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HEPATOLOGY

Virological and clinical implication of core promoter C1752/V1753 and T1764/G1766 mutations in hepatitis B virus genotype D infection in Mongolia

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Abstract

Background and Aim: The aim of the present study was to reveal virological and clinical features of hepatitis B virus (HBV) genotype D infection.

Methods: One hundred and twenty-two Mongolian chronic liver disease (CLD) patients infected with HBV were subjected for serological HBV-markers screening and HBV-enzyme immunoassay (EIA) genotyping. Nucleotide sequences were analyzed for 48 HBV/D strains (23 isolated from hepatocellular carcinoma (HCC) and 25 from CLD patients).

Results: Prevalence of hepatitis B e antigen (HBeAg) positivity was low (25.9%) in young patients (≤30 years old) indicating early HBeAg seroclearance in HBV/D carriers. The T1764/G1766 double mutation was the most common basal core promoter (BCP) mutation (29.2%) and was frequent in HBeAg-negative patients (39.3%). Patients harboring T1764/G1766 mutants exhibited lower HBV-DNA and HBV core antigen (HBcAg) levels than those with wild-type BCP strains ($P = 0.024$, 0.049, respectively). C1752 and/or V (not T) 1753 mutation was significantly prevalent in HCC patients (HCC *vs* CLD; 52.2% *vs* 20%, $P = 0.033$). T1762/A1764 mutation was detected in 75.0% of HCC patients with high viral load (≥5 log copies/mL). Precore stop codon mutation A1896 was detected in (70.8%) of HBV/D-infected patients.

Conclusions: In Mongolians infected with HBV/D, C1752 and/or V1753 mutation was associated with HCC.

Introduction

Hepatitis B virus (HBV) infection concerns more than 350 million infected people in the world and is a global public health problem. The clinical outcome of HBV infection varies greatly from acute self-limiting disease and inactive carriers up to chronic liver disease (CLD) including liver cirrhosis and hepatocellular carcinoma (HCC).[1] Accumulated evidence indicated that the most important viral factors contributing to development of HCC include genotype,[2] specific genetic mutations[3,4] and coinfections with other hepatitis viruses.[5–7]

Eight genotypes of HBV (A–H) are identified based on the comparison of complete genomes, and most of the genotypes have a distinct geographic distribution.[8–10] Controversial data concerning the clinical characteristics of HBV genotype D infection; HBV/D was associated with severe liver disease, and a higher prevalence of genotype D than genotype A was observed in HCC patients in comparison to asymptomatic carriers.[11] In contrast, other reports indicated the lack of association between this genotype and distinct clinical phenotype.[12–14]

The HBV genomic mutations occur due to a spontaneous error rate of viral reverse transcriptase and evolving of HBV genome under the antiviral pressure of host immune response.[15] Specific mutations may affect the translation of hepatitis B e antigen (HBeAg), as well as the replication of HBV and thus may modify the clinical outcome of HBV infection and contribute to HCC development.[16–18]

Previous studies clearly demonstrated that the coinfection with HBV and hepatitis D virus (HDV) and/or HCV is common in Mongolia, and is significantly associated with development of HCC in Mongolian HBV carriers.[5–7] The age-adjusted incidence rate of HCC in this country was estimated to be 61.8–98.93 per 100 000 men, representing one of the highest age-adjusted incidence rates in the world[19] and HBV/D infection was established in 50–80% of HCC patients according to the recent reports.[5,6] In the present study, we investigated potential association of the basal core promoter (BCP) and precore genomic region characteristics of HBV/D with virological and clinical features of the infection, particularly with development of HCC.

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Methods

Patients

A group of 122 hepatitis B surface antigen (HBsAg)-positive Mongolian patients with chronic liver disease was analyzed in this study. Coinfection (HBV + HDV) and (HBV + HCV) was found in 51/122 (41.8%) and 14/122 (11.5%), respectively, and triple infection (HBV + HCV + HDV) was detected in 41/122 (33.6%).[5] All patients had their biochemical liver profile examined; alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using kits from HUMAN International (Wiesbaden, Germany) by the Raitmana Frenkle kinetic method on a Humalyzer 3000 (HUMAN International). The clinical diagnosis of HCC was confirmed by biochemical laboratory liver function tests, liver tumor marker, Alpha fetoprotein (AFP) and protein induced by vitamin K antagonist-II (PIVKA-II), ultrasound, computer tomography, and/or liver biopsy with histopathological examination.[5] The patients with and without HCC were classified into two clinical groups, HCC and CLD, respectively.

Serological methods

Hepatitis B virus surface and e antigens were examined by chemiluminescent enzyme immunoassay (Lumipulse; Fujirebio, Tokyo, Japan). HBV genotypes were analyzed using two methods: EIA with pre-S2 epitopes specific monoclonal antibodies[20] (HBV genotype EIA; Institute of Immunology, Tokyo, Japan) and, where possible, by direct sequencing of enhancer II/core promoter and precore/core genomic regions with further phylogenetic analysis. Serum concentration of hepatitis B core antigen (HBcAg) and hepatitis B core-related antigen (HBcrAg) (HBcrAg is a precore/core gene product, comprising HBcAg and HBeAg) were measured using the chemiluminescence enzyme immunoassay (CLEIA) method as described previously.[21,22] Cut-off value for HBcAg positivity was set at 10 pg/mL and for HBcrAg positivity was set at 10 pg/mL.

HBV-DNA quantification

HBV-DNA was quantified using real-time detection polymerase chain reaction (RTD-PCR) as previously reported.[23] The method was applied with slight modification as described previously.[18] The detection limit of this assay was 100 copies/mL.

HBV genome PCR-amplification and sequencing

HBV-DNA was extracted from 0.1 mL serum using QIAamp DNA Blood Mini Kit (QIAGEN Inc., Hilden, Germany). The enhancer II/core promoter and precore regions of the HBV genome were amplified by PCR with a forward primer (IS2-2: 5′-CAT GGA GAC CAC CGT GAA CGC-3′ [nt 1607–1627]) and reverse primer (HBV1917R: 5′-CTC CAC AGA AGC TCC AAA TTC TTT A-3′ [nt 1942–1918]). PCR was initiated by the hot-start technique. The PCR reaction was undertaken for 45 cycles (94°C for 1 min, 60°C for 1 min and 72°C for 1 min) followed by an extension reaction for 7 min. Complete genomes were amplified by primer sets described previously.[24] PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA, USA) in an ABI 3100 DNA automated sequencer (Applied Biosystems).

Sequence analysis

Sequences were aligned using the CLUSTALW software program (Thompson *et al.* 1997).[25] Phylogenetic trees were constructed using neighbor-joining (NJ) analysis with the six-parameter distance correction method[26] with bootstrap test confirmation performed on 1000 resamplings on the online Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/).

Complete genome sequences were examined for the presence of intergenotypic recombination using the SIMPLOT software program as described previously[27] and were reconfirmed manually by visual inspection of the alignments.

Nucleotide sequences obtained and analyzed in this study were submitted to DDBJ with consecutive accession numbers AB270534–AB270584.

Statistical evaluation

Statistical analysis was performed with Fisher's exact test and the independent *t*-test for continuous variables using SPSS version 8.0 software packages (SPSS, Chicago, IL, USA). *P*-values (two-tailed) less than 0.05 were considered statistically significant.

Ethical considerations

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and its subsequent amendments. Informed consent was obtained from all patients.

Results

Characteristics of the patients and HBV genotypes

Genotyping by EIA determined HBV/D and HBV/A in 109/122 (89.3%) and 4/122 (3.3%), respectively, and 9/122 (7.4%) cases were untypable by EIA. No significant difference was observed in the genotype distribution between the HCC and CLD groups.

In order to confirm genotyping results and to investigate genetic characteristics of the strains, all the cases were subjected to DNA extraction, PCR using specific primers designed to amplify a part of the HBV genome including enhancer II, BCP, epsilon loop, and a part of the precore/core coding genes followed by direct sequencing. However, only 52/122 (42.6%) cases were amplified by PCR, including 51 of genotype D and one of genotype A as determined previously by EIA.

A phylogenetic analysis of the successfully sequenced strains confirmed one case with genotype A, and 48 cases with genotype D (tree not shown). However, the remaining three cases had discrepancy with the genotyping results by EIA (HBV/D) exhibiting phylogenetic clustering with HBV/C reference sequences (tree not shown). In order to investigate whether this discrepancy was due to coinfection with different genotypes or an intergenotypic recombination event, these cases were subjected for complete genome sequencing. Two cases were successfully amplified, sequenced and were subjected to Bootscan analyses using SIMPLOT software. The

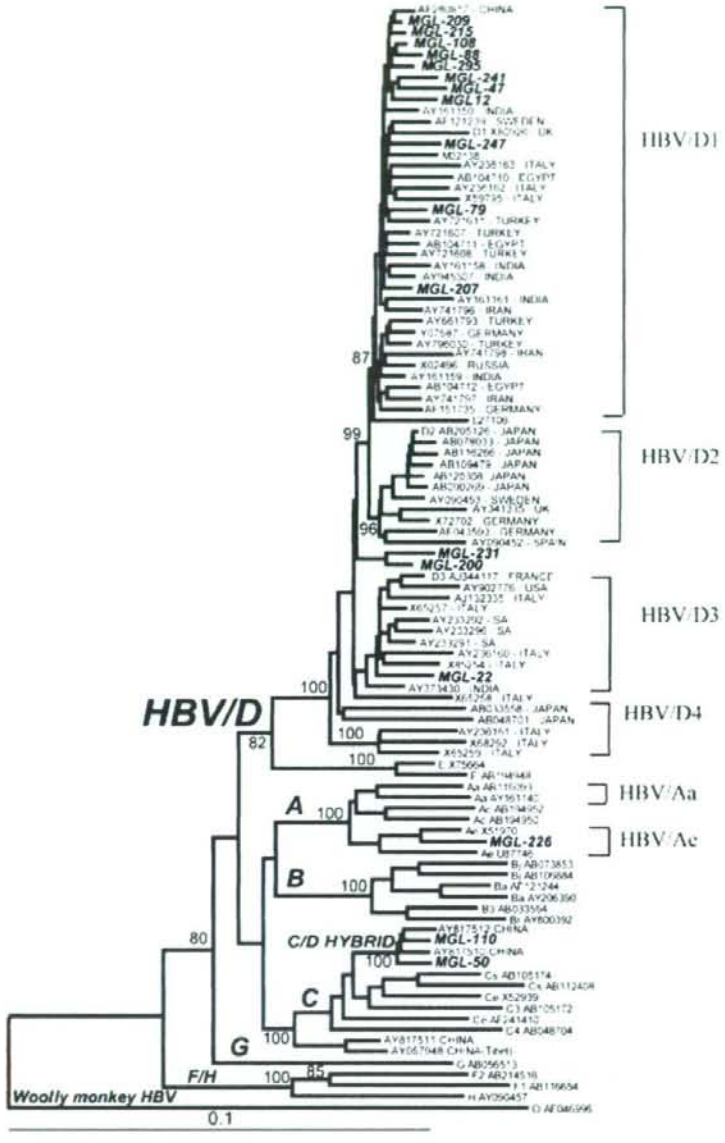

Figure 1 Phylogenetic NJ (Neighbour-joining) tree constructed using 100 complete genome sequences of hepatitis B virus (HBV). Seventeen strains isolated from Mongolia in this study are indicated in bold. Reference sequences retrieved from GenBank/EMBL/DDBJ are indicated with their accession numbers. The origins (in parentheses) are indicated for genotype D and C/D hybrid strains. Shown in the tree roots, bootstrap values higher than 80% were considered as significant. Genotypes (A to H and C/D hybrid) are indicated on the cluster roots. The outgroup consisted of HBV strain isolated from Woolly Monkey.

result indicated that both represented a hybrid HBV variant with a preS2/S region homologous with genotype D and the other regions homologous with genotype C. The breakpoints of the recombination were estimated around the positions of nucleotides 10 and 799.

The phylogenetic tree constructed using the complete genome sequences indicated that the two Mongolian strains were related to the previously reported Northern China C/D hybrid strains having the same recombination breakpoints[28] (Fig. 1). One of the two confirmed C/D hybrid cases had HCC.

The complete genome was also successfully amplified from the one HBV/A case in this study and, phylogenetically, it was related to the previously reported HBV/Ae subgenotype. In addition, 14 HBV/D strains were selected at random from CLD and HCC patients and subjected to complete genome sequencing. As shown in Fig. 1, 11 strains were grouped together with HBV/D1 references, one strain grouped with the HBV/D3 cluster, and the remaining two strains were not joined to any subgenotype groups while still corresponding to HBV/D.

Table 1 Clinical characteristics and viral mutations of patients with HBV/D

	Overall ($n = 48$)	CLD ($n = 25$)	HCC ($n = 23$)	*P*-value*
Age (years)†	46.1 ± 15	37.5 ± 13.3	55.3 ± 10.8	<0.0001
Gender (male)‡	22 (45.8%)	12 (48%)	10 (43.5%)	NS
HBeAg (positive)‡	20 (41.7%)	12 (48%)	8 (34.8%)	NS
ALT (IU/L)†	105.8 ± 62.9	87.7 ± 59.0	125.5 ± 62.2	0.036
AST (IU/L)†	93.1 ± 61.7	70.6 ± 53.2	117.7 ± 62.0	0.007
HBV-DNA (log copies/mL)†	4.4 ± 1.8	4.9 ± 1.9	3.9 ± 1.5	0.060
HBcAg (log pg/mL)†	1.5 ± 1.7	2.0 ± 1.9	0.8 ± 1.2	0.014
HBcrAg (log pg/mL)†	3.1 ± 1.7	3.6 ± 1.8	2.5 ± 1.3	0.020
Mutation prevalence‡				
T1653	7 (14.6%)	4 (16%)	3 (13%)	NS
G1727	7 (14.6%)	2 (8%)	5 (21.7%)	NS
C1752/ or V (not T)1753	17 (35.4%)	5 (20%)	12 (52.2%)	0.033
A1757	42 (87.5%)	23 (92%)	19 (82.6%)	NS
T1762/A1764	6 (12.5%)	3 (12%)	3 (13%)	NS
T1764/G1766	14 (29.2%)	6 (24%)	8 (34.8%)	NS
A1896	34 (70.8%)	16 (64%)	18 (78.3%)	NS
A1915	29 (60.4%)	17 (68%)	12 (52.2%)	NS

*Chronic liver disease group (CLD) *vs* hepatocellular carcinoma group (HCC).
†Mean ± SD.
‡*n* (%).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBcAg, hepatitis B core antigen; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NS, not significant.

Characteristics of HBV/D

To reveal specific mutations in association with HCC among patients with HBV/D, 48 DNA-positive genotype D cases were analyzed. Table 1 summarizes clinical characteristics and prevalence of the featured viral mutations among the 48 patients. The patients in the HCC group were significantly older than in the CLD group ($P < 0.0001$). Aminotransferase (ALT, AST) levels were significantly higher in the HCC group compared to the CLD group ($P = 0.036$, 0.0.007), respectively. HBcAg and HBcrAg levels were significantly higher in the CLD group compared to the HCC group ($P = 0.014$, 0.020), respectively. The level of HBV-DNA was relatively higher in the CLD group than in the HCC group (4.9 *vs* 3.9 log copies/mL, $P = 0.060$).

Comparing several mutations in enhancer II, core promoter, and precore regions, the most frequent BCP mutation was G1764T/C1766G (29.2%), followed by T1753V (not T) (23%), and A1762T/G1764A (12.5%), then A1752C (10%) (Table 1). Interestingly, G1757A substitution was prevalent in the overall population (87.5%) with no significant difference between HCC (92%) and CLD (82.6%) groups.

The V1753 mutation was frequent in HCC patients (34.7%) compared to CLD patients (12%) but did not reach a level of statistical significance ($P = 0.088$), probably due to the small number of samples. However, either or both mutations C1752/V1753 were detected in 17/48 (35.4%). The frequency of either or both C1752/V1753 mutation was significantly higher in the HCC group (52.2%) than in the CLD group (20%, $P = 0.033$). The same trend was also observed in HBeAg-negative patients (HCC *vs* CLD = 53.3% *vs* 15.3%, $P = 0.054$). The frequency of the T1764/G1766 double mutation was comparable between HCC (34.8%) and CLD (24%) groups. However, the T1762/A1764 double mutation was less frequent in both HCC (13%) and CLD (12%) groups. Precore stop codon mutation, G1896A, was detected in 70.8% (34/48) of HBV/D-infected patients including 64% (16/25) in the CLD group and 78.3% (18/23) in the HCC group.

Among the HBeAg-negative patients, the frequency of the precore stop codon mutation A1896 (75%) and BCP mutation T1764/G1766 (39.2%) was higher compared to those positive for HBeAg (65% and 15%), respectively. Studying the electropherogram of HBV strains in HBeAg-positive patients harboring A1896 mutants showed ambiguous patterns of G together with A at nt1896, indicating the presence of both wild-type and mutant strains in those patients.

Coinfection and mutations in association with HCC

It is important to note that only 13.1% of the serum samples tested were from HBV mono-infected patients whereas the remaining serum samples were from those coinfected with HBV + HDV (42%), HBV + HCV + HDV (34%) and HBV + HCV (12%). Therefore, there was a possibility that the observed association of a particular mutation with HCC was the result of coinfection-related bias. To elucidate this, we compared the prevalence of the mutations between HCC and CLD patients carrying any one particular pattern of infection; that is mono-infection with HBV or double-infection with HBV + HDV, or HBV + HCV, or triple-infection with HBV + HDV + HCV.

Among patients carrying HBV + HDV double-infection, we were able to observe that C1752/V1753 (either or both) mutations were significantly associated with HCC (HCC: 75.0% *vs* CLD: 18.2%, $P = 0.023$). The association of C1752/V1753 (either or

Table 2 Virological characteristics and prevalence of viral mutations between CLD and HCC patients in relation to HBV DNA

HBV-DNA (log copies/mL)	HCC		CLD	
	≥5 $n = 4$ (17.4%)	<5 $n = 19$ (82.6%)	≥5 $n = 10$ (40%)	<5 $n = 15$ (60%)
Age (years)	42.3 ± 14.2	58.1 ± 7.9	31.9 ± 13.1	41.3 ± 12.4
HBeAg (positive)	3 (75%)	5 (26.3%)	8 (80%)*	4 (26.7%)
HBcAg (positive)	3 (75%)**	2 (10.5%)	10 (100%)*	4 (26.7%)
Nucleotide substitutions				
T1653	0	3 (15.8%)	2 (20%)	2 (13.3%)
G1727	1 (25%)	4 (21.1%)	0	2 (13.3%)
C1752 and/or V1753	3 (75%)	9 (47.4%)	2 (20%)	3 (20%)
A1757	3 (75%)	16 (84.2%)	10 (100%)	13 (86.7%)
T1762/A1764	3 (75%)**,***	0	2 (20%)	1 (6.7%)
T1764/G1766	0	8 (42.1%)	1 (10%)	5 (33.3%)
A1896	3 (75%)	15 (78.9%)	4 (40%)	12 (80%)
A1915	2 (50%)	10 (52.6%)	7 (70%)	10 (66.7%)

*$P < 0.05$, in CLD group (patients with HBV-DNA ≥5 log copies/mL) *vs* (patients with HBV-DNA <5 log copies/mL).

**$P < 0.05$, in HCC group (patients with HBV-DNA ≥5 log copies/mL) *vs* (patients with HBV-DNA <5 log copies/mL).

***$P < 0.05$ (HCC patients with HBV-DNA ≥5 log copies/mL) *vs* (CLD patients with DNA <5 log copies/mL).

CLD, chronic liver disease group; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma.

both) mutations with HCC was also significant when patients with HBV + HDV dual-infection were combined with those with HBV + HDV + HCV triple-infection (HCC: 57.9% *vs* CLD: 18.8%, $P = 0.036$). However, in patients with HBV mono-infection, as well as those with HBV + HCV dual-infection or HBV + HDV + HCV triple-infection, we were unable to observe a significant association of this mutation with HCC, due to the small number of cases. Furthermore, no mutation or mutation pattern was observed in association with HBV mono-infection or coinfection, when we analyzed HBV BCP nucleotide sequence alignment (data not shown).

Virological characteristics of HBV infection in patients harboring BCP variants

The virological characteristics and prevalence of viral mutations were compared between patients with different HBV viral load in CLD and HCC groups (Table 2). The prevalence of HBeAg and HBcAg positivity was higher in both clinical groups with high HBV-DNA levels (≥5 log copies/mL). The frequency of double-mutation T1762/A1764 was significantly higher in the HCC group with HBV-DNA ≥5 log copies/mL (75%) compared to the HCC group with <5 log copies/mL (0%, $P = 0.002$) and CLD with <5 log copies/mL (6.7%, $P = 0.016$) (Table 2). Of note, the HBV-DNA level was significantly higher in HCC patients with T1762/A1764 mutants (6.1 ± 0.7 log copies/mL) compared to HCC with wild-type BCP strains (3.8 ± 2.0 log copies/mL) ($P = 0.021$), whereas there was no significant difference in the HBV-DNA level between CLD patients with the T1762/A1764 mutation (5.3 ± 0.6 log copies/mL) and those with wild-type BCP strains (5.5 ± 2.1 log copies/mL). The frequency of C1752 and/or V1753 tended to be higher in HCC with HBV-DNA ≥5 log copies/mL (75%) than in CLD with ≥5 log copies/mL (20%) and CLD with <5 log copies/mL (20%) ($P = 0.095$, 0.071, respectively).

Virological characteristics of BCP mutants are shown in Fig. 2. The mean level of HBV-DNA was higher in patients with the T1762/A1764 double mutation (5.7 ± 0.7 log copies/mL) than in those with wild-type BCP strains (4.9 ± 2.1 log copies/mL) with no significant difference (Fig. 2a), whereas in patients with T1764/G1766 mutants, both HBV-DNA levels (3.6 ± 1.1 log copies/mL) and HBcAg levels (0.9 ± 1.1 log pg/mL) were significantly lower than in those with wild-type BCP strains ($P = 0.024$, 0.049, respectively) (Fig. 2).

Discussion

Previous studies have demonstrated that coinfection with HBV and HDV and/or HCV is significantly associated with HCC development in Mongolian HBV carriers.[5–7] The main objective of the present study was to investigate the presence of a particular HBV mutation (or mutation pattern) in association with HCC in HBV genotype D-infected patients in this country. Using direct sequencing, we demonstrated that the C1752/V1753 (either or both) mutations were significantly associated with HCC in the studied cohort. This was also observed when only patients with coinfection were analyzed, suggesting that this mutation associated with HCC independently from coinfection.

The V1753 mutation was recently reported as a predictive factor for HCC among HBeAg-positive HBV/C1 carriers.[29] An *in vitro* study reported a higher replication capacity of HBV with C1753/T1762/A1764 than with T1762/A1764 mutations.[30]

Regarding the molecular aspect, C1753 is considered one of the 'hot spot' mutations of the HBx-encoding gene.[3,31] One of the functions of the HBx protein is transactivation of HBV-DNA transcription, as well as a number of cellular genes.[32] It was reported that C1753 enhanced the transactivation and antiproliferation activity of HBx protein in HBV/B[31] and, thereby, may contribute to carcinogenesis via the induction of a late G1 cell-cycle block,[33]

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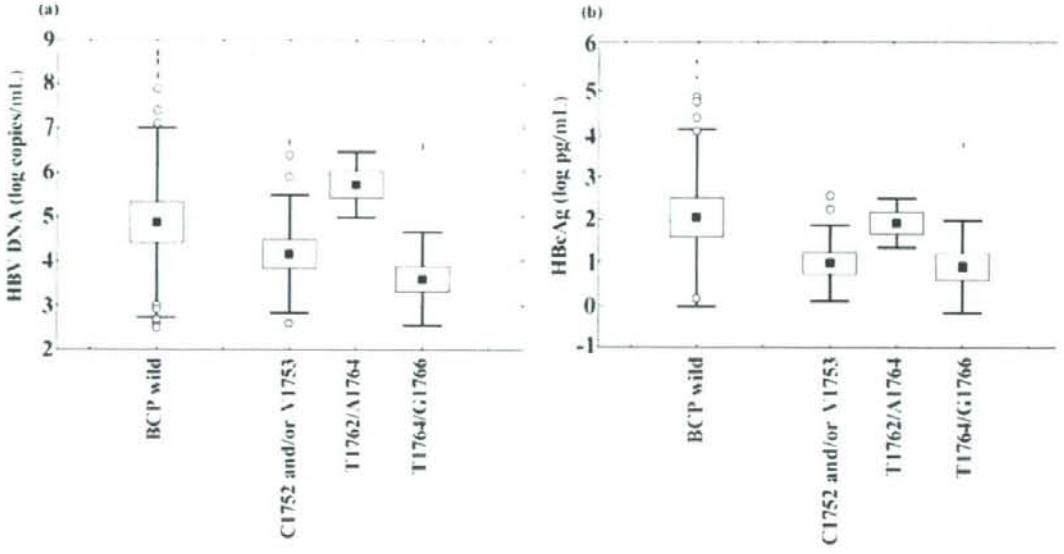

Figure 2 Comparison of (a) hepatitis B virus (HBV)-DNA levels (indicated in log copies/mL) and (b) HBV core antigen (HBcAg) levels (indicated in log pg/mL) among patients harboring basal core promoter wild-type strains (BCP wild); patients with C1752 and/or V1753 mutants (C1752 and/or V1753); patients with double-mutation T1762 and A1764 (T1762/A1764); and patients with double-mutation T1764 and G1766 (T1764/G1766). Box-whiskers, boxes and bars represent mean value, standard error and standard deviation, respectively.

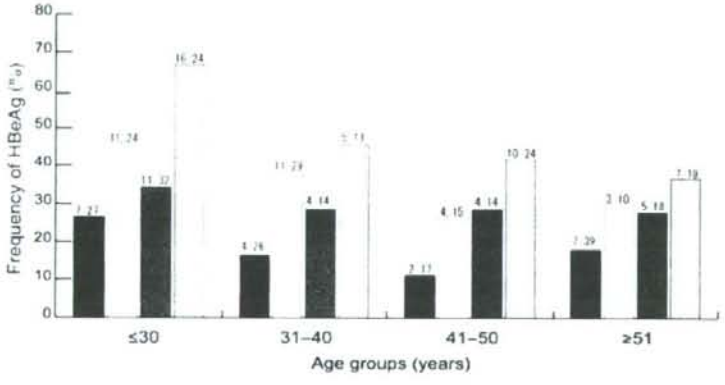

Figure 3 Age-specific seroprevalence of hepatitis B e antigen (HBeAg) in Mongolian patients with hepatitis B virus (HBV)/D enrolled in the present study compared with that observed previously in HBV/D, HBV/Aa (African/Asian type) and HBV/Ae (Europe/USA type).[18] (■), HBV/D (Mongolia); (■), HBV/D (India); ( ), HBV/Aa (Africa/Asia); (■), HBV/Ae (Europe).

enhancement of HBV replication[34] leading to higher frequency of HBV integration into the human host genome.[35] However, further prospective study is needed to clarify the pathogenic role of C1752 and/or V1753 in HCC development among HBV/D-infected patients. The current study was limited by the low prevalence of patients with detectable HBV/DNA, probably due to the high frequency of coinfection and triple-infection in the studied cohort. Both HCV and HDV could inhibit HBV replication as reported previously by *in vitro*[36] and *in vivo* studies.[37,38]

Interestingly, our cohort study showed early seroclearance of HBeAg among HBV/D-infected patients in Mongolia compared to previous data as shown in Fig. 3.[18] Previous reports described different viral mutations in association with early clearance of HBeAg in serum among different genotypes; in particular, the mutation in the so-called 'Kozak sequence' immediately upstream of the core initiation codon may interfere with the translation of the HBeAg precursor in HBV/Aa,[39] and the A1896 precore stop codon mutation in HBV/D.[18] Among studied HBV/D-infected patients, the frequency of precore stop codon mutation A1896 was higher among HBeAg-negative patients than those positive for HBeAg, which is in agreement with previous studies,[18,40] suggesting that this mutation is one of the most important factors of

HBeAg early seroconversion in HBV/D-infected individuals. Another mutation found in association with HBeAg-negative status in the studied cohort was the T1764/G1766 double mutation of the BCP region.

A recent report has indicated that the T1764/G1766 double mutation is characteristic of the HBV/D strain and it was associated with a higher viral load.[41] The present study is in agreement with the previous studies regarding the observation that the T1764/G1766 double mutation is a feature of genotype D strains. However, in the present study, this mutation was associated with lower HBV-DNA and HBcAg levels compared to wild-type BCP strains ($P = 0.024$, 0.049, respectively). The discrepancy between the previous and present studies might be due to the difference in the clinical characteristics of studied patients because HBeAg-negative patients were enrolled in the previous study. As has been shown, HBeAg-negative patients with wild strains of core promoter had lower HBV-DNA levels, as the virus replication is sufficiently suppressed by the immune system, whereas HBeAg-negative patients with core promoter variants will escape host immune response and thus maintain higher HBV replication.[42] Finally, the present study is the first to indicate the lack of association of the T1764/G1766 double mutation with HCC in HBV/D-infected patients by comparative analysis of the clinical groups.

The high incidence of T1762/A1764 in HBV/Ba and HBV/C is usually complementary to its presence as a predictive factor for HCC in patients infected with either of these genotypes.[4,43–45] The featured double-mutation T1762/A1764 was infrequently found in our study; overall, 13% of the patients were harboring this mutant, with a similar frequency in both clinical groups (CLD and HCC). The low frequency of double-mutation T1762/A1764 in HBV/D was also reported previously in Iran,[41] USA and India.[18]

Different pathogenic mechanisms have been suggested contributing T1762/A1764 mutants to HCC. Previous reports indicated HBeAg reduction, but high viral replication by T1762/A1764 mutants *in vitro*,[46] in contrast to clinical studies that argued against the enhancement effect of T1762/A1764 on viral replication in regard to T1762/A1764-related hepatocarcinogensis.[39,47] Our study revealed this mutation in 75% of HCC patients with high viral load (≥5 log copies/mL) which might indicate the association of this mutation with enhanced viral replication in HCC patients. However, the number of cases was small and further study is required to confirm this trend.

In conclusion, the association of either or both C1752/V1753 with HCC was indicated in HBV/D-infected patients. High prevalence of BCP T1764/G1766 mutation and precore A1896 may together contribute to early seroclearance of HBeAg in patients with HBV/D. Further large-scale studies are needed to investigate these trends.

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Case Report

Early emergence of entecavir-resistant hepatitis B virus in a patient with hepatitis B virus/human immunodeficiency virus coinfection

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The efficacy of entecavir for patients with hepatitis B virus/human immunodeficiency virus coinfection has not been fully elucidated. Here we examined a patient coinfected with both viruses in whom entecavir-resistant hepatitis B virus appeared. The 60-year-old Japanese male with the coinfection received antiretroviral therapy including lamivudine. The therapy initially suppressed replication of both viruses, followed by reactivation of the hepatitis B virus alone by 2 years of therapy. He subsequently received entecavir therapy in addition to the antiretroviral regimen. After entecavir administration, the hepatitis B virus DNA level was slightly reduced, but then increased after 6 months of entecavir therapy. In the sequencing analysis of hepatitis B virus, no drug resistance-associated amino acid substitutions were observed in the reverse transcriptase (rt) domain before antiretroviral therapy. The lamivudine-resistant amino acid substitutions at rt173, rt180 and rt204 were detected before entecavir administration, and further the entecavir-resistant rt202 substitution was observed after 6 months of entecavir therapy. The full-length hepatitis B sequences showed that the viral strain derived from the patient belonged to genotype H. In summary, this report describes a patient with hepatitis B virus/human immunodeficiency virus coinfection who received entecavir therapy in addition to an antiretroviral regimen and showed the early emergence of entecavir-resistance hepatitis B virus. In entecavir therapy for patients infected with both viruses, great care should be taken with respect to the emergence of entecavir-resistant hepatitis B virus, especially in patients with pre-existing lamivudine-resistant virus.

Key words: coinfection, drug-resistant hepatitis B virus, entecavir, hepatitis B virus, human immunodeficiency virus, lamivudine

INTRODUCTION

CHRONIC CARRIERS OF hepatitis B virus (HBV) number more than 350 million worldwide.[1] Chronic HBV infection is seen in approximately 10% of human immunodeficiency virus (HIV)-infected patients,[2] and coinfection with HBV and HIV is a serious health problem due to the shared mode of transmission. Since the prognosis of HIV-infected patients can be dramatically improved by highly active antiretroviral therapy (HAART), one of the major causes of mortality in HIV-infected patients is chronic liver disease due to HBV infection.[3]

Lamivudine (LAM, also abbreviated to 3TC), one of the antiretroviral drugs, has also been used for the reduction of HBV replication and improvement of HBV-related liver diseases.[4,5] However, the anti-HBV effect of LAM is hampered by the emergence of LAM-resistant mutant virus in cases of HBV monoinfection and HBV/

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HIV coinfection.[6,7] The LAM-resistant HBV strain is based on point mutation occurring within the reverse transcriptase (rt) domain of the polymerase gene. A methionine-to-valine/isoleucine amino acid substitution at rt204 (rtM204V/I) is known to confer LAM resistance.[8,9] A leucine-to-methionine substitution at rt180 (rtL180M) and a valine-to-leucine substitution at rt173 (rtV173L) have also been shown to appear in association with LAM resistance.[8,10,11] The emergence rate of LAM-resistant virus in patients coinfected with HBV and HIV has been reported to be approximately 50% after 2 years of therapy.[9]

Recently, entecavir (ETV) has been reported to be superior to LAM for the suppression of viral replication and disease activity in patients with HBV monoinfection who had not received previous treatment with other anti-HBV drugs (naïve patients).[12,13] ETV has also been shown to be effective in HBV-infected patients who had been treated with LAM and showed LAM resistance.[14] It has been demonstrated that ETV resistance occurs based with amino acid substitution(s) at rt184, rt202 and/or rt250, together with the LAM-resistant rtM204V/I and rtL180M substitutions.[15] The emergence rate of ETV-resistant virus after 3 years of therapy has been reported to be less than 1% in naïve patients and 15% in LAM-resistant patients with chronic HBV monoinfection.[16] However, the anti-HBV efficacy of ETV for HBV/HIV coinfection has not been fully clarified.

In this study, we examined a patient with concomitant HBV/HIV infection who underwent HAART including LAM, and showed the appearance of LAM-resistant HBV. Subsequent ETV administration did not lead to an adequate reduction of the HBV replicative level, followed by the early emergence of the ETV-resistant virus. We investigated the serial change in the drug resistance-associated mutation status within the rt domain of the HBV polymerase gene, as well as full-length nucleotide sequences of the ETV-resistant HBV strain derived from the patient.

CASE REPORT

Patient and serum sampling

A 60-YEAR-OLD JAPANESE heterosexual male first visited to the National Hospital Organization Osaka National Hospital in December 2001 due to a positive result from an HIV antibody (anti-HIV) test in voluntary HIV screening. From his anamnestic record, he had been admitted with type B acute hepatitis to another hospital 3 years earlier. Anti-HIV had been negative at that time. On his first visit, the anti-HIV positivity was confirmed by Western blot analysis. Antibodies to HIV-1 proteins, gp160, gp110/120, p68, p52, gp41, p40 and p34 were positive. As for antibodies to HIV-2 proteins, only an antibody to p68 was positive. According to these, he was judged to be infected with HIV-1. The HIV-RNA level was $10^{4.3}$ copies/mL, and the CD4+ T cell counts were 275/mm^3 (normal range, >300/mm^3). He tested positive for hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg), and negative for antibody to HBsAg (anti-HBs) and antibody to HBeAg (anti-HBe). The HBV-DNA level was $>10^{7.6}$ copies/mL, and the alanine aminotransferase (ALT) level was 106 IU/L. The patient was free of HIV-related symptoms and had no opportunistic infectious diseases. HAART with LAM (300 mg/day), zidovudine (AZT) (600 mg/day) and efavirenz (EFV) (600 mg/day) was started in April 2002. AZT and EFV were then substituted for sanilvudine (d4T) (60 mg/day) and avacavir (ABC) (600 mg/day) in July 2002 because of anemia and dizziness. By July 2002, HIV-RNA decreased to below the detection limit ($<10^{1.7}$ copies/mL), whereas the CD4+ T cell counts tended to rise up to >500/mm^3. In August 2006, fosamprenavir (FPV) (2400 mg/day) was commenced in place of d4T due to peripheral nerve palsy. Suppression of HIV-RNA below the detection limit continued at the end of follow-up, irrespective of repeated alterations in the therapeutic regimen of HAART. As for HBV status, HBV-DNA declined to $10^{3.9}$ copies/mL in April 2003 but increased again to $>10^{7.6}$ copies/mL in May 2005. To control HBV replication, ETV (0.5 mg/day) was added in October 2006. After the ETV administration, HBV-DNA slightly decreased from $>10^{7.6}$ to $10^{6.2}$ copies/mL in January 2007 but rose to $10^{7.2}$ copies/mL 3 months later. ALT remained abnormal and HBeAg continued to be positive throughout the follow-up period. The clinical course of the patient is summarized in Figure 1a.

For the nucleotide sequencing of HBV-DNA, the serum samples were obtained in December 2001 (before HAART), August 2006 (before ETV administration), and April 2007 (after 6 months of ETV therapy). These serum sampling points were designated as P1, P2 and P3 (see Fig. 1a). Serum samples were stored at −80 °C until use. Informed consent was obtained from the patient.

Virus markers and nucleotide sequencing

HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HIV were tested by chemiluminescent immunoassay. A

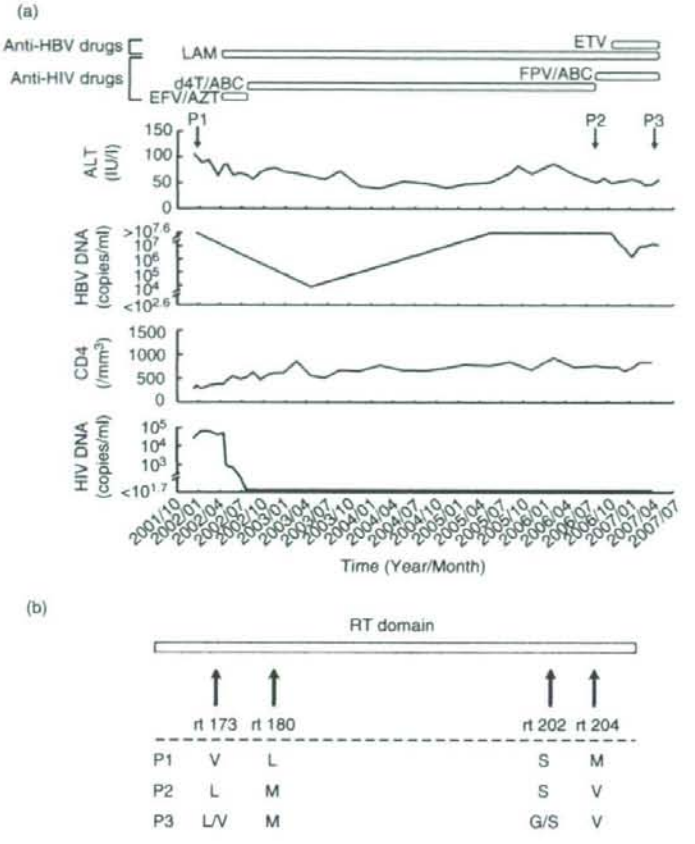

Figure 1 (a) Patient clinical course and serum sampling points. P1, P2 and P3 are the points at which serum samples were obtained. P1 was taken in December 2001 (before HAART), P2 in August 2006 (before ETV administration) and P3 in April 2007 (after 6 months of ETV therapy). ABC, avacavir; ALT, alanine aminotransferase; AZT, zidovudine; d4T, sanilvudine; EFV, efavirenz; ETV, entecavir; FPV, fosamprenavir; HBV, hepatitis B virus; HIV, human immunodeficiency virus; LAM, lamivudine. (b) Serial change in the status of drug resistance-associated amino acid substitutions.

confirmatory anti-HIV-1/2 testing was carried out by Western blot analysis. Serum HBV-DNA was detected by means of a PCR assay (Amplicor HB monitor; Roche Diagnostics, Basel, Switzerland) with a lower detection limit of $10^{2.6}$ (=400) copies/mL. Plasma HIV-RNA was quantified by a PCR assay (Amplicor HIV-1 monitor; Roche) whose lower detection limit was $10^{1.7}$ (=50) copies/mL.

The nucleotide sequences of HBV-DNA were determined by a method based on nested PCR and direct sequencing, as described elsewhere.[17] In this study, primers BF5-2 (5′-TCC TCA GGC CAT GCA GTG GA-3′, nt 3201-20) and BR8 (5′-TTG CGT CAG CAA ACA CTT GG-3′, nt 1195-76) were also used. Nucleotide sequences of the entire rt domain in the polymerase gene were examined in HBV strains derived from the P1 and P2 serum samples (GenBank accession nos. AB353765 and AB353766), whereas the full-length HBV-DNA was determined in the strain derived from the P3 serum sample (GenBank accession no. AB353764). The full-length HBV strain obtained in this study (designated as HBD103), the seven representative HBV strains of genotypes A–G and the eight previously isolated HBV strains of genotype H were aligned, and the phylogenetic tree was constructed. These analyses were done at the homepage of the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp).

Results of sequencing analysis of HBV

The serial change in the nucleotide sequences in the rt domain of the HBV polymerase gene was first examined

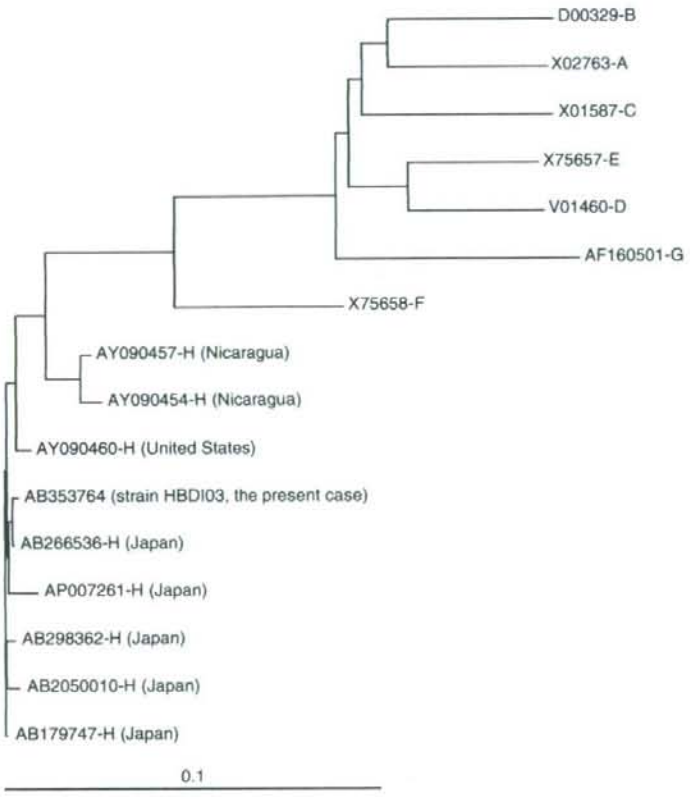

Figure 2 Phylogenetic tree analysis including the HBV strain HBDI03 obtained in this study, the seven representative HBV strains of genotypes A–G, and the eight previously isolated HBV strains of genotype H.

using serum samples obtained at P1–P3 (Fig. 1b). At point P1, no drug resistance-associated mutations were found in the rt domain, but three LAM resistance-associated substitutions, rtM204V, rtL180M and rtV173L, emerged at point P2. A serine-to-glycine substitution at rt202 (rtS202G), which has been shown to be one of the ETV resistance-associated substitutions,[15] was further observed at point P3, although rtS202G and rtV173L substitutions occurred incompletely. No other amino acid substitutions were seen in the rt domain of the HBV polymerase gene from point P1 to P3. Thus, in the patient with HBV/HIV coinfection, the emergence of the drug resistance-associated amino acid substitutions revealed a close relationship with the poor anti-HBV efficacy of LAM and ETV.

Next, the full-length nucleotide sequences of HBV were determined from the P3 serum sample of the patient with HBV/HIV coinfection showing ETV resistance. The full-length HBV strain HBDI03 comprised a total of 3215 nucleotide lengths. The phylogenetic tree was depicted using the HBV strain HBDI03, the seven representative HBV strains of genotypes A–G and the eight previously identified genotype H HBV strains. As shown in Figure 2, the HBV strain HBDI03 obtained in this study was classified as genotype H. When the nucleotide sequences of the strain HBDI03 were compared with the eight reported genotype H HBV strains, the strain HBDI03 showed a 97.2–99.8% identity with these strains. The unique amino acid substitutions in the strain HBDI03 were further investigated in comparison with these eight genotype H HBV strains. As shown in Table 1, four drug resistance-associated substitutions within the rt domain were observed, as described above. The two amino acid substitutions in the S gene were also caused by the same mutations of the drug resistance-associated rtV173L and rtM204V

Table 1 The unique amino acid substitutions in strain HBDI03 in comparison with eight previously isolated genotype H hepatitis B virus strains

Amino acid position	Consensus residue of genotype H	Residue unique to strain HBDI03
Polymerase		
519 (rt173)	V	L/V
526 (rt180)	L	M
548 (rt202)	S	G/S
550 (rt204)	M	V
Surface		
164	E	D/E
195	I	M
X		
32	W	G

Consensus residues of genotype H were from the eight reported hepatitis B virus (HBV) strains (GenBank accession nos. AY090454, AY090457, AY090460, AP007261, AB179747, AB205010, AB266536 and AB298362).

changes. As for the remaining one amino acid substitution in the X gene, the substituted glycine residue observed in the HBDI03 strain was a common one in the representative HBV strains of genotypes A–G at the corresponding codon position. Taken together, the HBDI03 strain did not appear to have any distinctive features other than the presence of the drug-associated amino acid substitutions.

DISCUSSION

RECENTLY, ETV HAS been widely accepted as an effective drug for the treatment of HBV monoinfection because of its stronger inhibitory effect on HBV replication and lower emergence rate of drug-resistant mutant virus compared to LAM.[12–14] ETV-resistant HBV has been demonstrated to be established by amino acid substitution(s) at rt184, rt202 and/or rt250, in addition to the LAM-resistant rtM204V/I and rtL180M substitutions.[15] The emergence rate of ETV-resistant virus has been reported to be higher in LAM-resistant patients than in naïve patients.[16] There has so far been little evidence concerning the anti-HBV efficacy of ETV for patients with HBV/HIV coinfection. In particular, LAM-resistant HBV has been shown to emerge frequently in patients with HBV/HIV coinfection who received LAM therapy as a component of HAART.[7] The therapeutic efficacy of ETV on LAM-resistant HBV should be assessed in patients with HBV/HIV coinfection. In this study, we examined a patient with HBV/HIV coinfection who had LAM-resistant HBV induced by HAART including LAM, and underwent subsequent ETV therapy. The patient showed a rather weak suppressive effect of ETV on HBV replication, followed by the emergence of ETV-resistant HBV in the early phase of therapy.

In the sequence analysis of the HBV genome, no drug-resistant HBV mutations were detected before HAART, but continuous LAM administration induced the LAM-resistant mutant HBV with rtM204V, rtL180M and rtV173L amino acid substitutions. Subsequent ETV therapy resulted in the emergence of an ETV-resistant virus possessing the rtS202G substitution in addition to the three LAM resistance-associated substitutions after no more than 6 months of ETV therapy, although the rtS202G and rtV173L substitutions were incomplete. In LAM-resistant patients with HBV monoinfection, the emergence rate of the ETV-resistant mutation has been reported to be merely 15% after 3 years of therapy.[16] In comparison with this, ETV-resistant HBV appeared in an extremely early phase of therapy in our patient with HBV/HIV coinfection. According to this, ETV resistance is speculated to be established earlier in patients with HBV/HIV coinfection than in those with HBV monoinfection, although concomitant HIV infection has not thus far been suggested to result in a higher incidence of the drug-resistant HBV strain in the treatment with other anti-HBV drugs in chronic HBV infection. The latent immune deficiency caused by HIV infection might prevent HBV eradication through a host immune response, resulting in poor anti-HBV efficacy of ETV. Alternatively, simultaneous usage of multiple antiretroviral drugs might in some way contribute to the emergence of ETV-resistant HBV.

Very recently, it has been shown that ETV possesses modest anti-HIV activity both *in vitro* and *in vivo* and can induce the drug-resistant mutant HIV strain in patients with HBV/HIV coinfection.[18] This suggests that ETV may not be appropriate for the treatment of patients with HBV/HIV coinfection in whom HAART is not needed. On the other hand, ETV is considered to be beneficial for patients with HBV/HIV coinfection undergoing a stable continuation of HAART. In particular, the therapeutic efficacy of ETV may be more promising in patients without LAM-resistant HBV than in those with it. Although the present case of the patient under discussion, who already displayed LAM-resistant HBV due to the preceding HAART, did not support the usefulness of ETV therapy because of the early emergence of ETV-resistant HBV, further studies with a large number of

patients should be completed to assess the antiviral efficacy and deliberate clinical application of ETV therapy for HBV/HIV coinfection.

Both adefovir dipivoxil (ADV) and tenofovir disoproxil fumarate (TDF) have recently been shown to effectively inhibit HBV replication in patients with HBV/HIV coinfection, irrespective of LAM resistance.[19,20] ADV exerts only anti-HBV activity and is available for patients with HBV/HIV coinfection who have no need for HAART or who are receiving a stable HAART regimen. In contrast, TDF can be used as a component of HAART because of its valuable antiviral activity against both HBV and HIV. Accordingly, ADV and TDF are currently useful drugs for patients with HBV/HIV coinfection and may be subsequent therapeutic options for the patient reported in this study.

Our patient was found to be infected with HBV of genotype H, a globally rare genotype. To date, the full-length sequences of eight genotype H HBV strains have been reported from the USA, Nicaragua and Japan (see Fig. 2). Of them, one strain has been obtained from a Japanese patient with chronic HBV monoinfection who underwent ETV therapy as a naïve patient and showed ETV resistance later.[21] The relevance of the genotype frequency to the therapeutic efficacy of ETV should be studied extensively in HBV-infected patients treated with ETV.

In Japan, genotypes B and C are prevalent in chronic HBV carriers who acquire the infection mainly through the mother-to-child transmission route. In contrast, the foreign HBV strains other than genotypes B and C have been shown to be involved in a considerable proportion of patients with acute HBV infection.[22] Infection of such foreign types of HBV possibly occurs through sexual contacts in Japan. In our patient with HBV/HIV coinfection who had genotype H HBV of foreign origin, it is speculated that acute HBV infection occurring 3 years before his first visit led to the transition to chronicity. The time of HIV infection cannot be defined due to the lack of HIV-RNA testing during the period of acute HBV infection. The possibility of simultaneous infection with HBV and HIV cannot be excluded, despite the negative result of anti-HIV at that time, because the test may have taken place during the immunological window period of HIV infection.

In summary, we have introduced a patient with HBV/HIV coinfection who underwent ETV therapy in addition to the HAART regimen and showed ETV resistance in the early phase of therapy. Our finding suggests that, in ETV therapy for patients with HBV/HIV infection, great care should be taken against the emergence of ETV-resistant HBV, especially in patients with pre-existing LAM-resistant HBV.

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Short Communication

Prevalence of hepatitis B virus infection in Japanese patients with HIV

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Patients with HIV infection are frequently infected with hepatitis viruses, which are presently the major cause of mortality in HIV-infected patients after the widespread use of highly active antiretrovirus therapy. We previously reported that approximately 20% of HIV-positive Japanese patients were also infected with hepatitis C virus (HCV). Hepatitis B virus (HBV) infection may also be an impediment to a good course of treatment for HIV-infected patients, because of recurrent liver injuries and a common effectiveness of some anti-HIV drugs on HBV replication. However, the status of co-infection with HIV and HBV in Japan is unclear. We conducted a nationwide survey to determine the prevalence of HIV–HBV co-infection by distributing a questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan. Among the 5998 patients reported to be HIV positive, 377 (6.4%) were positive for the hepatitis B surface antigen. Homosexual men accounted for two-thirds (70.8%) of the HIV–HBV co-infected patients, distinct from HIV–HCV co-infection in Japan in which most of the HIV–HCV co-infected patients were recipients of blood products. One-third of HIV–HBV co-infected patients had elevated serum alanine aminotransferase levels at least once during the 1-year observation period. In conclusion, some HIV-infected Japanese patients also have HBV infection and liver disease. A detailed analysis of the progression and activity of liver disease in co-infected patients is needed.

Key words: co-infection, hepatitis B, HIV, liver disease.

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major public health problem worldwide, along with hepatitis C virus (HCV) and HIV infections. In the USA, the estimated prevalence of HBV is less than 1%, but approximately 1 million people are persistently infected.[1] The prevalence of HIV in the USA is also <1%, and the virus is estimated to have infected approximately 800 000 people.[2] Because of the common transmission routes, that is, parenteral transmission routes, many people with HIV infection are also infected with HBV. Among the HIV-positive people in the USA, the prevalence of HBV co-infection is 6–14%.[1,2] Before the introduction of highly active antiretroviral therapy (HAART) in 1996, most patients with HIV infection died of HIV-associated opportunistic infections, such as *Pneumocystis jiroveci* pneumonia and cytomegaloviral infection. Since the widespread use of HAART, the mortality associated with HIV infection has declined. However, the reduction in mortality due to opportunistic infection, has left patients co-infected with HIV and hepatitis viruses faced with the menace of progressive liver diseases due to HBV infection,[3,4] in addition to HCV infection.[5]

HBV co-infection or superinfection of HIV-infected patients leads to several problematic situations. First, HBV infection tends to develop into persistent infection in HIV-infected patients,[1,6,7] which is a rare event in healthy adults, although it substantially depends on the genotype of HBV.[8] It results in the acceleration of the development of cirrhosis and eventually hepatocellular carcinoma. Second, some nucleoside reverse transcriptase inhibitors (NRTI) used in HAART also have

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inhibitory effects on the replication of HBV.[9–12] A careless administration or discontinuation of NRTI on HIV–HBV co-infected patients may cause reactivation and/or aggravation of hepatitis B. In addition, the administration of anti-HBV drugs in HIV–HBV co-infection may lead to the development of drug resistance.[11,12] Third, liver injury occurs more frequently in patients on HAART who are co-infected with HIV and HBV than those infected with HIV only.[9,10]

Importantly, co-infection with HIV and HCV increases the morbidity and mortality of HIV-infected patients in Japan,[13] where the prevalence of HIV infection is increasing linearly, and is exceptionally high among developed countries.[14] There are more than 14 000 HIV-positive people in Japan as of 2006, according to the AIDS National Survey in Japan,[14] and approximately 0.8 million chronic HBV carriers.[15] However, the prevalence of co-infection with HIV and HBV in Japan has not been clarified to date. Therefore, we conducted a nationwide study by distributing a postal mail-based questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan.

PATIENTS AND METHODS

IN THE QUESTIONNAIRE, the following information was obtained from the hospitals regarding the number of patients who visited the hospitals at least once between January and December in 2006: (i) the number of HIV-positive patients; (ii) the number of hepatitis B surface antigen (HBsAg)-positive patients among (i); (iii) the number of patients among (ii) who were determined at least once to have a serum alanine aminotransferase (ALT) level higher than 100 IU/L; (iv) the number of HIV-positive patients that contracted HIV from blood products; (v) the number of HBsAg-positive patients among (iv), (vi) the number of patients among (v) who were determined at least once to have a serum ALT level higher than 100 IU/L; (vii) the number of HIV-positive patients among homosexual men, (viii) the number of HBsAg-positive patients among (vii), (ix) the number of patients among (viii) who were determined at least once to have a serum ALT level higher than 100 IU/L; (x) the number of HIV-positive patients that contracted HIV through intravenous drug use (xi) the number of HBsAg-positive patients among (x), (xii) the number of patients among (xi) who had at least one determination of a serum ALT level more than 100 IU/L; (xiii) the number of HIV-positive patients whose transmission routes were classified as "others"; (xiv) the number of HBsAg-positive patients among (xiii); and (xv) the number of patients among (xiv) who were determined at least once to have a serum ALT level higher than 100 IU/L.

The questionnaire was sent to the 372 hospitals belonging to the HIV/AIDS Network of Japan by mail. Answers were mostly returned by mail and in some cases by fax. The list of the hospitals in the HIV/AIDS Network of Japan can be viewed at http://www.acc.go.jp/mLhw/mLhw_frame.htm.

RESULTS

THE QUESTIONNAIRE WAS sent to all 372 hospitals that were on the list of the hospitals in the HIV/AIDS Network of Japan in January 2006. Two hundred and seven hospitals (55.6%) responded within the indicated period. In total, 5998 patients were reported to be HIV positive. The collection rate of 55.6% was higher than that (47.8%) for a questionnaire HIV–HCV co-infection study carried out in 2003.[15] It may appear rather low, particularly considering the number of reported HIV-positive people in 2006, which was approximately 14 000, according to the AIDS National Survey in Japan.[14] However, not all of the HIV-positive people were going to hospitals, and the answers to the questionnaire were obtained from most of the major hospitals in the HIV/AIDS Network in big cities around Japan. This suggests that not all, but a majority of HIV-positive Japanese patients were enrolled in the study.

Among the 5998 patients reported to be HIV positive, 377 (6.3%) patients were positive for HBsAg (Table 1). Of these 377 patients, 122 (32.4%) had elevated serum ALT levels at least one time during the 1-year observation period.

The HBV prevalence rates, when fractionated by the routes of transmission, were as follows: among the 508 HIV-positive patients who contracted HIV from blood products, such as unheated concentrated coagulation factors, only 30 (5.9%) were HBsAg positive, which shows a marked contrast to the prevalence of HCV in this cohort (Fig. 1).[16] Among the 23 intravenous drug users, three (13.0%) were HBsAg positive. Among the 3213 HIV-positive patients who were homosexual men, 267 (8.3%) were HBsAg positive. In the remaining 2254 patients who were HIV-positive and whose route of HIV transmission was classified as "others", most contracted HIV heterosexually. This number (2254) showed a substantial increase from the 1316 obtained in the questionnaire for the HIV–HCV co-infection study in 2003, while the total number of HIV-positive patients increased from 4877 to 5998.[16] Among these, 77 (3.4%)

Table 1 Prevalence rates of hepatitis B virus infection among HIV-positive patients

Routes of transmission	No. patients	HBsAg positive (% in HIV positive according to route)	ALT >100 IU/L (% in HBsAg positive according to route)
Blood products	508 (5.9%)	30 (40.0%)	12
Homosexual men	3213 (8.3%)	267 (32.2%)	86
Drug addicts	23 (13.0%)	3 (66.7%)	2
Others (heterosexual etc.)	2254 (3.4%)	77 (28.6%)	22
Total	5998	377 (6.3%)	122 (32.4%)

ALT, serum alanine aminotransferase; HBsAg, hepatitis B surface antigen.

were HBsAg positive. In terms of the route of HIV infection, 267 (70.8%) of the 377 patients were homosexual men among the HIV–HBV co-infected patients. This shows a contrast to the status of HIV–HCV co-infection, in which the majority of HIV–HCV co-infected Japanese patients contracted both viruses from blood products.[16]

There were one or more HIV-positive patients in 154 (74.4%) of the 207 hospitals in the HIV/AIDS Network of Japan (Table 2). Twenty four (11.6%) of 207 hospitals had 20–49 HIV-positive patients, and 16 (7.7%) hospitals had 50 or more HIV-positive patients. There were one or more patients who were co-infected with HIV and HBV in 64 (30.9%) of the 207 hospitals. There were 10 or more HIV–HBV co-infected patients in nine (4.3%) hospitals, all of which had 50 or more HIV-positive patients (Table 2). HIV–HBV co-infected patients were concentrated in specific hospitals in big cities around Japan. In particular, in the Kanto area, HIV–HBV co-infected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area.

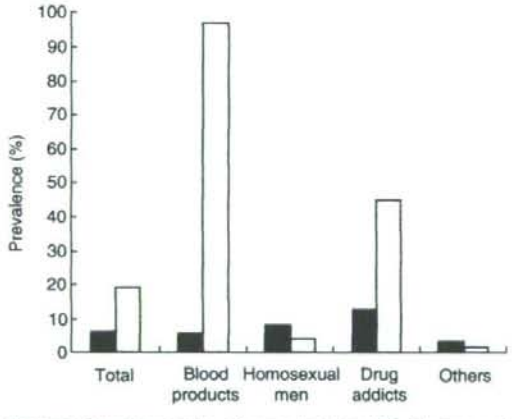

Figure 1 Prevalence rates of persistent hepatitis B virus and hepatitis C virus infections in the HIV-positive population sorted by the HIV risk group. (■), HBsAg, hepatitis B surface antigen; (□), anti-HCV, antibody to hepatitis C virus. *Prevalence rates of anti-HCV are obtained from Koike K *et al.*[16]

DISCUSSION

ALONG WITH THE increase in the number of HIV-infected patients in Japan, co-infection with HIV and hepatitis viruses has become a major medical issue. HBV infection of HIV-positive patients raises several difficult problems: HBV infection tends to develop into persistent infection, even in adults; some NRTI used in HAART also have inhibitory effects on the replication of HBV, the improper administration, or discontinuation of which may lead to drug resistance; and HIV–HBV co-infected patients on HAART have liver injuries more frequently than HIV-monoinfected patients. It is important to determine the status of HBV infection in HIV-positive patients.

According to the statistics of the Ministry of Health, Labor, and Welfare of Japan, the number of reported HIV-positive people was slightly over 14 000 in 2006.[14] In the present study, 6.4% of HIV-positive patients were positive for HBsAg, the most reliable marker for ongoing HBV infection. It might have been advantageous if

Table 2 Number of hospitals categorized according to the number of patients infected with HIV and those co-infected with HIV and hepatitis B virus (HBV)

No. HIV (+)/ HBV (+)	No. HIV(+)				
	0	1–19	20–49	50+	Total
0	53	76	13	1	143
1–9	0	38	11	6	55
10+	0	0	0	9	9
Total	53	114	24	16	207

serum HBV-DNA levels were determined, but unfortunately, HBV-DNA level determination was not a routine laboratory test in most hospitals. In addition, considering that the antibody to the hepatitis B core antigen might be the only marker of ongoing HBV infection in some immuno-compromised patients, it would also be advantageous if this viral marker were available. These issues should be investigated in future studies. Comments from hospitals to the questionnaire included one indicating that not all HIV-positive patients underwent a test for serum HBsAg, suggesting the actual prevalence of HBsAg in HIV-infected patients might be higher than 6.4%.

In a previous questionnaire study of HIV–HCV co-infection, the prevalence of HCV infection among HIV-infected patients was 19.2%;[16] the prevalence of HBV infection (6.4%), is one-third of it. The lower positivity for HBsAg than for the anti-HCV antibody among those who contracted HIV through blood products accounts for this difference: almost all (96.9%) of the patients who contracted HIV through blood products were also anti-HCV antibody positive.[16] It should be noted that among the homosexual male patients who were HIV positive, 8.3% were HBsAg positive, which is twice as high as that of the anti-HCV antibody in these populations. A higher prevalence of HBV infection as a sexually transmitted infection than that of HCV[17] may explain the high prevalence of HBV infection in HIV-positive homosexual men. Similarly, a HBV prevalence of 3.4% in heterosexually transmitted HIV-positive patients is higher than that of the general Japanese population of the same age.[15]

Of the 377 patients who were HBsAg positive, 122 (32.4%) had elevated serum ALT levels at least once in the 1-year observation period. In this type of study using a questionnaire, it is difficult to obtain the details of patients' data, including age, body weight, and the degrees of liver injuries and fibrosis. If detailed items were included in the questionnaire, then the collection rate would be low. This time, to obtain a high collection rate, we asked whether the patients with HBsAg showed an elevated ALT level higher than 100 IU/L at least once during the 1-year observation period. We thereby do not have details on liver disease in HIV–HBV co-infected patients in the current study. Nonetheless, one-third of HIV–HBV co-infected patients have moderate liver injuries, either chronic hepatitis B or adverse effects of drugs, and are waiting for an aid for the amelioration of liver disease. A detailed analysis of the progression and activity of liver disease in HIV–HBV co-infected patients is expected.

The collection rate of the present questionnaire from the hospitals belonging to the HIV/AIDS Network was 55.6% (207 of 372). This was higher than that (47.8%) in the HIV–HCV co-infection questionnaire study carried out in 2003. The reason for this increase is not clear, but presumably the questionnaire conducted in 2003 has raised awareness among hospital staff regarding the relevance of hepatitis virus and HIV co-infection in clinical practice.

In the current study, both Japanese patients and those of other nationalities/ethnicities were included in the study. Although the ratio of newly diagnosed HIV-positive foreign people has been declining to approximately 10% in 2006, the one in total HIV positive still accounts for approximately 25% in Japan. Because the rates of the HBV carrier are different among countries, it is ideal to analyze the HBV prevalence separately according to the nationalities/ethnicities. However, in the current survey to the hospitals in HIV/AIDS Network of Japan, nationality/ethnicity was not itemized in order to make the questionnaire simple. If we would attempt to obtain such data under the approval of the ethical committee in each hospital, the response rate to questionnaire would be extremely lowered.

To establish measures that decrease the morbidity and mortality of HIV–HBV co-infected patients, it is essential to determine the current status of co-infection. In the present study, the number and transmission routes of HIV–HBV co-infected patients in Japan were determined for the first time, although detailed information on the severity and progression of liver disease in HIV–HBV co-infected patients has not been obtained yet. Undoubtedly, this will be the first step towards improving the prognosis and quality of life of Japanese patients co-infected with HIV and HBV.

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