that is, they were cross-sectional studies, the association between different clinical events after seroconversion and specific HBV genomic mutations has not been clearly defined. To investigate this issue, complete HBV sequences were examined in eight IC and eight HCC patients before and after HBeAg seroconversion.

Materials and methods

Serum samples

Serum samples were obtained from 16 patients (eight patients were IC and the other eight were HCC patients) at the Nagoya City University Graduate School of Medical Sciences and National Hospital Organization Osaka National Hospital before and after seroconversion. Sixteen patients were infected with HBV genotype C. IC were defined as individuals who were hepatitis B surface antigen (HBsAg) positive with normal alanine aminotransferase (ALT) and α-fetoprotein levels over a 5-year period (with at least 12 evaluations at 3-month intervals) and without the presence of portal hypertension. HCC patients were diagnosed on the basis of results of abdominal ultrasonography, angiography, computed tomography, magnetic resonance imaging, or liver biopsy as well as by their having an elevated serum α-fetoprotein level (>400 ng/ml).

HBV Genotyping

HBV genotypes were determined by the restriction fragment length polymorphism method from the *S* gene sequence amplified by polymerase chain reaction (PCR)[13] or enzyme immunoassay (EIA) with monoclonal antibodies for distinct epitopes in the pre-S2 region products,[14] with commercial kits (HBV genotype EIA; Institute of Immunology, Tokyo, Japan). The genotypes were also confirmed by a phylogenetic tree analysis.

HBV DNA extraction

Serum samples were stored at −80°C until the assay. DNA was extracted from 100 μl of serum by using QIAamp DNA blood kits (Qiagen, Hilden, Germany).

Determination of the complete nucleotide sequences of HBV/C

The complete nucleotide sequences of 30 HBV/C isolates from 16 patients (HBV DNA in two serum samples from IC after seroconversion could not be amplified) were determined by a method reported previously[15] with a slight modification. In brief, two overlapping fragments of HBV genome were amplified by PCR, and eight overlapping HBV DNA fragments were amplified further by PCR with nested primers. Amplification was performed in a 96-well cycler (GeneAMP9600; Perkin-Elmer Cetus, Norwalk, CA, USA), and the PCR products were electrophoresed in 3.0% (wt/vol) agarose, stained with ethidium bromide, and observed under UV light.[3] Standard precautions were taken to avoid contamination during PCR. A negative control serum was also processed and included in each run to ensure specificity. Twelve overlapping HBV DNA fragments thus amplified were sequenced directly with a Prism BigDye kit (Applied Biosystems, Foster City, CA) in an ABI 3100 DNA automated sequencer.

Statistical analysis

Statistical analyses were performed with χ-squared and Fisher's exact tests for categorical variables. The Mann-Whitney *U* test was used for continuous variables, as appropriate. Differences were considered to be significant with P values <0.05. The statistical analysis software used was Stata software, version 8.0 (Statacorp LP, College Station, TX, USA).

Results

Table 1 compares age, ALT level, platelet count, HBV DNA, and rate of cirrhosis before and after HBeAg seroconversion, as well as age at seroconversion and the intervals between two sampling points for all patients. ALT level, platelet count, HBV DNA, and rate of cirrhosis after seroconversion were significantly higher among HCC patients than in IC.

The alignment of sequences covering the enhancer II and core promoter regions is shown in Fig. 1. We could not amplify HBV DNA in two serum samples from IC because of the small amount of HBV DNA in the samples. The box alpha and basal core promoter contained mutational hot spots, but box beta did not. The frequency of the T1653 mutation tended to be higher among HCC patients after seroconversion [IC vs. HCC: 1/6 (16.7%) vs. 5/8 (62.5%); $P = 0.086$] (Fig. 1 and Table 2), whereas the T1653 mutation did not differ between the two groups before seroconversion [IC vs. HCC: 1/8 (12.5%) vs. 2/8 (25%); $P = 0.522$]. The prevalence of the BCP double mutation was high among both IC and HCC patients after seroconversion [IC vs. HCC: 5/6 (83.3%) vs. 7/8 (87.5%); $P = 0.825$]. The prevalence of S1753 was low among IC and HCC patients before and after seroconversion (Fig. 1 and Table 2). The S1753 mutant was not recognized in the patients who were infected with the T1653 mutation clone. Deletion mutants of the core or pre-S region before seroconversion were significantly associated with HCC patients

Table 1. Comparison of clinical characteristics between IC and HCC patients before and after HBeAg seroconversion

Features	Before seroconversion			After seroconversion		
	Inactive carriers ($n = 8$)	HCC patients ($n = 8$)	Differences P value	Inactive carriers ($n = 8$)	HCC patients ($n = 8$)	Differences P value
Male, n (%)				4 (50)	7 (87.5)	0.106
Age (years)[a]	31.8 ± 8.4	40.0 ± 10.6	0.246	43.6 ± 10.0	51.6 ± 13.8	0.317
ALT (U/L)[a]	199.6 ± 220.5	234.6 ± 242.2	0.875	20.3 ± 7.2	40.4 ± 16.9	0.009*
Platelet count ($\times 10^4/mm^3$)[a]	17.5 ± 2.0	15.1 ± 4.3	0.268	17.5 ± 3.3	11.5 ± 5.4	0.027*
HBV DNA (LGE/ml)[a]	7.2 ± 0.5	7.2 ± 0.5	0.869	4.3 ± 0.7	5.7 ± 1.2	0.022*
Cirrhosis (%)	0 (0)	2 (25)	0.131	0 (0)	5 (62.5)	0.007*
Age at seroconversion (years)				37.8 ± 8.1	47.3 ± 14.2	0.226
Intervals between two sampling points (years)				11.8 ± 2.9	11.6 ± 4.4	1.0

IC, inactive carriers; HCC, hepatocellular carcinoma; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; HBV, hepatitis B virus; LGE, log genome equivalents
* Statistically significant
[a] Mean ± SD

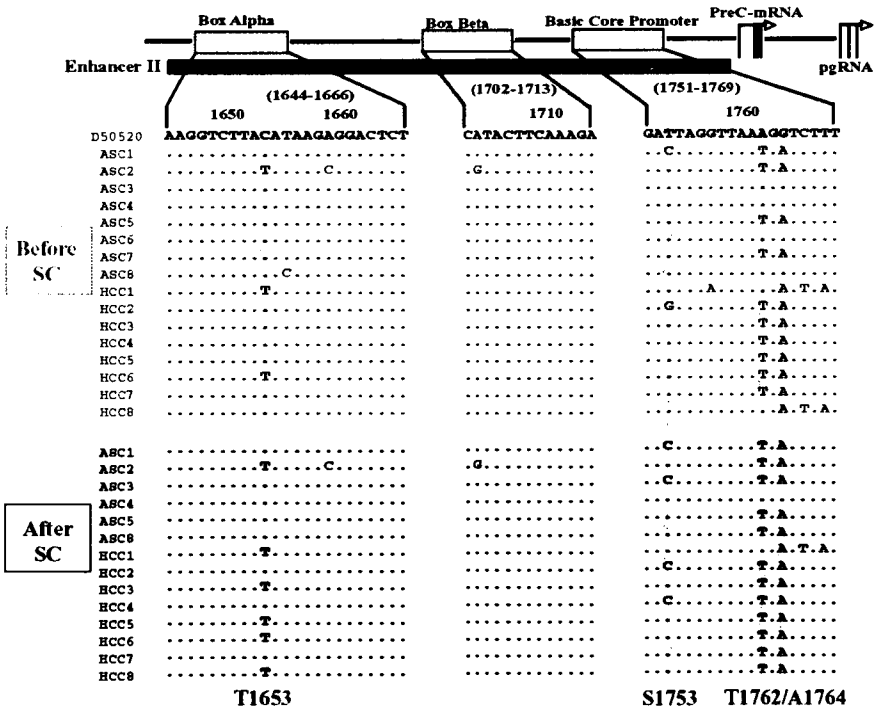

Fig. 1. Nucleotide sequences that cover enhancer II and core promoter regions in inactive carriers (IC) and hepatocellular carcinoma (HCC) patients. The wild-type sequence for genotype C is represented by D50520. *Dots* indicate nucleotides identical to the wild type. *SC*, seroconversion

(core deletion mutant: 0/8 [0%] vs. 4/8 [50%]; $P = 0.021$, pre-S deletion mutant 0/8 [0%] vs. 5/8 [62.5%]; $P = 0.007$, respectively) (Table 2).

The pre-S deletion mutant was detected in only one IC as a minor clone (Fig. 2), whereas among HCC patients, deletion mutants (major clones) were detected in five of eight (62.5%) patients before seroconversion, and in three of eight (37.5%) after seroconversion. Only two patients (patients 4 and 5) had pre-S deletion mutants before and after seroconversion. Two HCC patients (patients 1 and 2) had pre-S deletion mutants only before seroconversion (Fig. 3). Three HCC patients (patients 4–6) before seroconversion and one patient (No. 7) after seroconversion were coinfected with wild-type virus and pre-S deletion mutants (the deletion mutants were the major clones). Most deletions were identified in the 3′ terminus of the pre-S1 region and the 5′ terminus of the pre-S2 region.

Table 2. Comparison of HBV mutations between IC and HCC patients before and after HBeAg seroconversion

Features	Before seroconversion			After seroconversion		
	Inactive carriers ($n = 8$) n (%)	HCC patients ($n = 8$) n (%)	Differences P Value	Inactive carriers ($n = 8$) n (%)	HCC patients ($n = 8$) n (%)	Differences P Value
T1653 mutation	1 (12.5)	2 (25)	0.522	1/6 (16.7)	5/8 (62.5)	0.086
S1753 mutation	1 (12.5)	1 (12.5)	1.0	2/6 (33.3)	2/8 (25)	0.730
A1896 mutation	3 (37.5)	0 (0)	0.055	4/6 (66.7)	3/8 (37.5)	0.280
BCP double mutation	4 (50)	7 (87.5)	0.106	4/6 (66.7)	7/8 (87.5)	0.325
Core deletion Mutant	0 (0)	4 (50)	0.021*	0/6 (0)	0/8 (0)	1.0
Pre-S deletion Mutant	0 (0)	5 (62.5)	0.007*	1/8 (12.5)	3/8 (37.5)	0.248

BCP, basal core promoter
* Statistically significant

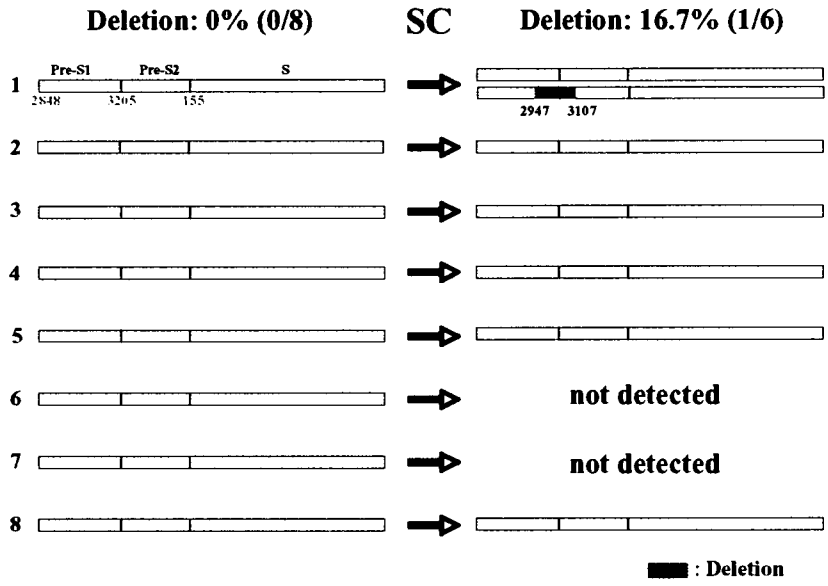

Fig. 2. Pre-S region deletion mutant in IC. The nucleotide sequences of the pre-S1, preS-2 and S are shown as bars. *Shading* of a bar indicates a deletion region. A pre-S deletion mutant was detected in only one IC after seroconversion as a minor clone (the *lower bar* shows the minor clone)

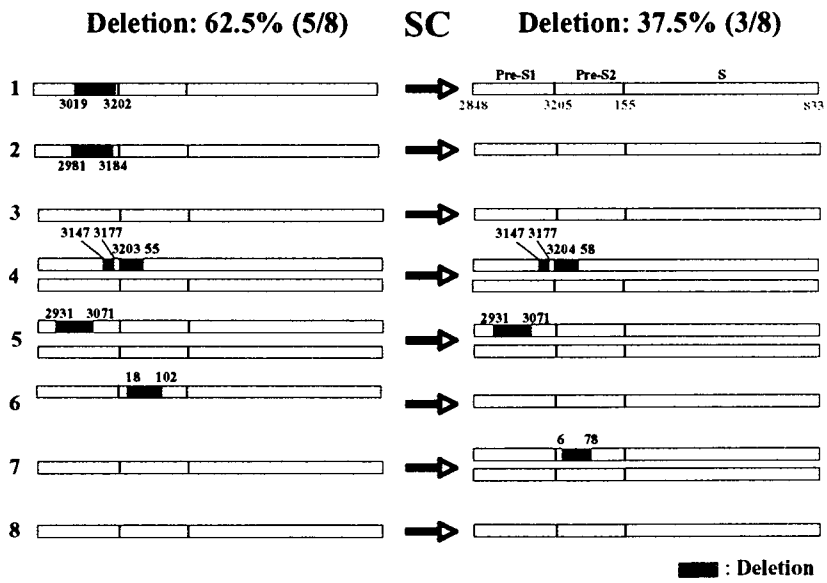

Fig. 3. The pre-S region deletion mutant in HCC patients. Pre-S deletion mutants were identified in five of eight (62.5%) HCC patients before seroconversion as a major clone (*upper bars* show major clones). Three of five deletion mutants before seroconversion were undetectable after seroconversion. Deletions were often in the C terminus of the pre-S1 domain and in the N terminus of the pre-S2 domain

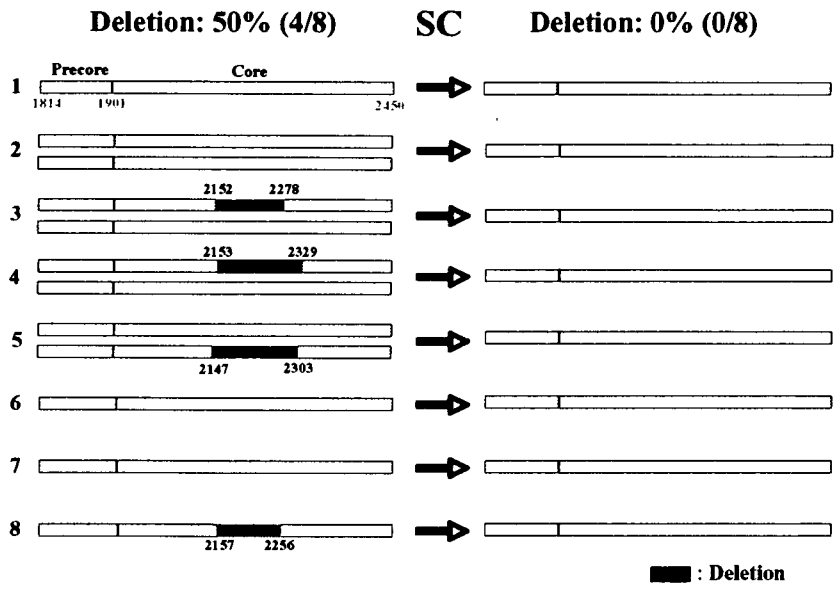

Fig. 4. Precore core region deletion mutant in HCC patients. The nucleotide sequences of precore and core are shown as bars. Core deletion mutants were identified in four of eight (50%) patients, only before seroconversion. All core deletion mutants around the center of the core domain were undetectable after seroconversion

Table 3. Clinical and virological characteristics among IC with HBV genotype C

Patient	Sex	Status of SC	Age (Years)	T1653 mutation	S1753 mutation	A1896 mutation	BCP double mutation	Core deletion mutant	Pre-S deletion mutant
1	M	Before	36	(−)	(+)	(−)	(+)	(−)	(−)
		After	45	(−)	(+)	(+)	(+)	(−)	(+)
2	M	Before	41	(+)	(−)	(+)	(+)	(−)	(−)
		After	55	(+)	(−)	(+)	(+)	(−)	(−)
3	M	Before	31	(−)	(−)	(+)	(−)	(−)	(−)
		After	46	(−)	(+)	(+)	(+)	(−)	(−)
4	M	Before	24	(−)	(−)	(−)	(−)	(−)	(−)
		After	34	(−)	(−)	(−)	(−)	(−)	(−)
5	M	Before	28	(−)	(−)	(+)	(+)	(−)	(−)
		After	35	(−)	(−)	(+)	(+)	(−)	(−)
6	M	Before	41	(−)	(−)	(−)	(−)	(−)	(−)
		After	56	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
7	M	Before	36	(−)	(−)	(−)	(+)	(−)	(−)
		After	49	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
8	F	Before	17		(−)	(−)	(−)	(−)	(−)
		After	29		(−)	(−)	(−)	(−)	(−)

SC, seroconversion

Figure 4 shows the deletion mutants in the precore/core region among HCC patients. Core deletion mutants were detected in four patients with HCC only before seroconversion. Core deletion mutants were identified around the center of the core region in these four patients. Three HCC patients (patients 3–5) before seroconversion were coinfected with wild-type virus and core deletion mutants. One HCC patient (patient 8) before seroconversion was infected with only the core deletion mutant.

Table 3 summarizes the virological characteristics of IC. We could not amplify HBV DNA in two serum samples of IC after seroconversion. No IC was infected with the core deletion mutant. The pre-S deletion mutant was identified in only one IC after seroconversion. BCP double mutations and A1896 mutations were identified in four of six (66.7%) IC after seroconversion. Table 4 shows the virological characteristics of HCC patients. The T1653 mutation was negatively correlated with the S1753 mutation in this population. The prevalence of T1762/A1764 was high in HCC patients. Of interest, a core or pre-S deletion mutant was detected in seven of eight (87.5%) HCC patients before seroconversion.

Table 4. Clinical and virological characteristics of HCC patients with HBV genotype C

Patient	Sex	Status of SC	Age (years)	T1653 mutation	S1753 mutation	A1896 mutation	BCP double mutation	Core deletion mutant	Pre-S deletion mutant
1	M	Before	40	(+)	(−)	(−)	(−)	(−)	(+)
		After	56	(+)	(−)	(+)	(−)	(−)	(−)
2	M	Before	31	(−)	(+)	(−)	(+)	(−)	(+)
		After	38	(−)	(+)	(+)	(+)	(−)	(−)
3	M	Before	38	(−)	(−)	(−)	(+)	(+)	(−)
		After	54	(+)	(−)	(+)	(+)	(−)	(−)
4	M	Before	55	(−)	(−)	(−)	(+)	(+)	(+)
		After	72	(−)	(+)	(−)	(+)	(−)	(+)
5	M	Before	28	(−)	(−)	(−)	(+)	(+)	(+)
		After	34	(+)	(−)	(−)	(+)	(−)	(+)
6	M	Before	36	(+)	(−)	(−)	(+)	(−)	(+)
		After	44	(+)	(−)	(−)	(+)	(−)	(−)
7	M	Before	35	(−)	(−)	(−)	(+)	(−)	(−)
		After	46	(−)	(−)	(−)	(+)	(−)	(+)
8	F	Before	57	(−)	(−)	(−)	(−)	(+)	(−)
		After	69	(+)	(−)	(−)	(+)	(−)	(−)

Discussion

Many previous studies have reported that the clinical course of chronic HBV infection may be modified by several specific viral mutations,[6,16–19] but most studies examined only serum samples collected from each patient at one time point. In this study, we compared viral mutations in IC and HCC patients before and after HBeAg seroconversion. ALT, HBV DNA levels, and rate of cirrhosis were significantly higher among the HCC patients than among IC only after seroconversion. Platelet count was lower among HCC patients than among IC only after seroconversion. Interestingly, even though clinical characteristics did not differ before seroconversion, deletion mutants of the core or pre-S region were significantly more associated with HCC patients than with IC. Core deletion mutants detected before seroconversion become undetectable in serum samples derived from the same patients after seroconversion. As well, pre-S deletion mutants were undetectable in three patients after seroconversion. However, the core and pre-S deletions being undetectable after seroconversion by direct sequencing does not exclude the possibility that they remained as minor clones. Preikschat et al.,[9] who sequenced cloned HBV genomes, reported the existence of deletion mutants as only minor clones; deletions and insertions in core promoter/enhancer II regions, deletions in the *C* gene, or deletions in the pre-S region were distributed on individual genomes in various combinations. Although it is unclear why major deletion mutants decreased after seroconversion, both core and pre-S deletion mutants may be predictive factors for HCC at an early stage in chronic HBV carriers.

Recently, Chen et al.[7] reported that combinations of HBV mutations (deletion in the pre-S region and/or mutations in the BCP and/or PC regions) were strongly associated with liver disease progression; a combination of mutations rather than a single mutation was associated with the development of progressive liver diseases, and pre-S deletion and BCP mutations in particular were significantly associated with the development of progressive liver diseases. In the present study, BCP mutation was identified frequently in HCC patients but also frequently in IC. The combination of a pre-S deletion with other mutants was not significantly associated with the development of HCC, owing to the small sample size in this study.

In our previous case–control study, a BCP double mutation was frequently found in each clinical group (40 IC, 40 chronic hepatitis, and 40 HCC patients), but the frequency of the T1653 mutation was significantly higher among HCC patients than among IC.[20] In this study, the T1653 mutation was identified in five of eight (62.5%) HCC patients after seroconversion and in only one of six (16.7%) IC after seroconversion, suggesting that the T1653 mutation is one of the factors promoting HCC development. However, the combination of pre-S or core deletion mutants with the T1653 mutation was not significantly associated with HCC development.

Both pre-S and core regions play an essential role in the immune response interaction because they contain B- and T-cell epitopes. Pre-S deletion and core deletion mutants thus allow escape from the host's immune function. In this study, most pre-S and core deletion regions in the HCC group encompassed B- and T-cell epitopes: most of the pre-S deletions in the 3′-terminus of the pre-S1 region and the 5′-terminus of the pre-S2 region

in HCC patients, and all of the core deletions around the center of the core region in HCC patients. These pre-S and core deletion sites including B- and T-cell epitopes[21-25] were consistent with those reported in previous papers describing patients infected with pre-S and core deletion mutants.[7,26-28]

Previous studies have shown that pre-S deletion mutants tend to accumulate at the later stage of HBV infection[3,4,29,30] and have demonstrated a marked decrease in the synthesis and secretion of small surface protein leading to retention of envelope protein and viral particles within hepatocytes, resulting in the ground-glass appearance of hepatocytes.[4] Recently, Hsieh et al.[31] identified two types of ground-glass hepatocytes containing two types of mutant L proteins with deletion within the pre-S1 and pre-S2 regions, respectively. They found that these pre-S deletion mutants accumulate in the endoplasmic reticulum (ER), resulting in strong ER stress. They concluded that the pre-S mutation causes ground-glass hepatocytes to induce oxidative DNA damage and mutations in hepatocytes in the late stages of HBV infection.

Yuan et al.[28] described the characteristics of a core deletion mutant: (1) Deletion often occurs within core amino acids 80 to 120. It does not usually extend into the partially overlapping polymerase. (2) The exact end points and sizes of deletions vary from variant to variant. (3) Deletions appear to be more often in frame than out of frame. (4) Internal deletions coincide with a potent T-cell epitope, suggesting an immune system escape function for this mutation. In the present study, the features of the core deletion mutant were mostly consistent with these characteristics.

In conclusion, our data showed a significant association of pre-S deletion and core deletion mutants before seroconversion with HCC development. The T1653 mutation after seroconversion was frequently found in HCC patients infected with HBV genotype C. These results suggest that these mutations may be a predictive factor for HCC development.

Acknowledgments. This work was supported by a Grant-in-Aid for Scientific Research C from the Ministry of Education, Culture, Sports, Science and Technology (18590741), a Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan, Toyoaki Foundation, and Foundation for Promotion of Cancer Center in Japan.

References

1. Parkin DM. International variation. Oncogene 2004;23:6329–40.
2. Naoumov NV, Schneider R, Grotzinger T, Jung MC, Miska S, Pape GR, et al. Precore mutant hepatitis B virus infection and liver disease. Gastroenterology 1992;102:538–43.
3. Sugauchi F, Ohno T, Orito E, Sakugawa H, Ichida T, Komatsu M, et al. Influence of hepatitis B virus genotypes on the development of preS deletions and advanced liver disease. J Med Virol 2003;70:537–44.
4. Fan YF, Lu CC, Chen WC, Yao WJ, Wang HC, Chang TT, et al. Prevalence and significance of hepatitis B virus (HBV) pre-S mutants in serum and liver at different replicative stages of chronic HBV infection. Hepatology 2001;33:277–86.
5. Huy TT, Ushijima H, Win KM, Luengrojanakul P, Shrestha PK, Zhong ZH, et al. High prevalence of hepatitis B virus pre-s mutant in countries where it is endemic and its relationship with genotype and chronicity. J Clin Microbiol 2003;41:5449–55.
6. Kao JH, Chen PJ, Lai MY, Chen DS. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. Gastroenterology 2003;124:327–34.
7. Chen BF, Liu CJ, Jow GM, Chen PJ, Kao JH, Chen DS. High prevalence and mapping of pre-S deletion in hepatitis B virus carriers with progressive liver diseases. Gastroenterology 2006; 130:1153–68.
8. Marschenz S, Endres AS, Brinckmann A, Heise T, Kristiansen G, Nurnberg P, et al. Functional analysis of complex hepatitis B virus variants associated with development of liver cirrhosis. Gastroenterology 2006;131:765–80.
9. Preikschat P, Gunther S, Reinhold S, Will H, Budde K, Neumayer HH, et al. Complex HBV populations with mutations in core promoter, C gene, and pre-S region are associated with development of cirrhosis in long-term renal transplant recipients. Hepatology 2002;35:466–77.
10. Ferrari C, Cavalli A, Penna A, Valli A, Bertoletti A, Pedretti G, et al. Fine specificity of the human T-cell response to the hepatitis B virus preS1 antigen. Gastroenterology 1992;103:255–63.
11. Kuroki K, Floreani M, Mimms LT, Ganem D. Epitope mapping of the PreS1 domain of the hepatitis B virus large surface protein. Virology 1990;176:620–4.
12. Milich DR, McLachlan A, Moriarty A, Thornton GB. Immune response to hepatitis B virus core antigen (HBcAg): localization of T cell recognition sites within HBcAg/HBeAg. J Immunol 1987;139:1223–31.
13. Mizokami M, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, et al. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. FEBS Lett 1999;450:66–71
14. Usuda S, Okamoto H, Tanaka T, Kidd-Ljunggren K, Holland PV, Miyakawa Y, et al. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. J Virol Methods 2000;87:81–9.
15. Sugauchi F, Mizokami M, Orito E, Ohno T, Kato H, Suzuki S, et al. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. J Gen Virol 2001;82: 883–92.
16. Lin CL, Liao LY, Wang CS, Chen PJ, Lai MY, Chen DS, et al. Basal core-promoter mutant of hepatitis B virus and progression of liver disease in hepatitis B e antigen-negative chronic hepatitis B. Liver Int 2005;25:564–70.
17. Chu CJ, Keeffe EB, Han SH, Perrillo RP, Min AD, Soldevila-Pico C, et al. Prevalence of HBV precore/core promoter variants in the United States. Hepatology 2003;38:619–28.
18. Kajiya Y, Hamasaki K, Nakata K, Nakagawa Y, Miyazoe S, Takeda Y, et al. Full-length sequence and functional analysis of hepatitis B virus genome in a virus carrier: a case report suggesting the impact of pre-S and core promoter mutations on the progression of the disease. J Viral Hepat 2002;9:149–56.
19. Chu CM, Yeh CT, Lee CS, Sheen IS, Liaw YF. Precore stop mutant in HBeAg-positive patients with chronic hepatitis B: clinical characteristics and correlation with the course of HBeAg-to-anti-HBe seroconversion. J Clin Microbiol 2002;40:16–21.
20. Ito K, Tanaka Y, Orito E, Sugiyama M, Fujiwara K, Sugauchi F, et al. T1653 mutation in the box alpha increases the risk of hepatocellular carcinoma in patients with chronic hepatitis B virus genotype C infection. Clin Infect Dis 2006;42:1–7.

21. Maeng CY, Ryu CJ, Gripon P, Guguen-Guillouzo C, Hong HJ. Fine mapping of virus-neutralizing epitopes on hepatitis B virus PreS1. Virology 2000;270:9–16.
22. Penna A, Bertoletti A, Cavalli A, Valli A, Missale G, Pilli M, et al. Fine specificity of the human T cell response to hepatitis B virus core antigen. Arch Virol Suppl 1992;4:23–8.
23. Park JH, Cho EW, Lee YJ, Shin SY, Kim KL. Determination of the protective effects of neutralizing anti-hepatitis B virus (HBV) immunoglobulins by epitope mapping with recombinant HBV surface-antigen proteins. Microbiol Immunol 2000;44:703–10.
24. Jung MC, Diepolder HM, Spengler U, Wierenga EA, Zachoval R, Hoffmann RM, et al. Activation of a heterogeneous hepatitis B (HB) core and e antigen-specific CD4+ T-cell population during seroconversion to anti-HBe and anti-HBs in hepatitis B virus infection. J Virol 1995;69:3358–68.
25. Tsai SL, Chen MH, Yeh CT, Chu CM, Lin AN, Chiou FH, et al. Purification and characterization of a naturally processed hepatitis B virus peptide recognized by CD8+ cytotoxic T lymphocytes. J Clin Invest 1996;97:577–84.
26. Wakita T, Kakumu S, Shibata M, Yoshioka K, Ito Y, Shinagawa T, et al. Detection of pre-C and core region mutants of hepatitis B virus in chronic hepatitis B virus carriers. J Clin Invest 1991; 88:1793–801.
27. Akarca US, Lok AS. Naturally occurring core-gene-defective hepatitis B viruses. J Gen Virol 1995;76 (Pt 7):1821–6.
28. Yuan TT, Lin MH, Qiu SM, Shih C. Functional characterization of naturally occurring variants of human hepatitis B virus containing the core internal deletion mutation. J Virol 1998;72:2168–76.
29. Tai PC, Suk FM, Gerlich WH, Neurath AR, Shih C. Hypermodification and immune escape of an internally deleted middle-envelope (M) protein of frequent and predominant hepatitis B virus variants. Virology 2002;292:44–58.
30. Yamamoto K, Horikita M, Tsuda F, Itoh K, Akahane Y, Yotsumoto S, et al. Naturally occurring escape mutants of hepatitis B virus with various mutations in the S gene in carriers seropositive for antibody to hepatitis B surface antigen. J Virol 1994;68: 2671–6.
31. Hsieh YH, Su IJ, Wang HC, Chang WW, Lei HY, Lai MD, et al. Pre-S mutant surface antigens in chronic hepatitis B virus infection induce oxidative stress and DNA damage. Carcinogenesis 2004;25:2023–32.

Hepatology Research 2007 doi: 10.1111/j.1872-034X.2007.00292.x

Original Article

Initial viral response is the most powerful predictor of the emergence of YMDD mutant virus in chronic hepatitis B patients treated with lamivudine

Nao Kurashige,[1] Naoki Hiramatsu,[1] Kazuyoshi Ohkawa,[1] Tsugiko Oze,[1] Yuko Inoue,[1] Mika Kurokawa,[1] Takayuki Yakushijin,[1] Takumi Igura,[1] Shinichi Kiso,[1] Tatsuya Kanto,[1] Tetsuo Takehara,[1] Shinji Tamura,[1] Akinori Kasahara,[1] Masahide Oshita,[2] Taizo Hijioka,[3] Kazuhiro Katayama,[4] Harumasa Yoshihara,[5] Eijirou Hayashi,[6] Yasuharu Imai,[7] Michio Kato[8] and Norio Hayashi[1]

[1]*Osaka University,* [2]*Osaka Police Hospital,* [3]*National Organization Osaka Minami Medical Center,* [4]*Osaka Kouseinenkin Hospital,* [5]*Osaka Rousai Hospital,* [7]*Ikeda City Hospital,* [8]*National Hospital Organization Osaka National Hospital, Osaka, and* [6]*Kinki Central Hospital, Hyogo, Japan*

Aim: Lamivudine (LAM) has been widely used to treat chronic hepatitis B (CHB) patients, but the emergence of a LAM-resistant virus greatly limits its therapeutic efficacy. In this study, we tried to identify factors affecting the emergence of a LAM-resistant virus in CHB patients treated with LAM.

Methods: The subjects were 190 CHB patients in continuous LAM therapy (139 males, mean age 50 years, 87 HBeAg-positive). The mean duration of follow-up was 39 months (range 12–104). The initial viral response (IVR) was defined as HBV DNA < 4.0 logcopies/mL, and the initial biochemical response (IBR) as normalization of alanine aminotransferase (ALT) (<40 IU/L) at 6 months.

Results: IVR was positive in 86% of the patients. The cumulative emergence rates of LAM-resistant virus were 10% at 1 year, 30% at 2 years and 46% at 3 years. In univariate analysis, factors contributing to the emergence of LAM-resistant virus were baseline HBV DNA > 6.5 logcopies/mL ($P = 0.0044$), HBeAg-positivity ($P = 0.0062$), IBR ($P = 0.01$) and IVR ($P < 0.0001$). The cumulative emergence rates of LAM-resistant virus in IVR-positive and -negative patients were 4% and 41% at 1 year, and 41% and 79% at 3 years. In multivariate analysis, only IVR was an independent factor affecting the emergence of LAM-resistant virus ($P < 0.0001$).

Conclusion: IVR is a useful factor for predicting the emergence of LAM-resistant virus in CHB patients treated with LAM. For IVR-negative patients, therapeutic options other than LAM monotherapy should be used because of the high incidence of the emergence of LAM-resistant virus.

Key words: chronic hepatitis B, initial viral response, lamivudine monotherapy, lamivudine-resistant virus

INTRODUCTION

MORE THAN 350 million people are chronically infected with hepatitis B virus (HBV) worldwide.[1] Chronic HBV infection eventually leads to the development of cirrhosis and hepatocellular carcinoma (HCC), and raises the risk of hepatic disease-related death. Nucleos(t)ide analogs are widely used to suppress HBV replication and the progression of HBV-related liver diseases. Lamivudine (LAM), the first approved nucleoside analog for chronic HBV infection, has been shown to suppress viral replication and disease activity.[2] In addition, LAM therapy has recently been reported to reduce the incidence of HCC, the risk of major complications and to improve survival.[3,4] However, the relatively high incidence of LAM resistance is a serious problem in the case of LAM therapy for chronic HBV infection. The emergence of LAM-resistant HBV is linked to the reappearance of active viral replication, followed by the worsening of liver disease.

LAM-resistant HBV is based on point mutation within the YMDD motif of the reverse transcriptase domain of

Correspondence: Dr Naoki Hiramatsu, Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita City, Osaka 565-0871, Japan. Email: hiramatsu@gh.med.osaka-u.ac.jp
Received 24 August 2007; revision 9 October 2007; accepted 14 October 2007.

HBV (YMDD mutation).[5,6] The emergence rates of the mutant virus have been reported to be 24% at 1 year and 70% at 4 years from the start of treatment.[7]

Recent work has shown that newly developed nucleos(t)ide analogs, such as adefovir dipivoxil (ADV) and entecavir (ETV), are also useful agents for controlling patients with chronic HBV infection.[8–11] In particular, the drug-resistant mutant virus has been reported to appear less frequently in cases of treatment with ADV and ETV than with LAM.[12,13] For this reason, LAM has been replaced by ADV and ETV for the treatment of chronic hepatitis B. However, there are still a considerable number of patients with chronic HBV infection who are already on continuous LAM therapy. Thus, further clarification is needed of what factors influence the emergence of the LAM-resistant HBV in LAM treatment for chronic HBV infection.

For a more precise evaluation, we investigated baseline and on-treatment factors affecting the emergence of LAM-resistant mutant virus in patients with chronic hepatitis B treated with LAM.

METHODS

Patients and treatment

THIS STUDY WAS conducted at nine institutions in the Osaka area of Japan (Osaka Police Hospital, Osaka Minami Medical Center, Osaka Kouseinenkin Hospital, Osaka Rousai Hospital, Kinki Central Hospital, Ikeda City Hospital, Osaka National Hospital, Otemae Hospital and Osaka University Hospital). The subjects were 190 consecutive patients with chronic hepatitis B who underwent continuous LAM therapy for more than 12 months. All patients tested positive for hepatitis B surface antigen (HBsAg) or had detectable levels of HBV DNA in their sera by the polymerase chain reaction (PCR)-based method (for 100 patients)[14] or the transcription-mediated amplification (TMA) method (for 90 patients).[15] Exclusion criteria were patients with antihepatitis C antibody, antihuman immunodeficiency virus antibody and other forms of liver diseases (alcoholic liver disease, drug-induced liver disease and autoimmune hepatitis). Forty-one (22%) patients had previously received interferon (IFN)-α therapy for 24 weeks.

All patients were treated with 100 mg of LAM daily. After the beginning of the therapy, liver function tests and HBV DNA were measured every other month for the first 6 months and every two months thereafter. HBeAg and anti-HBe were tested every 6 months. In 33 patients (18%), combination therapy with IFN was carried out for the initial 6 months. Three or six mega-units of natural IFN-á were administered daily for the first 2 weeks and three times a week thereafter, followed by LAM monotherapy. The mean follow-up period of the 190 patients was 39 (range 12–104) months. The LAM-resistant YMDD mutant virus was detected by the PCR-enzyme-linked minisequence (ELMA) assay[16] when the virological or biochemical breakthrough was observed. The YMDD mutant virus was found in 86 (45%) patients during follow-up. Fifty-eight of these patients underwent ADV therapy in addition to ongoing LAM treatment and were excluded from the follow-up when ADV administration began. In this study, the initial viral response (IVR) was defined as HBV DNA < 4.0 logcopies/mL, and the initial biochemical response (IBR) as normalization of alanine aminotransferase (ALT) (<40 IU/L) after 6 months of therapy.

Table 1 Patient characteristics

Gender (male/female)	139/51
Age (years)	50 ± 11
Chronic hepatitis/liver cirrhosis	113/77
Hepatocellular carcinoma	14 (7%)
AST (IU/L)	122 ± 157
AST (IU/L)	177 ± 236
ALT (≤1/1–2/2–5/>5 × ULN)	22/53/65/50
Platelet (10^4/mm^3)	12.6 ± 5.1
Prothrombin time (%)	71.5 ± 16.6
HBV DNA (logcopies/mL)	6.5 (3.0–7.6<)
HBeAg (positive/negative)	87/103
Combination with interferon	33 (17%)
Duration of treatment (months)	38.9 ± 17.5

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; ULM, upper limit normal.

The patients' clinical characteristics are shown in Table 1. There were 139 males and 51 females, ranging in age from 25 to 75 (mean 50) years. Of them, 113 (59%) patients were diagnosed as having chronic hepatitis and the remaining 77 patients (41%) as having cirrhosis according to liver histology and/or the imaging procedure. HCC was developed in 14 (7%) patients. The aspartate aminotransferase (AST) at baseline was 122 ± 157 IU/l, and the ALT at baseline was 177 ± 236 IU/L. Abnormal ALT was observed in 168 (88%) patients. Eighty-seven patients (46%) tested positive for HBeAg. The median HBV DNA at baseline was 6.5 (range 3.0 to 7.6<) logcopies/mL.

HBV testing

HBsAg, hepatitis B e antigen (HBeAg) and antihepatitis B e antibody (anti-HBe) were examined by chemiluminescent immunoassay or enzyme immunoassay.

The HBV DNA level was measured by the PCR-based method (Amplicor HBV monitor; Roche Diagnostics, Tokyo, Japan)[14] or the TMA method (TMA-HPA; Fujirebio, Tokyo, Japan),[15] which have lower detection limits of 2.6 and 3.7 logcopies/mL, respectively. The LAM-resistant YMDD mutant virus was examined by the PCR-ELMA method.[16]

Statistical analysis

Comparisons of categorical and continuous variables between groups were done by the χ^2-test, Student's *t*-test and Mann–Whitney's *U*-test. The cumulative emergence rates of LAM-resistant virus were evaluated with the Kaplan–Meier's curve and the differences between groups were analyzed by the log-rank test. For multivariate analysis to investigate factors affecting the cumulative emergence rate of LAM-resistant virus, Cox proportional hazard regression analysis was carried out. A *P*-value of less than 0.05 (two-tailed) was considered to be statistically significant.

RESULTS

Therapeutic efficacy and the emergence of LAM-resistant mutant virus

AMONG THE 190 patients with chronic hepatitis B who underwent continuous LAM therapy, reduction of HBV DNA to less than 4 logcopies/mL was observed in 86% (163/190) at 6 months, 89% (151/170) at 1 year, 88% (83/94) at 2 years and 89% (48/54) at 3 years of the treatment. Normalization of ALT was achieved by 77% (146/190) at 6 months, 83% (141/170) at 1 year, 81% (76/94) at 2 years and 83% (45/54) at 3 years. Among the 87 HBeAg-positive patients, HBeAg was cleared in 22% (19/86) at 6 months, 26% (21/80) at 1 year, 22% (11/50) at 2 years and 43% (16/37) at 3 years. As for the virological and biochemical response at 6 months of therapy, 163 (86%) of the patients achieved IVR, whereas IBR was seen in 146 (77%) of patients.

When the various patient characteristics were compared between IVR-positive and -negative patients (Table 2), HBV DNA at baseline tended to be lower in patients showing IVR (median 6.5 [range 3.0 to 7.6<] logcopies/mL) than in those who did not show IVR (median 7.3 [range 4.3 to 7.6<] logcopies/mL) ($P < 0.0001$). IVR-negative patients had higher HBeAg positivity at baseline than IVR-positive patients (81% vs 40%, $P = 0.01$). As for the emergence of LAM-resistant mutant virus during follow-up, it was detected more frequently in IVR-negative patients (21/27, 78%) than in IVR-positive patients (65/163, 40%) ($P = 0.002$).

Among the 190 patients examined in this study, the emergence of LAM-resistant YMDD mutant virus occurred in 86 (45%) patients during follow-up. The cumulative probabilities of the emergence of the YMDD mutant virus were 10% at 1 year, 30% at 2 years and 46% at 3 years.

Factors affecting the emergence of LAM-resistant mutant virus

Factors affecting the cumulative probability of the emergence of the YMDD mutant virus were investigated using

Table 2 Comparison of patient characteristics between IVR-positive and -negative patients

	IVR ($n = 163$)	Non-IVR ($n = 27$)	*P*-value
Gender (male/female)	118/45	21/6	NS
Age (years)	50 ± 11	48 ± 12	NS
Chronic hepatitis/liver cirrhosis	91/72	22/5	NS
Hepatocellular carcinoma	13 (8.0%)	1 (4%)	NS
AST (IU/L)	131 ± 167	69 ± 34	NS
ALT (IU/L)	190 ± 252	100 ± 55	NS
ALT (≤1/1–2/2–5/>5 × ULN)	21/43/52/47	1/10/13/3	NS
HBV DNA (logcopies/mL)	6.5 (3.0–7.6<)	7.3 (4.3–7.6<)	<0.0001
HBeAg (positive/negative)	65/98	22/5	0.01
Combination with interferon	27 (17%)	6 (22%)	NS
Emergence of LAM-resistant viruses	65 (40%)	21 (78%)	0.002
Duration of treatment (months)	39.2 ± 17.2	37.3 ± 19.1	NS

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; IVR, initial viral response; LAM, lamivudine; NS, not significant; ULN, upper limit normal.

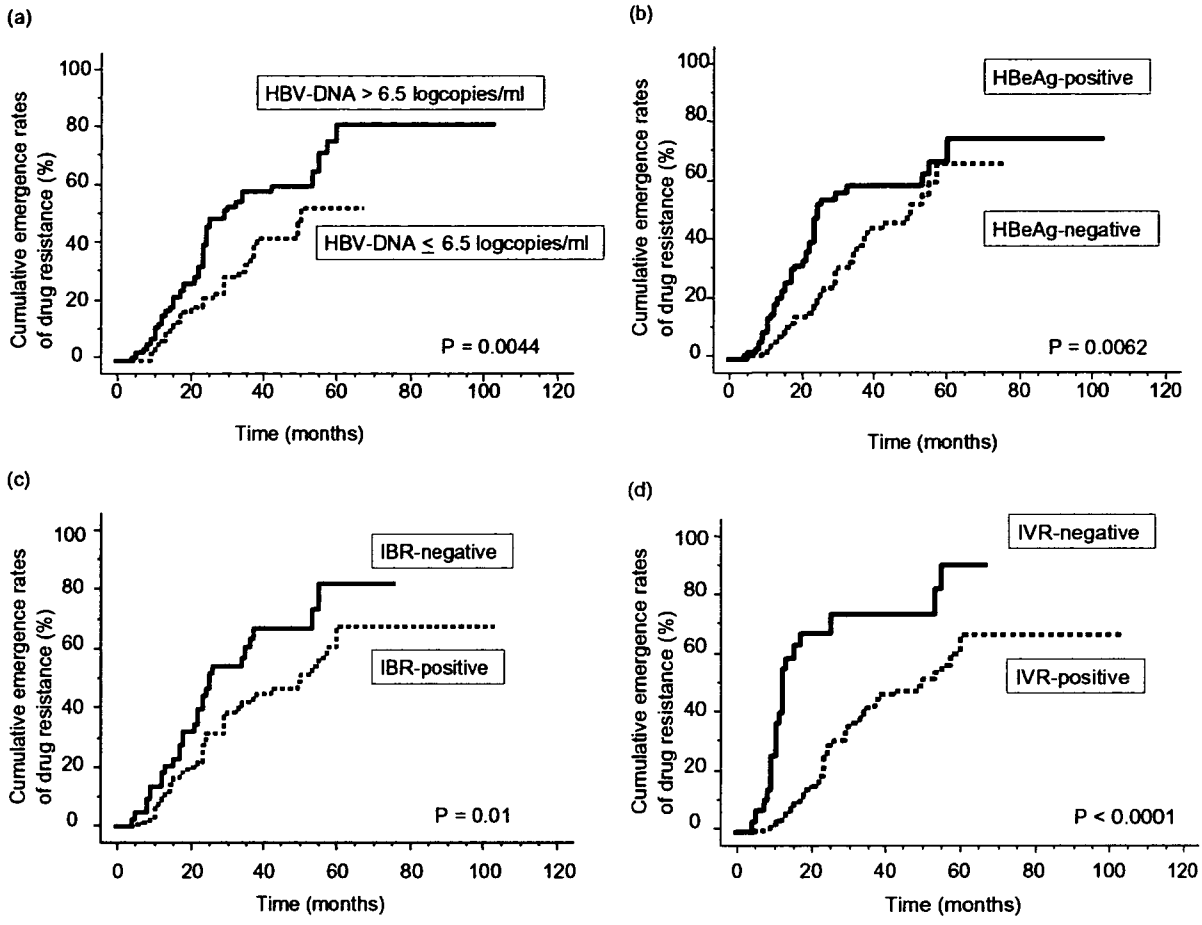

Figure 1 Cumulative emergence rate of lamivudine (LAM)-resistant virus in patients with chronic hepatitis B virus (HBV) infection treated with LAM according to: (a) HBV DNA at baseline; (b) hepatitis B e antigen (HBeAg) status; (c) the presence or absence of initial biochemical response (IBR); and (d) the presence or absence of initial viral response (IVR).

both univariate and multivariate analyses. Nine baseline and on-treatment factors – gender, age, liver disease (chronic hepatitis or cirrhosis), ALT at baseline, HBeAg positivity, HBV DNA at baseline, combination therapy with IFN-α, presence of IBR and presence of IVR – were examined. The cumulative emergence of LAM-resistant virus was significantly higher in patients with baseline HBV DNA > 6.5 logcopies/mL than in those with HBV DNA ≤ 6.5 logcopies/mL ($P = 0.0044$) (Fig. 1a). HBeAg-positive patients revealed a significantly higher emergence rate of the LAM-resistant virus than HBeAg-negative patients ($P = 0.0062$) (Fig. 1b). A significant difference was also seen in the cumulative emergence of the YMDD mutant virus between IBR-positive and -negative patients ($P = 0.01$) (Fig. 1c). Furthermore, the cumulative emergence of LAM-resistant mutant virus was much higher in the IVR-negative patients than in the IVR-positive patients ($P < 0.0001$) (Fig. 1d). The cumulative emergence rates of LAM-resistant virus in the IVR-positive and -negative patients were 4% and 41% at 1 year, 25% and 69% at 2 years, and 41% and 79% at 3 years, respectively. Gender, age, liver disease, ALT at baseline and combination therapy of IFN-α did not show a significant relation with the emergence of the YMDD mutant virus. When factors influencing the higher cumulative emergence of LAM-resistant virus were searched for by multivariate analysis, only the absence of IVR was selected as a significant independent factor ($P < 0.001$) (Table 3), with high HBV DNA, HBeAg positivity and the absence of IBR not being selected.

Table 3 Factors associate with emergence of LAM-resistant virus determined by multivariate analysis

	Hazard ratio	95% confidence interval	*P*-value
Gender			
0: male	1	0.497–1.455	0.55
1: female	1.176		
Age			
0: ≤50	1	0.640–1.700	0.87
1: >50	0.959		
Chronic hepatitis/liver cirrhosis			
0: CH	1	0.656–1.740	0.79
1: LC	0.935		
Pretreatment ALT (IU/L)			
0: ≤200	1	0.605–1.818	0.87
1: >200	0.953		
HBV DNA (logcopies/mL)			
0: ≤6.5	1	0.394–1.125	0.13
1: >6.5	1.502		
HBeAg			
0: negative	1	0.499–1.337	0.42
1: positive	1.225		
Combination therapy with interferon			
0: no	1	0.410–1.303	0.29
1: yes	1.368		
IBR			
0: positive	1	0.483–1.312	0.37
1: negative	1.256		
IVR			
0: positive	1	0.159–0.536	<0.001
1: negative	3.425		

HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; IBR, initial biochemical response; IVR, initial viral response; LAM, lamivudine.

DISCUSSION

IN LAM THERAPY for patients with chronic HBV infection, the emergence of a LAM-resistant YMDD mutant virus is a serious problem, because it inevitably restricts the antiviral efficacy of LAM. To resolve this, detailed studies are needed to identify factors related to the emergence of the YMDD mutant virus. To date, a few investigators have suggested male gender, advanced age, high baseline ALT, the presence of severe acute exacerbation of the liver disease, high baseline HBV DNA and HBeAg-positivity as possible predictors of the emergence of LAM-resistant virus.[7,17,18] Lower body surface area was also reported as a significant factor for virological and biochemical therapeutic effect.[19] In the present study, we studied 190 patients with chronic hepatitis B treated with LAM and investigated baseline and on-treatment factors affecting the emergence of LAM-resistant mutant virus. Univariate analysis revealed that two baseline factors, high HBV DNA and HBeAg positivity, had a relation to the high incidence of the YMDD mutant virus, which is consistent with previous reports.[7,17,18] In addition, two on-treatment factors, IBR and IVR, were found to be correlated with the emergence of LAM resistance. Patients who did not show IVR had a 3.4-fold higher incidence of the emergence of the YMDD mutant virus than those who did show IVR. This agrees with a previous report that the HBV DNA level after 6 months of therapy may be a determinant for subsequent occurrence of a LAM-resistant mutant virus.[20] Multivariate analysis showed that only the absence of IVR was a significant factor contributing to the emergence of LAM-resistant virus. Baseline HBV DNA and HBeAg status were not selected as significant factors by multivariate analysis probably because of the tendency for higher HBV DNA and high frequency of HBeAg positivity in IVR-negative patients compared with IVR-positive patients. It is particularly interesting that the absence of IVR, rather than other baseline and on-treatment factors, was a powerful independent pre-

dictor for the emergence of the YMDD mutant virus in LAM therapy for chronic HBV infection. This means that IVR of an on-treatment factor is very important for good therapeutic effect and the stage for the next therapeutic strategy can thus be set in a new light with this information.

Our results showed that approximately one-seventh of the patients with chronic hepatitis B treated with LAM did not achieve IVR. In the non-IVR patients, the antiviral therapeutic regimen should be amended due to the frequent emergence of LAM-resistant virus. Recently, new nucleos(t)ide analogs have become available for the treatment of chronic HBV infection. ETV has been reported to be more effective for the reduction of HBV DNA and the less frequently induced drug-resistant mutant virus than LAM in "naïve" patients with chronic hepatitis B who had not previously received nucleos(t)ide analog therapy.[10,11] ETV was also effective in patients with chronic HBV infection showing LAM resistance,[21] but the emergence rate of the ETV-resistant virus was considerably higher in LAM-resistant patients than in naïve patients.[13,22] This is because the ETV-resistant HBV strain is established by LAM-resistant YMDD mutation plus additional mutation(s) at the amino acid position(s) 184, 202 and/or 250 within the reverse transcriptase domain of HBV.[22] According to these findings, switching from LAM to ETV may be useful for treating patients who do not achieve IVR on LAM administration. This should be done before the emergence of LAM-resistant YMDD mutant virus so as not to reduce the therapeutic efficacy of ETV. In clinical practice, there are still a number of patients who have already been on continuous LAM therapy, although the current first choice drug for patients with chronic HBV infection is ETV. In our opinion, foregoing patients without IVR or YMDD mutant viruses should be switched from LAM to ETV. The therapeutic efficacy of switching from LAM to ETV in non-IVR patients should be assessed by further study with a larger number of patients.

ADV and tenofovir disoproxil fumarate (TDF) have also been shown to exert antiviral efficacy in patients with chronic HBV infection with less frequent occurrence of drug-resistant mutant virus compared to LAM.[23] In addition, unlike the case of ETV, both ADV and TDF are known to be effective in LAM-refractory patients with chronic hepatitis B, as well as naïve patients.[23] Using ADV and TDF may be helpful for the treatment of non-IVR patients, especially after the establishment of LAM-resistant mutant virus.

In conclusion, our findings indicate that IVR may be a useful factor for predicting the emergence of LAM-resistant mutant virus in patients with chronic HBV infection treated with LAM. For patients who do not achieve IVR, therapeutic options other than LAM monotherapy should be promptly implemented because of the high incidence of the subsequent emergence of the YMDD mutant virus.

REFERENCES

1 Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepat* 2004; 11: 97–107.
2 Lai CL, Chien RN, Leung NW *et al.* A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; 339: 61–8.
3 Liaw YF, Sung JJ, Chow WC *et al.* Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med* 2004; 351: 1521–31.
4 Papatheodoridis GV, Dimou E, Dimakopoulos K *et al.* Outcome of hepatitis B e antigen-negative chronic hepatitis B on long-term nucleos(t)ide analog therapy starting with lamivudine. *Hepatology* 2005; 42: 121–9.
5 Allen MI, Deslauriers M, Andrews CW *et al.* Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. *Hepatology* 1998; 27: 1670–7.
6 Liaw YF, Chien RN, Yeh CT *et al.* Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy. *Hepatology* 1999; 30: 567–72.
7 Lai CL, Dienstag J, Schiff E *et al.* Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. *Clin Infect Dis* 2003; 36: 687–96.
8 Hadziyannis SJ, Tassopoulos NC, Heathcote EJ *et al.* Adefovir dipivoxil for the treatment of hepatitis B e antigen-negative chronic hepatitis B. *N Engl J Med* 2003; 348: 800–7.
9 Marcellin P, Chang TT, Lim SG *et al.* Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *N Engl J Med* 2003; 348: 808–16.
10 Chang TT, Gish RG, Man RD *et al.* A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006; 354: 1001–10.
11 Lai CL, Shouval D, Lok AS *et al.* Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. *N Engl J Med* 2006; 354: 1011–20.
12 Hadziyannis SJ, Tassopoulos NC, Heathcote EJ *et al.* Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis B for up to 5 years. *Gastroenterology* 2006; 131: 1743–51.
13 Colonno RJ, Rose R, Baldick CJ *et al.* Entecavir resistance is rare in nucleoside naïve patients with hepatitis B. *Hepatology* 2006; 44: 1656–65.
14 Dai CY, Yu ML, Chen SC *et al.* Clinical evaluation of COBAS amplicor HBV monitor test for measuring serum

HBV DNA and comparison with the quantiplex branched DNA signal amplification assay in Taiwan. *J Clin Pathol* 2004; 57: 141–5.
15 Kamisango K, Kamogawa C, Sumi M *et al.* Quantitative detection of hepatitis B virus transcription-mediated amplification and hybridization protection assay. *J Clin Microbiol* 1999; 37: 310–14.
16 Kobayashi S, Shimada K, Suzuki H *et al.* Development of a new method for detecting a mutation in the gene encoding hepatitis B virus reverse transcriptase active site (YMDD motif). *Hepatol Res* 2000; 17: 31–42.
17 Tsubota A, Arase Y, Suzuki F *et al.* Severe acute exacerbation of liver disease may reduce or delay emergence of YMDD motif mutants in long-term lamivudine therapy for hepatitis B e antigen-positive chronic hepatitis B. *J Med Virol* 2004; 73: 7–12.
18 Chang ML, Chien RN, Yeh CT *et al.* Virus and transaminase levels determine the emergence of drug resistance during long-term lamivudine therapy in chronic hepatitis B. *J Hepatol* 2005; 43: 72–7.
19 Nakamuta M, Kotoh K, Tanabe Y *et al.* Body surface area is an independent factor contributing to the effects of lamivudine treatment. *Hepatol Res* 2005; 31: 13–17.
20 Yuen MF, Sablon E, Hui CK *et al.* Factors associated with hepatitis B virus DNA breakthrough in patients receiving prolonged lamivudine therapy. *Hepatology* 2001; 34: 785–91.
21 Sherman M, Yurdaydin C, Sollano J *et al.* Entecavir for treatment of lamivudine-refractory, HBeAg-positive chronic hepatitis B. *Gastroenterology* 2006; 130: 2039–49.
22 Tenney DJ, Rose RE, Baldick CJ *et al.* Two-year assessment of entecavir resistance in lamivudine-refractory hepatitis B virus patients reveals different clinical outcomes depending on the resistance substitutions present. *Antimicrob Agents Chemother* 2007; 51: 902–11.
23 Bommel F, Wunsche T, Reinke P *et al.* Comparison of adefovir and tenofovir in the treatment of lamivudine-resistant hepatitis B virus infection. *Hepatology* 2004; 40: 1421–5.

Hepatology Research 2007 doi: 10.1111/j.1872-034X.2007.00307.x

Case Report

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

Aimi Kanada,[1] Tetsuo Takehara,[1] Kazuyoshi Ohkawa,[1,2] Michio Kato,[3] Tomohide Tatsumi,[1] Takuya Miyagi,[1] Ryotaro Sakamori,[1] Shinjiro Yamaguchi,[1] Akio Uemura,[1] Keisuke Kohga,[1] Akira Sasakawa,[1] Hayato Hikita,[1] Kiyomi Kawamura,[4] Tatsuya Kanto,[1,2] Naoki Hiramatsu[1] and Norio Hayashi[1]

[1]Department of Gastroenterology and Hepatology, [2]Department of Dendritic Cellular Research and Clinical Application, Osaka University Graduate School of Medicine, Yamadaoka, Suita and Departments of [3]Gastroenterology and [4]Immunology Infectious Disease, National Hospital Organization Osaka National Hospital, Hoenzaka, Chuo-ku, Osaka, Japan

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/

Correspondence: Professor Norio Hayashi, Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Suita 565-0871, Japan. Email: hayashin@gh.med.osaka-u.ac.jp
Received 21 August 2007; revision 31 October 2007; accepted 1 November 2007.

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

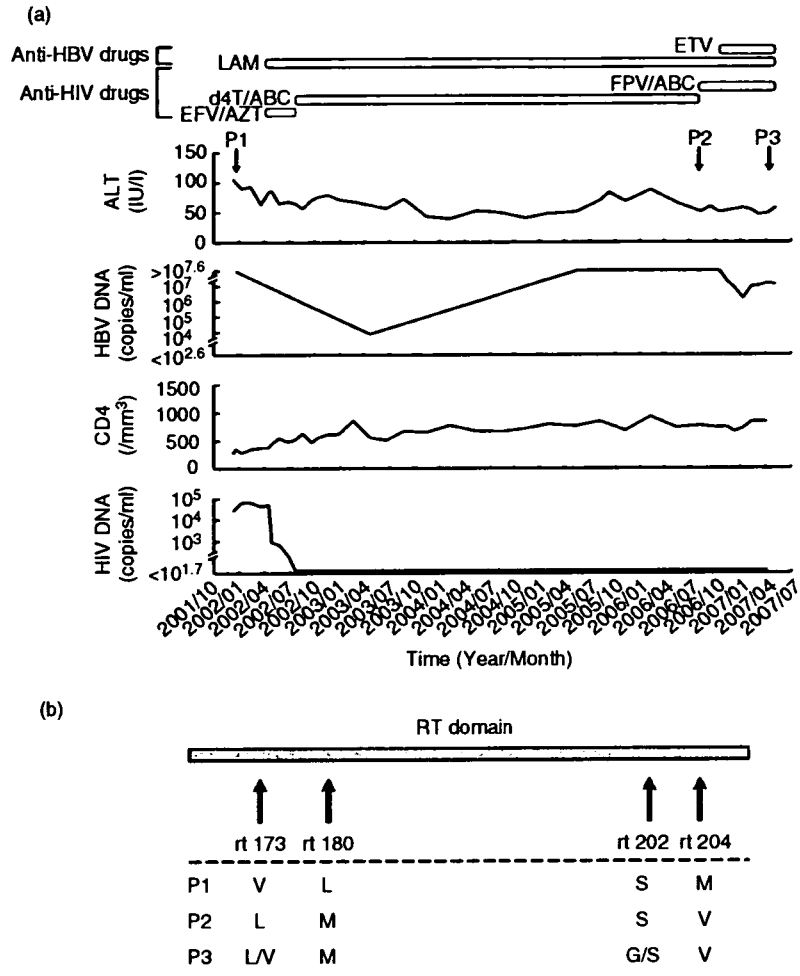

Figure 1 (a) Patient clinical course and serum sampling points. P1, P2 and P3 are the points at which serum samples were obtained. P1 was taken in December 2001 (before HAART), P2 in August 2006 (before ETV administration) and P3 in April 2007 (after 6 months of ETV therapy). ABC, avacavir; ALT, alanine 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 HBDI03), 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

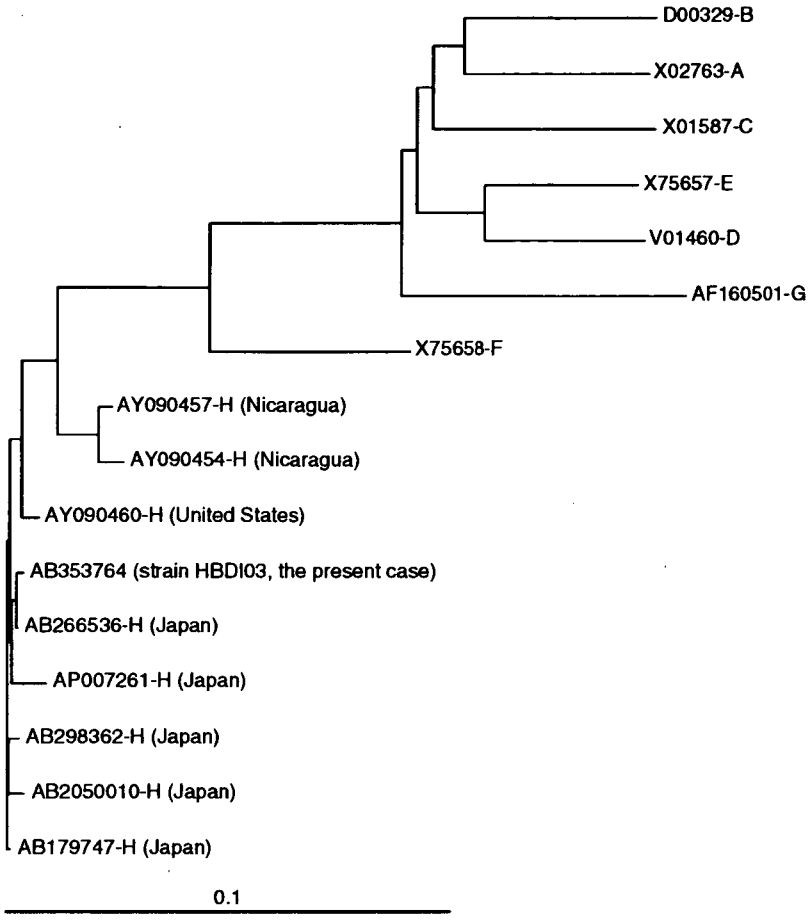

Figure 2 Phylogenetic tree analysis including the HBV strain HBDI03 obtained in this study, the seven representative HBV strains of genotypes A–G, and the eight previously isolated HBV strains of genotype H.

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.

REFERENCES

1 Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; 337: 1733–45.
2 Konopnicki D, Mocroft A, de Wit S *et al.* EuroSIDA Group. Hepatitis B and HIV: prevalence, AIDS progression, response to highly active antiretroviral therapy and increased mortality in the EuroSIDA cohort. *AIDS* 2005; 19: 593–601.
3 Thio CL, Seaberg EC, Skolasky R Jr *et al.* Multicenter AIDS Cohort Study. HIV-1, hepatitis B virus, and risk of liver-related mortality in the Multicenter Cohort Study (MACS). *Lancet* 2002; 360: 1921–6.
4 Lai CL, Chien RN, Leung NW *et al.* A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; 339: 61–8.
5 Benhamou Y, Katlama C, Lunel F *et al.* Effects of lamivudine on replication of hepatitis B virus in HIV-infected men. *Ann Intern Med* 1996; 125: 705–12.
6 Lai CL, Dienstag J, Schiff E *et al.* Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. *Clin Infect Dis* 2003; 36: 687–96.
7 Benhamou Y, Bochet M, Thibault V *et al.* Long-term incidence of hepatitis B virus resistance to lamivudine in human immunodeficiency virus-infected patients. *Hepatology* 1999; 30: 1302–6.
8 Allen MI, Deslauriers M, Andrews CW *et al.* Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. *Hepatology* 1998; 27: 1670–7.
9 Ono-Nita SK, Kato N, Shiratori Y *et al.* Susceptibility of lamivudine-resistant hepatitis B virus to other reverse transcriptase inhibitors. *J Clin Invest* 1999; 103: 1635–40.
10 Ono SK, Kato N, Shiratori Y *et al.* The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. *J Clin Invest* 2001; 107: 449–55.
11 Delaney WE, Yang H, Westland CE *et al.* The hepatitis B virus polymerase mutation rtV173L is selected during lamivudine therapy and enhances viral replication in vitro. *J Virol* 2003; 77: 11833–41.
12 Chang TT, Gish RG, de Man R *et al.* BEHoLD AI463022 Study Group. A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006; 354: 1001–10.
13 Lai CL, Shouval D, Lok AS *et al.* BEHoLD AI463027 Study Group. Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. *N Engl J Med* 2006; 354: 1011–20.
14 Sherman M, Yurdaydin C, Sollano J *et al.* AI463026 BEHoLD Study Group. Entecavir for treatment of