Chen DS. Genotypes and clinical phenotypes of hepatitis B virus in patients with chronic hepatitis B virus infection. J Clin Microbiol 2002;40:1207–1209.
13. Wai CT, Chu CJ, Hussain M, Lok AS. HBV genotype B is associated with better response to interferon therapy in HBeAg(+) chronic hepatitis than genotype C. HEPATOLOGY 2002;36:1425–1430.
14. Mayerat C, Mantegani A, Frei C. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? J Viral Hepatitis 1999; 6:299–304.
15. Thakur V, Guptan RC, Kazim SN, Malhotra V, Sarin SK. Profile, spectrum and significance of HBV genotypes in chronic liver disease patients in the Indian subcontinent. J Gastroenterol Hepatol 2002;17:165–170.
16. Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, et al. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J Virol 2002;76: 5985–5992.
17. Akuta N, Suzuki F, Kobayashi M, Tsubota A, Suzuki Y, Hosaka T, et al. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J Hepatol 2003;38:315–321.
18. Sugauchi F, Orito E, Sakugawa H, Ichida H, Gish RG, Chutaputti A, et al. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 2003;124: 925–932.
19. Bowyer SM, van Staden L, Kew MC, Sim JG. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. J Gen Virol 1997;78:1719–1729.
20. Sugauchi F, Kumada H, Acharya SA, Shrestha SM, Gamutan MTA, Khan M, et al. Epidemiological and virological differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. J Gen Virol 2004; 85:811–820.
21. Kimbi G, Kramvis A, Kew M. Distinctive Sequence characteristics of subgenotype A1 isolates of hepatitis B virus From South Africa. J Gen Virol 2004;85:1211–1220.
22. Kramvis A, Weitzmann L, Owiredu WK, Kew MC. Analysis of the complete genome of subgroup A' hepatitis B virus isolates from South Africa. J Gen Virol 2002;83:835–839.

23. Sugauchi F, Orito E, Kato H, Suzuki S, Kawakita S, Sakamoto Y, et al. Genotype, serotype, and phylogenetic characterization of the complete genome sequence of hepatitis B virus isolates from Malawian chronic carriers of the virus. J Med Virol 2003;69:33–40.
24. Kramvis A, Bukofzer S, Kew MC, Song E. Nucleic acid sequence analysis of the precore region of hepatitis B virus from sera of southern African black adult carriers of the virus. HEPATOLOGY 1997;25:235–240.
25. Kramvis A, Kew MC, Bukofzer S. Hepatitis B virus precore mutants in serum and liver of Southern African Blacks with hepatocellular carcinoma. J Hepatol 1998;28:132–141.
26. Baptista M, Kramvis A, Kew MC. High prevalence of 1762(T) 1764(A) mutations in the basic core promoter of hepatitis B virus isolated from black Africans with hepatocellular carcinoma compared with asymptomatic carriers. HEPATOLOGY 1999;29:946–953.
27. Ahn SH, Kramvis A, Kawai S, Spangenberg HC, Li J, Kimbi G, et al. Sequence variation upstream of precore translation initiation codon reduces hepatitis B virus e antigen production. Gastroenterology 2003;125: 1370–1378.
28. Botha JF, Ritchie MJ, Dusheiko GM, Mouton HW, Kew MC. Hepatitis B virus carrier state in black children in Ovamboland: role of perinatal and horizontal infection. Lancet 1984;1:1210–1212.
29. Dusheiko GM, Bowyer SM, Paterson A, Song E, Dibisceglie A, Kew MC. Clinical and serological events accompanying changes in hepatitis B viral replication: case reports. Liver 1985;5:77–83.
30. Usuda S, Okamoto H, Tanaka T, Kidd-Ljunggren K, Holland PV, Miyakawa Y, et al. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. J Virol Methods 2000;87:81–89.
31. Abe A, Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R, et al. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. J Clin Microbiol 1999;37:2899–2903.
32. Hasegawa I, Tanaka Y, Kramvis A, Kato T, Sugauchi F, Acharya SK, et al. A novel hepatitis B virus genotype A subtyping assay that distinguishes subtypes Aa from Ae and its application in epidemiological studies. J Virol 2004;78:7575–7581.
33. Sugauchi F, Mizokami M, Orito E, Ohno T, Kato H, Suzuki S, et al. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. J Gen Virol 2001;82:883–892.
34. Gojobori T, Ishii K, Nei M. Estimation of average number of nucleotide substitutions when the rate of substitution varies with nucleotide. J Mol Evol 1982;18:414–423.
35. Robertson B, Myers G, Howard C, Brettin T, Bukh J, Gaschen B, et al. Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. Arch Virol 1998;143:2493–2503.
36. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. HEPATOLOGY 1994;19:1513–1520.
37. Fattovich G, Giustina G, Schalm SW, Hadziyannis S, Sanchez-Tapias J, Almasio P, et al. Occurrence of hepatocellular carcinoma and decompensation in western European patients with cirrhosis type B. HEPATOLOGY 1995;21:77–82.
38. Lee WM. Hepatitis B virus infection. N Engl J Med 1997;337:1733–1745.
39. Lok AS, Heathcote EJ, Hoofnagle JH. Management of hepatitis B: 2000—summary of a workshop. Gastroenterology 2001;120:1828–1853.
40. Kew MC, Macerollo P. Effect of age on the etiologic role of the hepatitis B virus in hepatocellular carcinoma in blacks. Gastroenterology 1988;94: 439–442.
41. Buckwold VE, Xu Z, Chen M, Yen TS, Ou JH. 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.
42. Kozak M. Initiation of translation in prokaryotes and eukaryotes. Gene 1999;234:187–208.
43. Nassal M, Rieger A. A bulged region of the hepatitis B virus RNA encapsidation signal contains the replication origin for discontinuous first-strand DNA synthesis. J Virol 1996;70:2764–2773.
44. Hou J, Lin Y, Waters J, Wang Z, Min J, Liao H, et al. Detection and significance of a G1862T variant of hepatitis B virus in Chinese patients with fulminant hepatitis. J Gen Virol 2002;83:2291–2298.
45. Murakami S, Cheong JH, Kaneko S. Human hepatitis virus X gene encodes a regulatory domain that represses transactivation of X protein. J Biol Chem 1994;269:15118–15123.
46. Li J, Buckwold VE, Hon MW, Ou JH. Mechanism of suppression of hepatitis B virus precore RNA transcription by a frequent double mutation. J Virol 1999; 73:1239–1244.
47. Kao JH, Chen PJ, Lai MY, Chen DS. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. Gastroenterology 2003;124:327–334.
48. Bressac B, Kew M, Wands J, Ozturk M. Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. Nature 1991;350: 429–431.