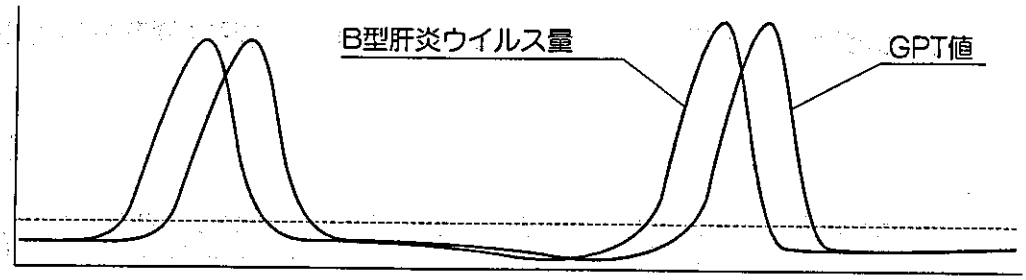
Q4 肝機能が悪くないのに定期検査を受ける必要がありますか？

慢性B型肝炎の人は、肝機能（GPT、GOTなど）の値が正常と異常をくりかえす場合があります。ウイルスがいるといわれたけれども、肝機能が正常だから大丈夫というのは誤りです。肝機能が正常であっても、ウイルスがいる限りは、知らない間に肝硬変や肝がんに進むことがありますので、最低でも半年に1回は定期的に検査をする必要があります。



慢性B型肝炎では採血する時期によって肝機能が正常値になったりします

Q5 他の人にうつさないために、日常生活で気をつけることはどんなこと？

次のことを守っていれば周囲の人への感染はありませんので、日常生活で神経質になる必要はありません。

1　カミソリや歯ブラシなどの共用は避けましょう。
2　血液や分泌物がついたものは、くるんで捨てるか、洗い流しましょう。
3　けが、皮膚炎、鼻血などは、できるだけ自分で手当てしましょう。
4　乳幼児に、口うつしで食物を与えないようにしましょう。
5　献血はしないようにしましょう。

Q6 治療は必要ですか？治るんですか？

精密検査で肝臓の状態がどうなっているかを調べてから、治療が必要かどうかを決めます。慢性肝炎で肝機能が異常とわかれば、肝硬変や肝がんにならないようにするために治療が必要です。最近では飲み薬で特効薬が開発されました。B型肝炎ウイルスが消えてしまうことは稀ですが、肝硬変や肝がんになることを防ぐことが出来ます。慢性肝炎で治療も検査も要らないということはありません。

病気のことでわからないことがありましたら、
主治医または市町村保健師に御相談ください。

【お願い】

市町村保健師が、今後も年１回程度、検査や治療についての悩みをうかがい、ご支援をさせていただくために、連絡をいたします。御了承下さい。

石川県肝炎対策検討会
事務局　石川県健康福祉部健康推進課
TEL. 076(225)1436

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Identification of Rare Polymerase Variants of Hepatitis B Virus Using a Two-Stage PCR With Peptide Nucleic Acid Clamping

Waka Ohishi, Hiroo Shirakawa, Yoshiiku Kawakami, Sonoko Kimura, Masaya Kamiyasu, Susumu Tazuma, Toshio Nakanishi, and Kazuaki Chayama*

Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

Emergence of lamivudine-resistant variants, with amino acid substitutions in the Tyr-Met-Asp-Asp (YMDD) motif of hepatitis B virus (HBV) reverse transcriptase, is a serious problem in antiviral therapy. Presence of YMDD motif variants in patients who had never been treated with lamivudine has been reported recently. However, no analysis of nucleotide and amino acid sequences of these variants has been performed. In the present study, using polymerase chain reaction (PCR) with peptide nucleic acid (PNA) clamping, we detected many new variants, such as Tyr-Arg-Asp-Asp (YRDD), Tyr-Met-Asp-Asn (YMDN). Many of them had stop codon(s) in overlapping *HBs* gene. Although the biological activity of these HBV polymerase variants remains to be determined, our results showed that numerous quasispecies are created during virus replication. A typical lamivudine-resistant Tyr-Val-Asp-Asp (YVDD) variant was detected in only one of 62 (1.6%) anti-HBe patients with HBV infection before administration of lamivudine. This variant did not have the L528M mutation, which is often associated with YVDD variants, and lamivudine therapy in this patient suppressed HBV replication. Thus, care should be taken when interpreting the results of detection of YMDD variants, especially when the sensitivity of the assay is very high. Amplification of rare variants by PCR with PNA seems a useful tool to examine the emergence of drug-resistant variants as well as naturally occurring mutants, such as the hepatitis B e antigen (HBeAg) stop codon and vaccine escape mutants. Examination of rare variants should enhance the understanding of the mechanism for emergence of drug-resistant HBV variants and help in developing strategies for new antiviral drugs. ***J. Med. Virol. 72:558–565, 2004.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: hepatitis B virus; peptide nucleic acid; lamivudine; quasispecies; YMDD variant

INTRODUCTION

Chronic viral infections are serious health problems worldwide. Antiviral therapy using nucleotide analogs is effective in reducing viral load and disease activity. Lamivudine, (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (3TC) and famciclovir, 2-[2-(2-amino-9H-purin-9-yl) ethyl]-1,3-propanediol diacetate have been shown to reduce hepatitis B virus (HBV) DNA load and reduce inflammatory activity in the liver [Honkoop et al., 1997a; Lai et al., 1997, 1998; Nevens et al., 1997; Dienstag et al., 1999; Suzuki et al., 1999; Liaw et al., 2000] although their effects on patient survival have not yet been determined.

The emergence of drug-resistant variants is one of the most serious problems associated with antiviral therapy. Lamivudine is the most widely used anti-HBV drug worldwide. It is known to inhibit both HBV and human immunodeficiency virus (HIV) reverse transcriptase [Honkoop et al., 1997a; Lai et al., 1997, 1998; Nevens et al., 1997; Dienstag et al., 1999; Suzuki et al., 1999; Liaw et al., 2000]. Although the initial effect of the drug is excellent in suppressing HBV replication and reducing alanine aminotransferase activity, the emergence

*Correspondence to: Prof. Kazuaki Chayama, Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan.
E-mail: chayama@hiroshima-u.ac.jp

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of drug-resistant variants reduces considerably the effect of the drug [Honkoop et al., 1997b; Allen et al., 1998; Chayama et al., 1998; Niesters et al., 1998; Liaw et al., 1999; Ono-Nita et al., 1999a,b]. Lamivudine-resistant variants have unique amino acid substitutions in the Tyr-Met-Asp-Asp (YMDD) motif of the reverse transcriptase. One class of the variants, Tyr-Val-Asp-Asp (YVDD) variant, is usually associated with additional amino acid substitutions in the B domain of the reverse transcriptase, L528M. The other variant, Tyr-Ile-Asp-Asp (YIDD) variant, only occasionally exhibits this additional mutation. The YVDD variant associated with L528M shows the highest replication rate in the presence of lamivudine [Gutfreund et al., 2000; Ono-Nita et al., 2001].

YMDD variants emerge after a certain period of lamivudine therapy. This suggests that variants evolve during such therapy because the rate of nucleotide substitutions of the reverse transcriptase of HBV is quite high, though still controversial [Orito et al., 1989; Melegari et al., 1998]. However, some patients show relatively early emergence of drug-resistant strains, suggesting the existence of such strains prior to therapy. Viruses exist in the sera of infected patients as quasi-species. It is assumed that some of the quasispecies are inactive biologically because a proportion of these variants have stop codons that are thought to be fatal for the virus life cycle. Whether all the remaining variants without stop codons actually replicate in hosts is not known. To date, it has been difficult to analyse rare variants, for example, drug-resistant variants, before therapy, because they exist as rare quasispecies among the vast majority of wild-type virus. Previous studies that reported the presence of lamivudine-resistant variants in patients who received no lamivudine therapy [Kobayashi et al., 2001; Kirishima et al., 2002] did not determine the exact nature of such variants.

The aim of the present study was to detect these rare variants by using polymerase chain reaction (PCR) with peptide nucleic acid (PNA) clamping as reported previously [Kirishima et al., 2002]. PNA is a DNA analogue in which the ribose-phosphodiester backbone of DNA has been replaced by *N*-(2-aminoethyl) glycine linkages [Nielsen et al., 1991; Cherny et al., 1993; Egholm et al., 1993; Zhong et al., 1999]. The PNA anneals strongly to DNA like a complementary DNA, but with higher affinity. The annealing of the PNA to the target sequence prevents amplification of DNA in the PCR if the amplification conditions are appropriately optimised. We investigated nucleotide and amino acid sequences of resistant viral variants of HBV including YMDD variants that have amino acid substitutions in the YMDD motif of reverse transcriptase by using two-stage PCR with PNA clamping.

MATERIALS AND METHODS

Patients

Sixty-two Japanese adult patients with acute or chronic HBV infection before or without lamivudine therapy were studied. These patients were negative for hepatitis B e antigen (HBeAg), but positive for anti-hepatitis B e antibody (anti-HBe). We preferentially selected these anti-HBe positive patients because recent reports [Kobayashi et al., 2001; Kirishima et al., 2002] showed that YMDD variants were often detected in anti-HBe positive patients. Nineteen of our patients were treated with lamivudine. All serum samples analysed in this study were collected prior to therapy with informed consent and stored at −80°C. All patients were negative for markers of hepatitis C virus (HCV) and HIV. The clinicopathological profiles of these patients are shown in Table I.

Blood Analysis

Hepatitis B surface antigen (HBsAg) was determined by enzyme immunoassay (Roche Diagnostics, Basel, Switzerland). HBeAg and anti-HBe were tested by radioimmunoassay (Abbott Diagnostics, Chicago, IL). HBV DNA was measured by branched DNA signal amplification technology (Chiron Corp., Emeryville, CA), and the results were expressed as 10^6 genomic equivalents (Meq) per millilitre. The lower limit of the assay was 0.7 MEq/ml. Antibody against HCV was tested by the third-generation enzyme immunoassay (Roche Diagnostics). HBV genotype was determined using the method of Naito et al. [2001].

Detection of YMDD Variants by Two-Stage PCR With PNA Clamping and Restriction Fragment Length Polymorphism (RFLP)

HBV DNA was extracted from 100 μl of serum using SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μl of H_2O. Nested PCR was performed using a first primer set (PLF1 and BR123) and a second PCR primer set (PLF2 and PLR2). Nucleotide sequences of primers and the sequence of PNA used in this study are listed in Table II. PNA-mediated PCR clamping was optimised by changing the PNA annealing

TABLE I. Clinicopathological Features of Anti-HBe Positive Patients

Variable	No lamivudine therapy (n = 62)
Age (years)	48.5 ± 10.0
Sex (M/F)	48/14
Alanine aminotransferase (IU/L)[a]	55 (21–7,941)
HBV DNA (Meq/ml)	
ND	6
<0.7	34
≥0.7 to <100	17
≥100	5
Genotype (B:C:F:ND)	5:51:1:5
Clinicopathologic diagnosis	
Chronic hepatitis	26
Liver cirrhosis	8
Hepatocellular carcinoma	19
Acute hepatitis	1
Fulminant hepatitis	6
Normal	2

[a]Median (range).

TABLE II. Primers and Peptide Nucleic Acid (PNA) Used in the Present Study

Primer	Nucleotide sequence	Nucleotide
PLF1	Sense: 5′-GGTATGTTGCCCGTTTGTCC-3′	458–477
BR123	Antisense: 5′-TTCCAATTACATATCCCAT-3′	895–877
PLF2	Sense: 5′-CCTATGGGAGTGGGCCTCAG-3′	637–656
PLR2	Antisense: 5′-CCAATTACATATCCCATGAAGTTAAGGGA-3′	893–865
YNSspI	Sense: 5′-TTTCCCCCACTGTTTGGCTTTCAGTAATAT-3′	711–740
BR109	Antisense: 5′-AAGGGAGTAGCCCCAACGTT-3′	870–851
TMApaLI	Antisense: 5′-CAGACTTGGCCCCCAATACCACATCGTGCA-3′	769–740
PNA552	Antisense: H_2N-CACATCATCCATATAACT-CON_2H	750–733

Nucleotide sequence position numbers are those of Norder et al. [1994].

temperature from 68 to 75°C and the PNA concentration from 1.25 to 10 μM. Optimised PCR with PNA clamping was performed in a total volume of 25 μl, consisting of reaction buffer (100 mM Tris-HCl, [pH 8.3], 50 mM KCl, and 15 mM $MgCl_2$), 0.2 mM of each dNTP, 1 μl of the DNA solution, 12.5 pmol of each primer set, 150 pmol of PNA 552, and 1 U of Taq DNA polymerase (Gene Taq, Wako Pure Chemicals, Tokyo, Japan) with 0.2 μg of anti-Taq high (Toyobo Co., Osaka, Japan). The amplification conditions included initial denaturation at 95°C for 4 min, 25 cycles of amplification (denaturation at 95°C for 45 sec, PNA annealing at 73°C for 2 min, annealing and extension of primer at 63°C for 50 sec), followed by final extension at 63°C for 7 min. Detection of YVDD and YIDD variants was performed using primers TMApaLI and YNSspI, respectively, as described previously [Chayama et al., 1998].

The error rate of the Taq DNA polymerase was estimated to be 1.76×10^{-5} per site by amplifying about 100 copies of plasmid using the same conditions described above, followed by cloning and sequencing.

Cloning and Sequence Analysis of Rare Polymerase Variants

In this study, 1 μl of the above second PCR product amplified by two-stage PCR with PNA was amplified using primers PLF2 and BR109. Thirty-five cycles of PCR (94°C for 1 min; 58°C for 1 min; 72°C for 1.5 min) were performed after 4 min of initial denaturation at 94°C, followed by final extension for 7 min at 72°C. PCR-amplified DNA was purified after 2% agarose gel electrophoresis and cloned into pGEM-T Easy Vector (Promega Co., Madison, WI). Sequencing was performed using the ABI PRISM™ 310NT Genetic analyser (Applied Biosystems, Tokyo, Japan) with Big Dye terminator version 3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequences of independent clones from five of 62 anti-HBe patients were determined and analysed.

RESULTS

Detection of YMDD Variants in Serum Samples From 62 Anti-HBe Patients by Two-Stage PCR With PNA Clamping

By adding PNA in two-stage PCR and optimising the amplification conditions for suppression of wild-type virus, we were able to detect YVDD variants mixed in a larger amount of wild-type sequences. We used clones with nucleotide sequences corresponding to YMDD wild-type and YVDD variant as described previously [Chayama et al., 1998]. The nucleotide sequences of these plasmids in these motifs are TAT ATG GAT GAT and TAT GTG GAT GAT, respectively. As shown in Figure 1, YMDD variants were detected when mixed with the same amount of wild-type in the absence of PNA. In contrast, by introducing PNA, only 10^2 copies of YMDD variants were detected when mixed with 10^6 copies of wild-type. In fact, our method detected the YMDD variant mixed with the wild-type at a ratio of 1:10,000, and was 10,000-fold more sensitive than conventional RFLP without PNA (Fig. 1).

Using this method, YVDD variants were detected in only one of 62 (1.6%) serum samples from anti-HBe patients. Further analyses of nine serial serum samples obtained from this patient revealed that another sample before the start of lamivudine therapy was positive for YVDD variants. Unexpectedly, the patient was later treated with lamivudine and showed a good response to the therapy during at least 23 months, and HBV DNA was not detected by two-stage PCR with PNA clamping after such treatment (data not shown).

Analyses of Nucleotide and Amino Acid Sequences of Clones Obtained From a Serum Sample of a Patient who Possessed YVDD Variant

We examined quasispecies in one of the two serum samples that tested positive for the YVDD variants by cloning and sequencing after amplification of HBV DNA by two-stage PCR with PNA clamping. Analyses of amino acid sequences of 43 clones showed the presence of multiple rare variants such as YRDD and YMDN that had not been previously reported (Fig. 2). Identified nucleotide substitutions showed preferential transitions, particularly transitions from G to A (Fig. 2A). Thirty-seven of 43 clones had nucleotide substitutions of methionine and asparagine residues but none had substitutions of tyrosine residues. Only one of 43 clones sequenced had the YVDD sequence. This variant did not have the L528M mutation that has been reported to be often associated with the YVDD variant (Fig. 2B). Thirteen of 43 clones had YIDD amino acid sequences. However, nucleotide sequences of these YIDD variants (TAT ATA GAT GAT) (Fig. 2A) differed from the reported lamivudine-resistant variants (TAT ATT

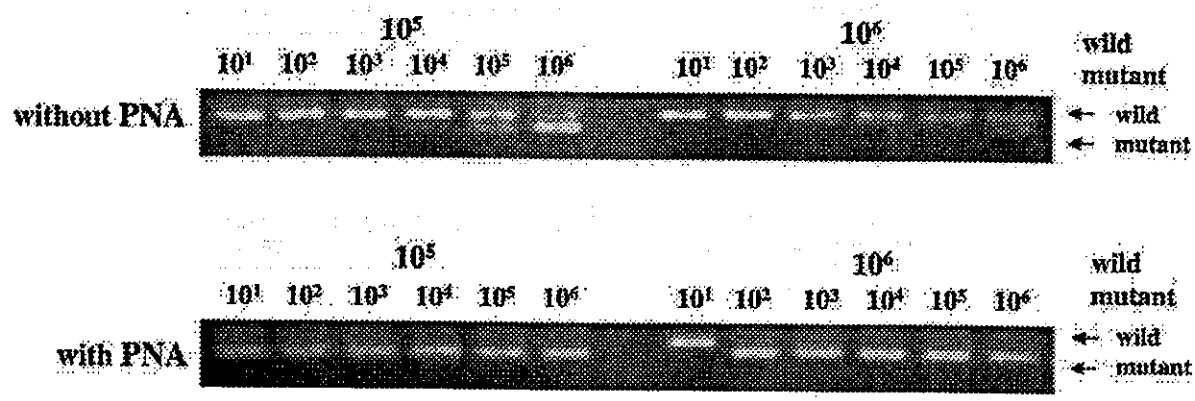

Fig. 1. Detection of the Tyr-Met-Asp-Asp (YMDD) variant by two-stage polymerase chain reaction (PCR) with peptide nucleic acid (PNA). 10^1–10^6 copies of Tyr-Val-Asp-Asp (YVDD) variant plasmid were mixed with 10^5 or 10^6 copies of wild-type plasmid. After amplification using primers PLF2 and TMApaL I, the amplified DNA was digested with restriction enzyme ApaLI and separated in a 3% agarose gel. The restriction fragment length polymorphism (RFLP) method involving PCR with the PNA clamping could detect only 10^2 copies of YVDD variants in the presence of 10^6 copies of the wild-type hepatitis B virus (HBV). Remarkably, PNA completely blocked 10^5 copies of wild-type HBV in the presence of 10^1–10^6 copies of the mutant type (YVDD variants).

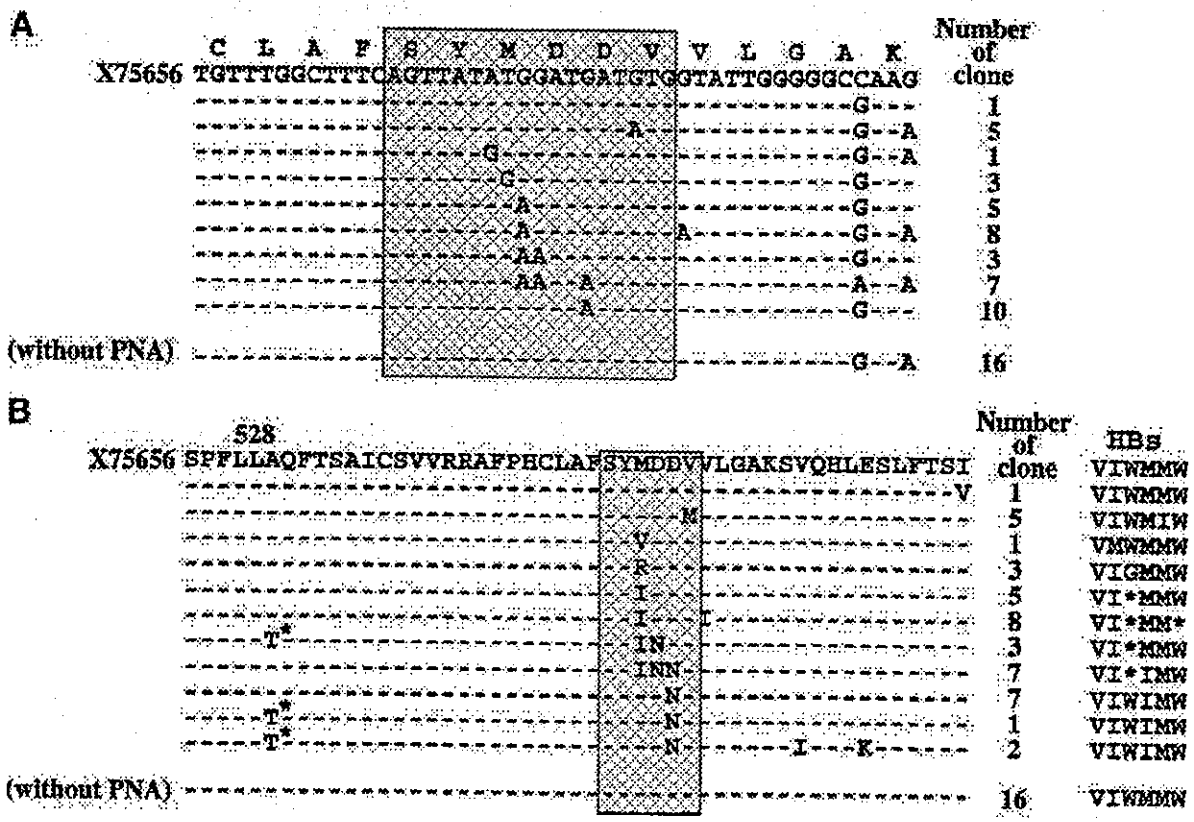

Fig. 2. Nucleotide (A) and amino acid (B) sequences of 43 clones obtained from a serum sample of a patient who possessed YVDD variant before the start of lamivudine therapy. Dark boxes indicate nucleotide and amino acid sequences where the PNA was assumed to anneal to the wild-type sequence of the YMDD motif. Nucleotide and amino acid sequences of 16 clones amplified without PNA are also shown. Created stop codons in the overlapping envelope (S) gene are labeled with asterisks.

GAT GAT) and were undetectable by RFLP because the amplified DNA fragment from this sequence using primers for the detection of the lamivudine-resistant YIDD variant (YNSspI) did not produce an SspI restriction site. These YIDD variants had stop codon(s) in the overlapping *HBs* gene, suggesting that all 13 YIDD variants might be biologically inactive. Eight of these 13 clones had an additional stop codon just adjacent to the YMDD motif. Similarly, Tyr-Ile-Asn-Asp (YIND) and Tyr-Ile-Asn-Asn (YINN) variants had stop codons in the *HBs* gene. Figure 2B shows the amino acid sequences surrounding the YMDD motif. There are a few amino acid substitutions in this area, and some of them had an additional stop codon (Fig. 2B).

We also determined the nucleotide sequences of DNA fragments amplified using similar amplification conditions without PNA. Although six of 43 clones obtained after amplification with PNA had the wild-type YMDD sequence, all 16 clones obtained without PNA did not have any nucleotide and amino acid substitutions in the YMDD motif (Fig. 2).

Analyses of Nucleotide and Amino Acid Sequences of Clones Obtained From Four of 62 Anti-HBe Patients With Chronic HBV Infection

We then examined the nucleotide and amino acid sequences of 41 clones obtained from four of 62 patients with chronic HBV infection, by cloning and sequencing after two-stage PCR with PNA. As shown in Figure 3, multiple YMDD variants with nucleotide and amino acid substitutions were identified in Patient 1. In contrast, none of ten clones obtained from Patient 2 had amino acid substitutions in the YMDD motif. There were many stop codons in the overlapping S gene in clones obtained from Patient 1, but none of 10 clones obtained from Patient 2 had any stop codon in the polymerase and S gene (Fig. 3). Further analyses of nucleotide and amino acid sequences of clones from remaining two patients (Patients 3 and 4) showed previously unknown variants such as Tyr-Met-Asn-Asn (YMNN). Many stop codons in the overlapping S gene were noted in clones obtained from Patient 3, but none of 11 clones obtained from Patient 4 had any stop codon (data not shown). Of 41 clones analysed, only ten clones had a wild-type sequence. Interestingly, none of the 41 clones had amino acid substitution of the tyrosine residue of the YMDD motif. Only two of these 41 clones had amino acid substitution of the serine residue, just one amino acid upstream of the YMDD motif, despite the fact that PNA was designed to anneal to the nucleotide sequence of this serine residue. Guanine to adenine transition was predominant in Patients 1, 3, and 4, but only a thymine to cytosine transition was seen in Patient 2 (Fig. 3).

Estimated Mis-Incorporation Rate of Taq DNA Polymerase Employed in This Study

We also studied the error rate of Taq DNA polymerase employed in this study by using cloned HBV plasmid DNA under exactly the same conditions. We sequenced 6,665 bases from 31 clones (215 bases of each clone) and found only ten nucleotide substitutions. They included seven C to T substitutions (six were at the same position), two G to A and one T to C substitution at different positions. All of these substitutions were situated outside the PNA annealing site. The estimated mis-incorporation rate was only 1.76×10^{-5} substitutions per site.

DISCUSSION

Viruses, especially RNA viruses and retroviruses, exist in the hosts as quasispecies. HBV, despite being a DNA virus, replicate via an RNA intermediate [Summers and Mason, 1982; Miller et al., 1984; Will et al., 1987; Bavand and Laub, 1988] as retroviruses. HBV shows high-mutation frequency [Orito et al., 1989] partly because of the lack of proof-reading enzymes that assure fidelity of DNA replication [Holland et al., 1982]. The only method to analyse the quasispecies has been cloning of the genome of these viruses, with or without PCR amplification, and nucleotide sequencing. In this process, only major strains can be detected but no studies have focused on rare strains. For example, even analysis of the hypervariable region of the HCV revealed the existence of only a limited number of species [Koizumi et al., 1995; Pawlotsky et al., 1998]. The fact that amino acid sequences of the rare YMDD variants identified in this study had never been reported suggests that previous studies had observed only the relatively abundant strains. The importance of such rare strains during emergence of drug-resistant strains or vaccine escape variants remains to be elucidated [Carman et al., 1990; Fujii et al., 1992; Okamoto et al., 1992; Waters et al., 1992; Zuckerman, 2000]. Further studies using two-stage PCR with PNA clamping as described in this study should clarify such issues.

Mis-incorporation by Taq DNA polymerase must be taken into account in such analyses. We studied the error rate of Taq DNA polymerase and found that the mis-incorporation rate was only 1.76×10^{-5} substitutions per site. Furthermore, no substitutions were found in the PNA annealing site from clones amplified with PNA in the conditions described herein. These results indicate that almost all variants identified in this study actually exist in all five of the patients studied in the present study.

Drug-resistant variants usually emerge after a certain period of antiviral therapy [Honkoop et al., 1997; Allen et al., 1998; Chayama et al., 1998; Niesters et al., 1998; Liaw et al., 1999; Ono-Nita et al., 1999a,b]. It is debatable whether these strains actually existed prior to antiviral therapy were selected by antiviral drugs [Melegari et al., 1998] or whether resistant strains evolved from non-resistant virus by nucleotide substitution(s) during replication under pressure from the drug.

In this study, both the YVDD, which is reported to be resistant to lamivudine, and YIDD variants were identified in one patient before the start of lamivudine therapy. The detection rate of variants in this study is

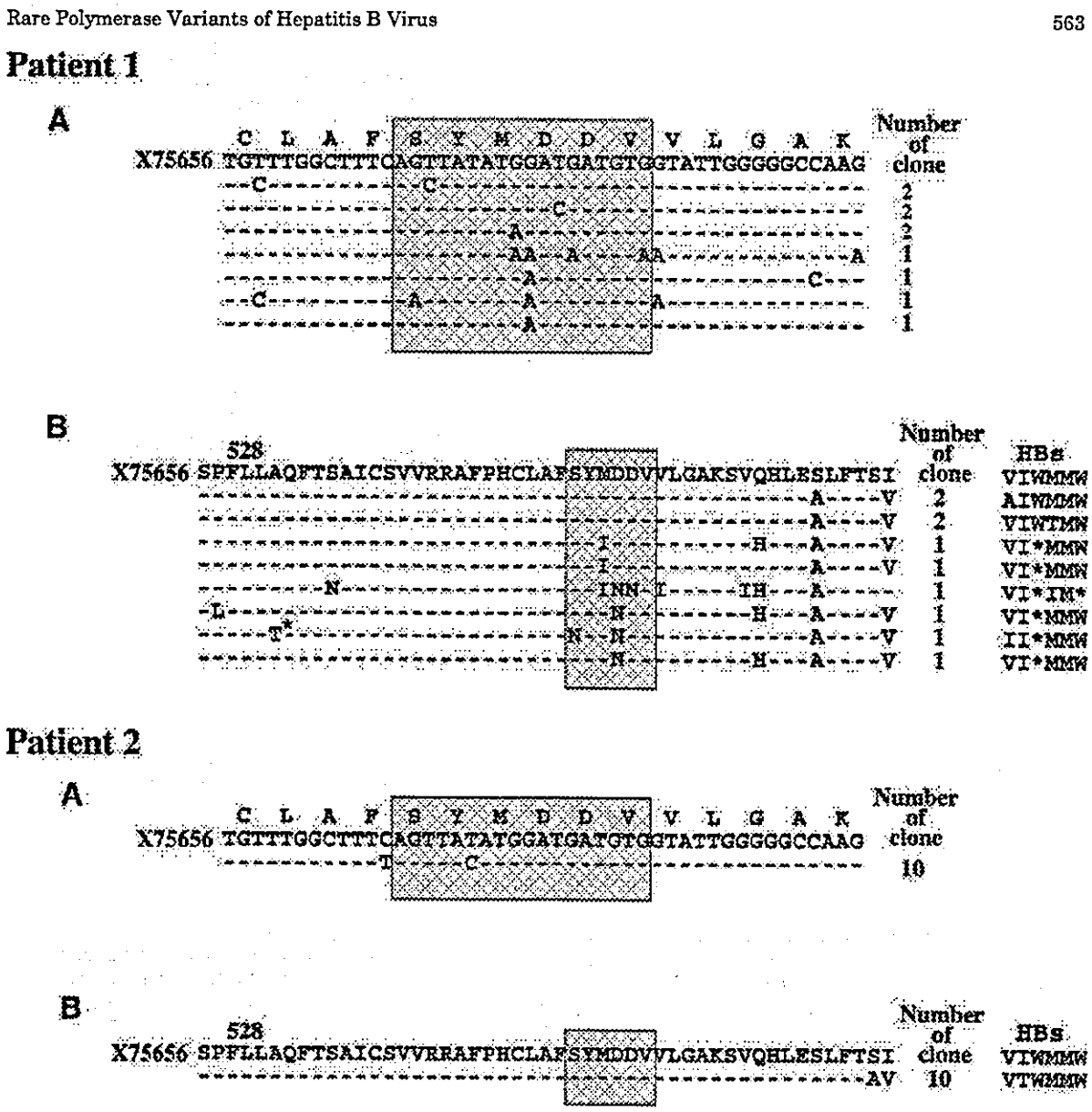

Fig. 3. Nucleotide (A) and amino acid (B) sequences of clones obtained from two patients with anti-HBe (Patients 1 and 2). Created stop codons in the overlapping envelope (S) gene are labeled with asterisks.

quite different from those reported in previous studies. For example, Kirishima et al. [2002] used the same PNA method to detect the YMDD variant and found four of 18 subjects were positive for the variant. Since our method has similar or even better sensitivity than that of Kirishima et al., this difference might be due to different background of the patients'. The YVDD variant was detected in only one of 43 clones obtained from this patient (Fig. 2). Since RFLP showed that only part of the amplified DNA was digested with the enzyme ApaLI, the prevalence of YVDD variants in this patient was assumed to be very low. Furthermore, the variant detected was distinct from the double mutation variant (M552V and L528M) that had been reported to have the strongest resistance to lamivudine [Gutfreund et al., 2000; Ono-Nita et al., 2001]. This might explain the excellent response to lamivudine in this patient. Alternatively, such variants might be defective viruses that lack the ability to replicate because of mutations in a different area that was not analysed in the present study. The data, however, show that care should be taken when interpreting results of detection of YMDD variants, especially when the sensitivity of the assay is very high. Whether such variants can evolve to drug-resistant variants needs to be studied in a larger number of patients, as the amount of HBV DNA fluctuates in

patients. Variants with the amino acid sequence YIDD identified in this patient had stop codon(s) in the overlapping *HBs* gene. Whether these variants, including the remaining newly identified sequences with stop codon(s) in the overlapping *HBs* gene, can replicate with trans-complementation by co-existing intact virus needs further studies. The enzymatic activity of reverse transcriptase/DNA polymerase in these variants, especially those without the HBs stop codon, should also be studied further.

One of the interesting findings of this study was that certain transition patterns were predominant in some patients. For example, 55 of 59 nucleotide substitutions in a patient who possessed YVDD variant before the start of lamivudine therapy were guanine to adenine transitions (Fig. 2A). In contrast, only a thymine to cytosine transition was identified in Patient 2 (Fig. 3A). A possible relationship between amino acid sequence/function of polymerase and patterns of nucleotide substitutions in these patients should be investigated. Amino acid substitutions in clones obtained from five patients were also distributed unevenly in the YMDD motif. None of 84 clones analysed from five patients had amino acid substitutions in the tyrosine molecule in this motif, and only two of 84 clones had amino acid substitution of the serine molecule just upstream of the YMDD motif. This uneven distribution of amino acid substitutions might be related to the function of the polymerase. Whether these rare polymerase variants are actually functional is important for designing new antiviral nucleotide analogs.

In conclusion, rare viral quasispecies were identified by using a sensitive method. The method might be further applied to study other nucleotide substitutions of other viruses. Analyses of rare variants might be useful for further understanding of the mechanism for emergence of drug-resistant HBV variants and for developing strategies for new antiviral drugs.

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

Yasuhito Tanaka,[1] Izumi Hasegawa,[1,2] Takanobu Kato,[1] Etsuro Orito,[2] Noboru Hirashima,[3] Subrat K. Acharya,[4] Robert G. Gish,[5] Anna Kramvis,[6] Michael C. Kew,[6] Namiko Yoshihara,[7] Santosh Man Shrestha,[8] Mobin Khan,[9] Yuzo Miyakawa,[10] and Masashi Mizokami[1]

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.

From the Departments of [1]Clinical Molecular Informative Medicine and [2]Internal Medicine and Molecular Science, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; [3]Department of Gastroenterology, Chukyo Hospital, Nagoya, Japan; [4]Sub Bagian Hepatologi, Department of Gastroenterology, All India Institute of Medical Sciences, New Delhi, India; [5]Hepatology and Gastroenterology, California Pacific Medical Center, San Francisco, CA; [6]MRC/CANSA/University Molecular Hepatology Research Unit, Department of Medicine, University of the Witwatersrand, Johannesburg, South Africa; [7]AIDS Research Center, National Institute of Health, Tokyo, Japan; [8]Liver Foundation Nepal, Kathmandu, Nepal; [9]Department of Hepatology, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh; [10]Miyakawa Memorial Research Foundation, Tokyo, Japan.

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Address reprint requests to: Masashi Mizokami, MD, PhD, Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan. E-mail: mizokami@med.nagoya-cu.ac.jp; fax: +81 52-842-0021.

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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 hemineste d 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 × 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

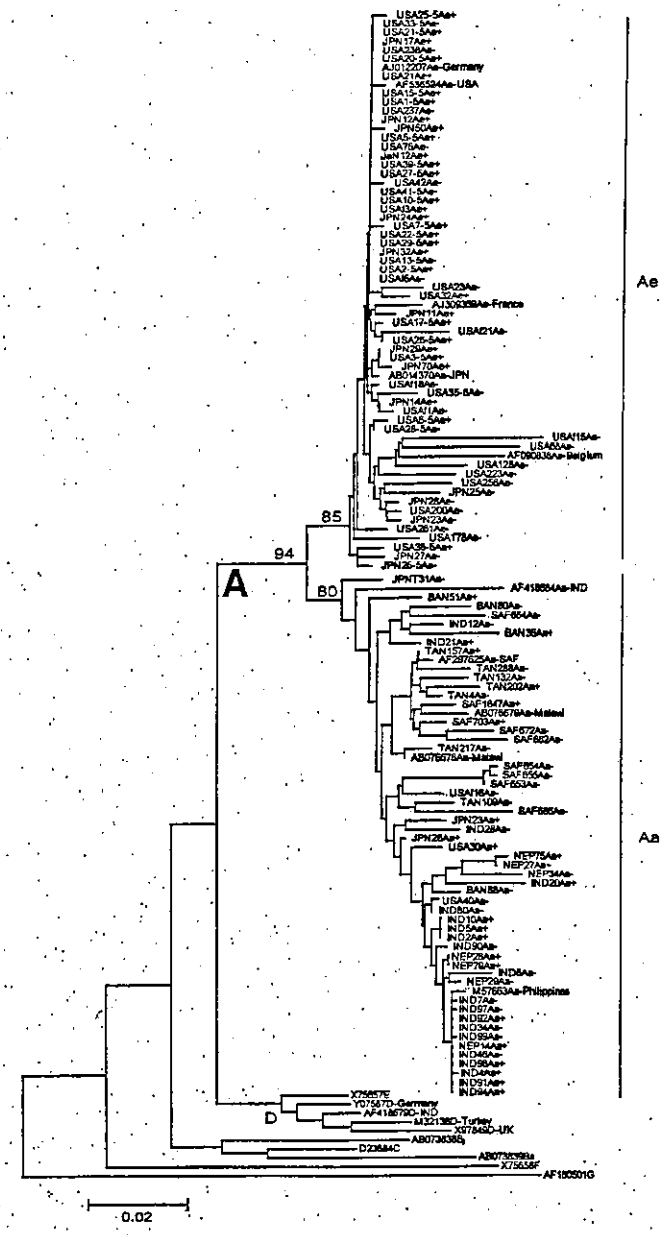

Fig. 1. A phylogenetic tree constructed on the X gene, precore region and core gene sequences. Together with 14 sequences of hepatitis B virus genotype A (HBV/A) and hepatitis B virus genotype D (HBV/D) isolates retrieved from the database, the 111 HBV/A sequences determined in the present study were 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 *P* Value
	Aa (n = 78)	Ae (n = 78)	D (n = 78)	
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-

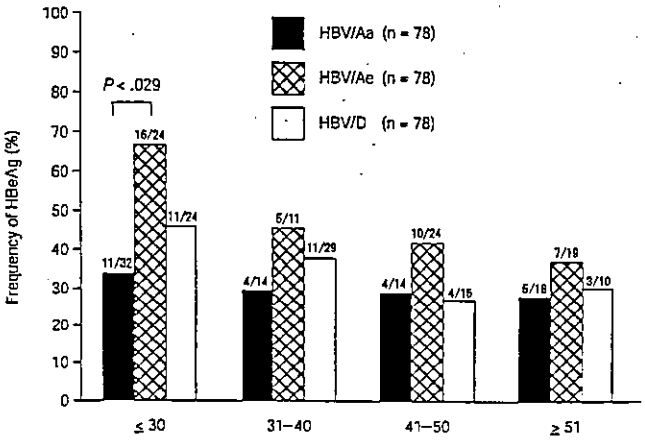

Fig. 2. Age-specific prevalence rates of hepatitis B e antigen (HbeAg) in carriers of hepatitis B virus genotype Aa (HBV/Aa), hepatitis B virus genotype Ae (HBV/Ae), or hepatitis B virus genotype D (HBV/D).

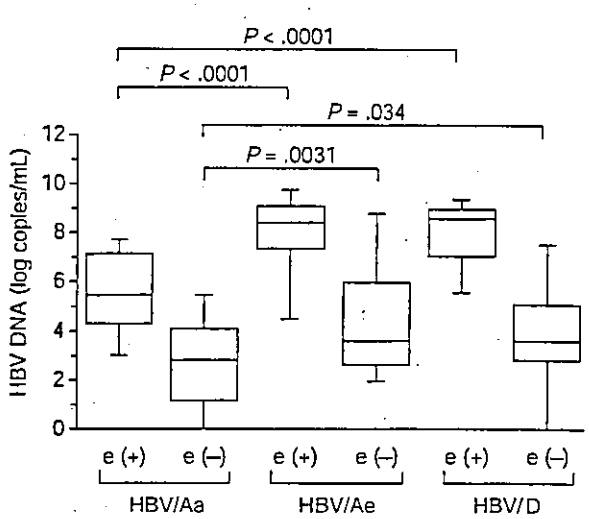

Fig. 3. Hepatitis B virus (HBV) DNA levels in carriers of hepatitis B virus genotype Aa (HBV/Aa), hepatitis B virus genotype Ae (HBV/Ae), and hepatitis B virus genotype D (HBV/D) with or without hepatitis B e antigen (HBeAg) in serum. HBV DNA was quantitated by real-time detection polymerase chain reaction, 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.

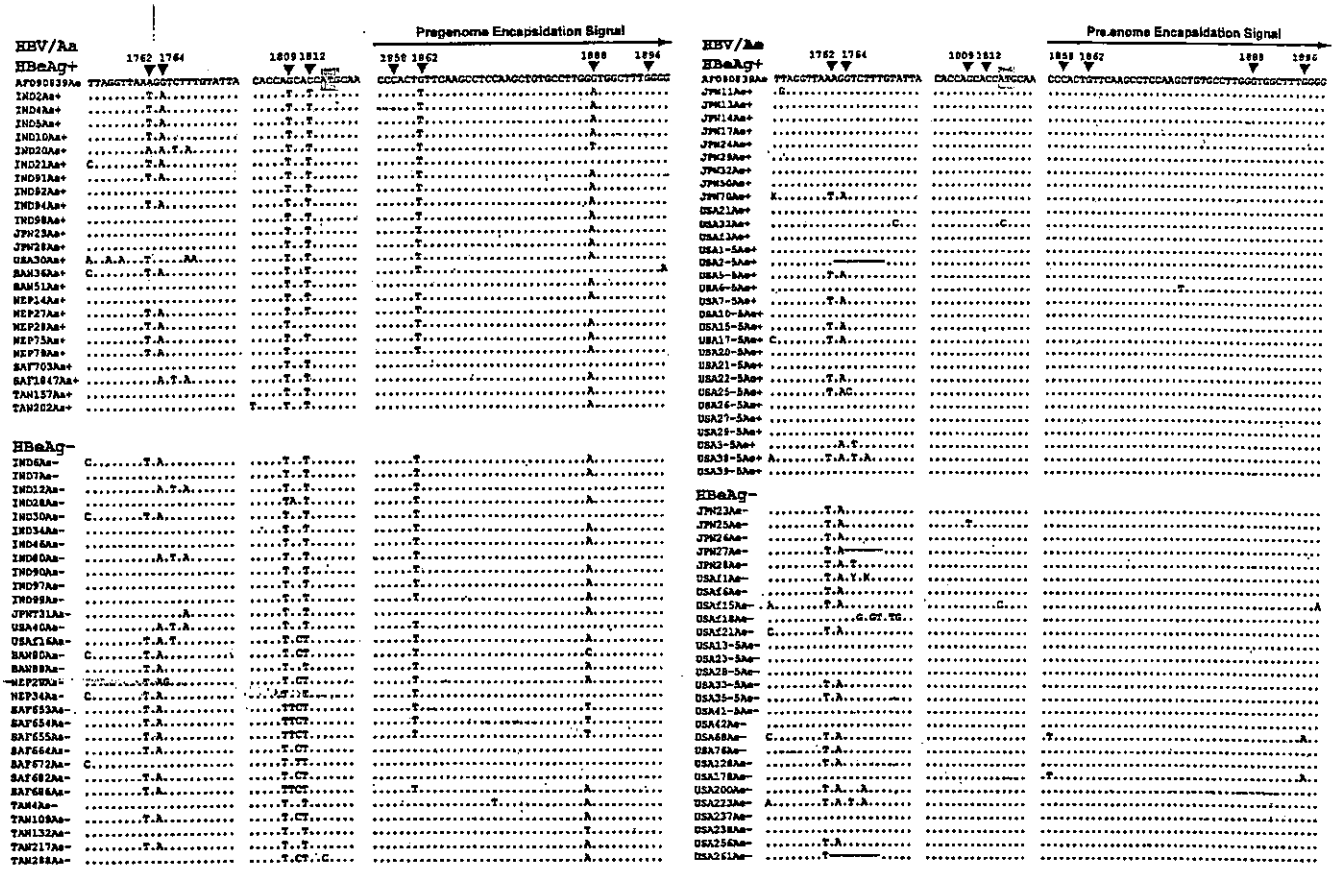

Fig. 4. Nucleotide sequences spanning the basic core promoter and pregenome encapsidation signal in hepatitis B virus (HBV) isolates of (A) subtype Aa and (B) subtype Ae. Positions of the double-mutation (nucleotide [nt] 1762 and 1764), two substitutions frequent in subtype Aa (nt 1809 and 1812), and 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).

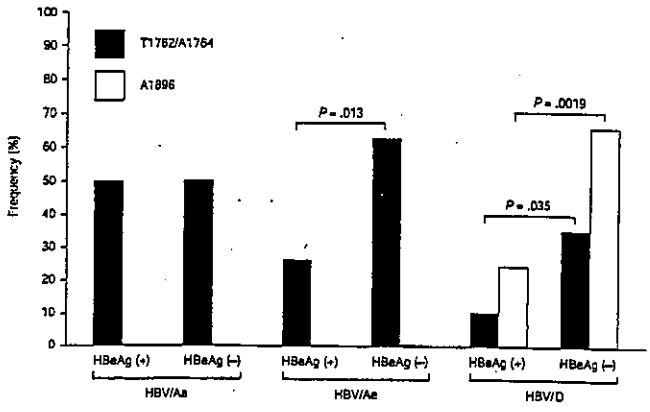

Fig. 5. The frequency of basic core promoter (BCP) mutation (T1762/A1764) and precore mutation (A1896) among carriers of hepatitis B virus genotype Aa (HBV/Aa), hepatitis B virus genotype Ae (HBV/Ae), and hepatitis B virus genotype D (HBV/D) with or without serum hepatitis B e antigen (HBeAg). The prevalence 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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Novel Hepatitis B Virus Genotype A Subtyping Assay That Distinguishes Subtype Aa from Ae and Its Application in Epidemiological Studies

Izumi Hasegawa,[1,2] Yasuhito Tanaka,[1] Anna Kramvis,[3] Takanobu Kato,[1] Fuminaka Sugauchi,[2] Subrat K. Acharya,[4] Etsuro Orito,[2] Ryuzo Ueda,[2] Michael C. Kew,[3] and Masashi Mizokami[1]*

Department of Clinical Molecular Informative Medicine[1] and Department of Internal Medicine and Molecular Science,[2] Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; MRC/CANSA/University Molecular Hepatology Research Unit, Department of Medicine, University of the Witwatersrand, Johannesburg, South Africa[3]; and Sub Bagian Hepatologi, Department of Gastroenterology, All India Institute of Medical Sciences, New Delhi, India[4]

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The eight genotypes of hepatitis B virus (HBV) have different geographical distributions, virological characteristics, and clinical manifestations. A unique subtype of HBV genotype A (HBV/A) was reported in sub-Saharan Africa, raising the possibility that patients infected with this subtype (HBV/Aa ["a" for African and Asian]) may have different clinical outcomes than other HBV/A isolates (HBV/Ae ["e" for European]). Comparison between 30 HBV/Aa and 30 HBV/Ae isolates indicated that almost all HBV/Ae isolates had G at nucleotide (nt) 1809 and C at nt 1812, whereas HBV/Aa isolates had T1809/T1812. Taking advantage of these two single nucleotide polymorphisms (SNPs), a novel subtype-specific PCR assay in the X/precore/core region was developed. This assay was combined with a restriction fragment length polymorphism assay using BglII in a different region (nt 1984 to 1989), which has a SNP distinguishing HBV/Aa from HBV/Ae, resulting in 100% specificity for the combined assay. Application of the subtyping assay using sera from 109 paid donors in the United States indicated significantly different distributions of HBV/A subtypes among races; African-Americans, Caucasians, and Hispanics had HBV/Ae, whereas Asians had mainly HBV/Aa, suggesting that the HBV/Aa isolates may have been imported by recent immigration from Asia. In conclusion, the specificity and sensitivity of the combined subtyping assay were confirmed, and its usefulness was demonstrated in a practical context.

Hepatitis B virus (HBV) is one of the most important causes of acute and chronic liver disease, such as fulminant hepatitis, cirrhosis, and hepatocellular carcinoma, throughout the world, especially in Asian and African countries. Eight genotypes of HBV, classified A to H (HBV/A to HBV/H), are distinguished by intergenotypic differences of more than 8% in the entire nucleotide sequence of approximately 3,200 nucleotides (nt) (3, 21, 22, 25). These genotypes have different geographical distributions, virological characteristics, and clinical manifestations (18, 20). Moreover, among the HBV isolates of the same genotype, there might also be differences of virological characteristics.

Recently, in Asian countries, two subtypes of HBV genotype B, designated Ba ("a" standing for Asia) and Bj ("j" for Japan) have been reported (27), and clinical differences between patients infected with HBV/Ba and HBV/Bj are being recognized (2, 28).

Similarly, a subtype of genotype A was reported and designated A′ in sub-Saharan African countries (5). A large number of HBV isolates from South Africa were found to cluster with this subtype A′ by phylogenetic analyses of both the pre-S2/S region (5) and the complete genome (13, 16). This subtype has also been found in HBV isolates from Malawi (29). In African countries, genotype A isolates, most of which are subtype A′, seem to be associated with low levels of HBV DNA in serum and a low prevalence of hepatitis B e antigen (HBeAg) in serum (14, 15). On the other hand, HBV carriers infected with isolates belonging to the remainder of genotype A, distributed widely in European countries and the United States, have a higher rate of sustained remission after seroconversion and a lower rate of death related to liver disease than other genotypes during long-term follow-up (8, 24, 33).

In this paper, we report a convenient assay with a subtype-specific PCR combined with a restriction fragment length polymorphism (RFLP) assay to distinguish these two subtypes of genotype A. Recently, a cross-sectional study in the United States showed a correlation of HBV genotypes not only with ethnicity but also with the place of birth of the carriers of the virus (6). By using the newly developed assay, we also examined the association between the subtypes of HBV/A and ethnicity in the United States. To avoid confusion, as previously proposed (30), the subgroup (subtype) A′, which was proposed by Bowyer et al. (5), is renamed subtype HBV/Aa ("a" standing for African and Asian), the other subtype found in Euro-

* Corresponding author. Mailing address: Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Kawasumi 1, Mizuho, Nagoya 467-8601, Japan. Phone: 81-52-853-8292. Fax: 81-52-842-0021. E-mail: mizokami@med.nagoya-cu.ac.jp.

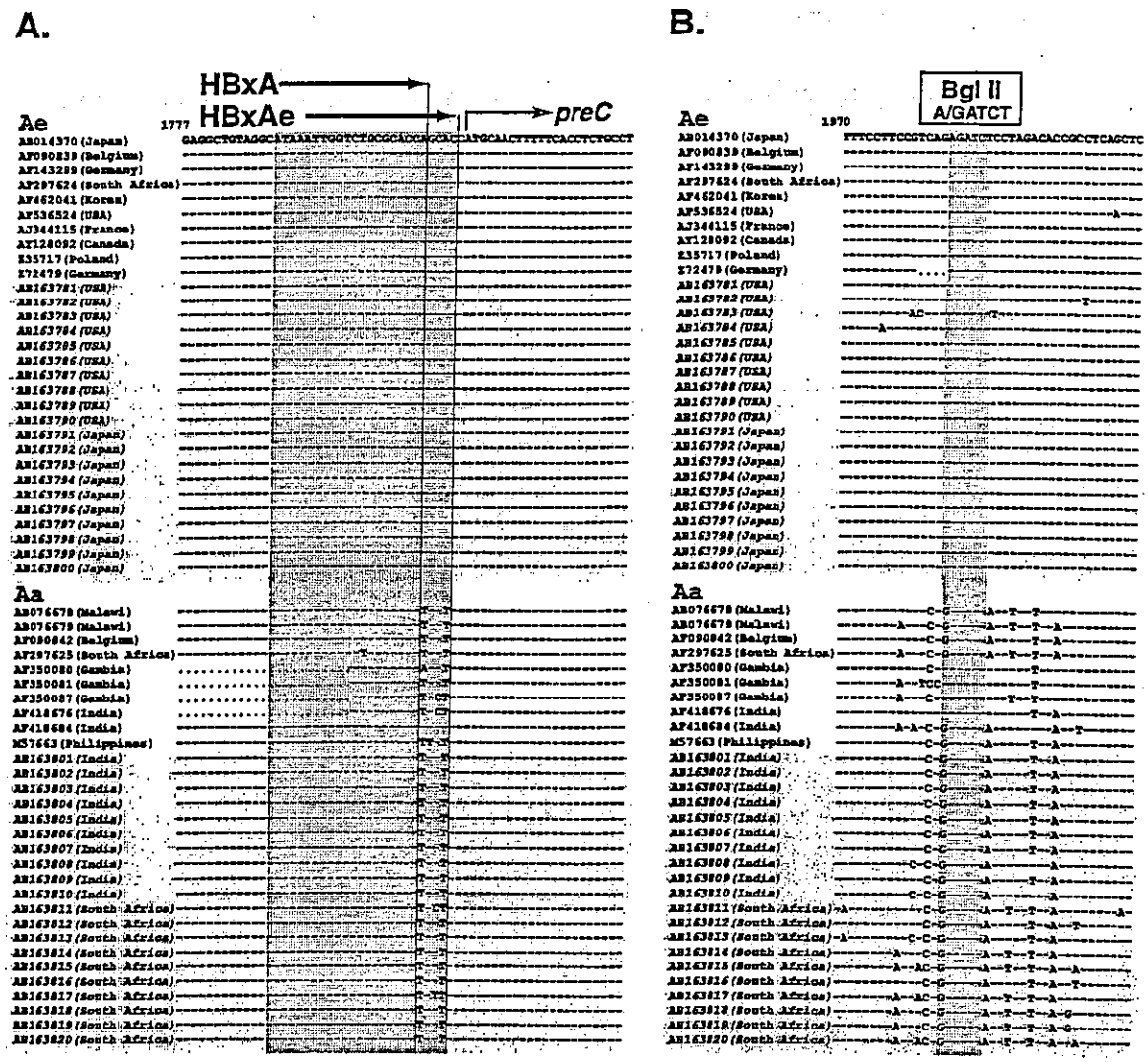

FIG. 1. Alignment of 40 HBV/A isolates sequenced in the present study with 20 reference isolates. (A) Alignment of sequences of HBV/A isolates within the subtype-specific region of the primer (nt 1777 to 1836). The positions of sense primers for the second-round PCR are shown by the arrows above alignments and are shaded in grey. (B) Alignment of sequences of HBV/A isolates within the core region containing a BglII restriction site (nt 1970 to 2009). The position of the enzyme restriction site is shaded in grey. The isolates sequenced in the present study are shaded in light grey.

pean countries and the United States is HBV/Ae ("e" for European and U.S. type), and genotype A (HBV/A) includes both HBV/Ae and HBV/Aa.

MATERIALS AND METHODS

Serum samples. Forty HBV/A-positive serum samples were collected from carriers in four countries (10 each from Japan, the United States, India, and South Africa) and were used to examine the specificity and sensitivity of our HBV/A subtype-specific PCR combined with RFLP assay. These serum samples were stored at −80°C until assayed.

For the practical testing of this assay, 333 sera from HBsAg-positive paid donors in the United States recruited by ProMedDx Inc. (Plainville, Mass.) were used in this study. Of the 333 samples, 139 belonged to HBV/A. After hepatitis C virus- and/or human immunodeficiency virus-infected samples and samples whose ethnicity was unknown were omitted, 109 samples obtained from 38 African-American, 16 Asian, 24 Caucasian, and 31 Hispanic subjects were examined. All of them had not received current antiviral therapy, and their alanine aminotransferase (ALT) levels in serum were normal. For controls, 32 HBV/A isolates from South African black asymptomatic carriers of HBV with normal ALT levels were used (age range, 18 to 44 years; 30 [94%] carriers were male). 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 subject.

Serological determination of HBeAg and HBV genotypes. HBeAg was detected by the chemiluminescent enzyme immunoassay (Ortho Clinical Diagnostics, Tokyo, Japan). The genotype of these samples was serologically confirmed as HBV/A by using commercial kits (HBV Genotypes EIA; Institute of Immunology) based on five monoclonal antibodies directed to the corresponding epitopes on the product of the pre-S2 region which are designated *b*, *m*, *k*, *s*, and *u* (31, 32).

Detection of HBV DNA and cloning. DNA was extracted from 100 μl of serum using a QIAamp DNA blood mini kit (QIAGEN Inc., Hilden, Germany). HBV DNA of X and precore/core genome sequences were amplified by a method reported previously (26). In brief, the first-round hemi-nested PCR was performed with sense primer HB7F (5′-GAG ACC ACC GTG AAC GCC CA-3′ [nt 1611 to 1630]) and antisense primer HB8R-2 (5′-ATA GGG GCA TTG GTC T-3′ [nt 2314 to 2299]; a modification of HB8R) for 5 min at 96°C, followed by denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C (an additional 5 min in the last cycle), in a 96-well cycler (GeneAmp