9. Lai CL, Gane E, Liaw YF, *et al.* Telbivudine versus lamivudine in patients with chronic hepatitis B. *N Engl J Med* 2007; **357**: 2576–88.
10. Marcellin P, Heathcote EJ, Buti M, *et al.* Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. *N Engl J Med* 2008; **359**: 2442–55.
11. Suzuki F, Suzuki Y, Tsubota A, *et al.* Mutations of polymerase, precore and core promoter gene in hepatitis B virus during 5-year lamivudine therapy. *J Hepatol* 2002; **37**: 824–30.
12. Akuta N, Suzuki F, Kobayashi M, *et al.* Virological and biochemical relapse according to YMDD motif mutant type during long-term lamivudine monotherapy. *J Med Virol* 2003; **71**: 504–10.
13. Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507–39.
14. Yatsuji H, Suzuki F, Sezaki H, *et al.* Low risk of adefovir resistance in lamivudine-resistant chronic hepatitis B patients treated with adefovir plus lamivudine combination therapy: two-year follow-up. *J Hepatol* 2008; **48**: 923–31.
15. Werle-Lapostolle B, Bowden S, Locarnini S, *et al.* Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. *Gastroenterology* 2004; **126**: 1750–8.
16. Wursthorn K, Lutgehetmann M, Dandri M, *et al.* Peginterferon alpha-2b plus adefovir induce strong cccDNA decline and HBsAg reduction in patients with chronic hepatitis B. *Hepatology* 2006; **44**: 675–84.
17. Kimura T, Rokuhara A, Sakamoto Y, *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; **40**: 439–45.
18. Kimura T, Ohno N, Terada N, *et al.* Hepatitis B virus DNA-negative dane particles lack core protein but contain a 22-kDa precore protein without C-terminal arginine-rich domain. *J Biol Chem* 2005; **280**: 21713–9.
19. Rokuhara A, Tanaka E, Matsumoto A, *et al.* Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen: a marker distinct from viral DNA for monitoring lamivudine treatment. *J Viral Hepat* 2003; **10**: 324–30.
20. Wong DK, Tanaka Y, Lai CL, *et al.* Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection. *J Clin Microbiol* 2007; **45**: 3942–7.
21. Suzuki F, Miyakoshi H, Kobayashi M, Kumada H. Correlation between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. *J Med Virol* 2009; **81**: 27–33.
22. 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.
23. Matsumoto A, Tanaka E, Rokuhara A, *et al.* Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: a multicenter retrospective study of 2795 patients. *Hepatol Res* 2005; **32**: 173–84.
24. Di Marco V, Marzano A, Lampertico P, *et al.* Clinical outcome of HBeAg-negative chronic hepatitis B in relation to virological response to lamivudine. *Hepatology* 2004; **40**: 883–91.
25. Lampertico P, Vigano M, Manenti E, *et al.* Low resistance to adefovir combined with lamivudine: a 3-year study of 145 lamivudine-resistant hepatitis B patients. *Gastroenterology* 2007; **133**: 1445–51.
26. Hosaka T, Suzuki F, Kobayashi M, *et al.* Development of HCC in patients receiving adefovir dipivoxil for lamivudine-resistant hepatitis B virus mutants. *Hepatol Res* 2010; **40**: 145–52.
27. Kubo S, Hirohashi K, Tanaka H, *et al.* Effect of viral status on recurrence after liver resection for patients with hepatitis B virus-related hepatocellular carcinoma. *Cancer* 2000; **88**: 1016–24.
28. Hung IF, Poon RT, Lai CL, *et al.* Recurrence of hepatitis B-related hepatocellular carcinoma is associated with high viral load at the time of resection. *Am J Gastroenterol* 2008; **103**: 1663–73.
29. Kim BK, Park JY, Kim do Y, *et al.* Persistent hepatitis B viral replication affects recurrence of hepatocellular carcinoma after curative resection. *Liver Int* 2008; **28**: 393–401.
30. Wu JC, Huang YH, Chau GY, *et al.* Risk factors for early and late recurrence in hepatitis B-related hepatocellular carcinoma. *J Hepatol* 2009; **51**: 890–7.
31. Jang JW, Choi JY, Bae SH, *et al.* The impact of hepatitis B viral load on recurrence after complete necrosis in patients with hepatocellular carcinoma who receive transarterial chemolipiodolization: implications for viral suppression to reduce the risk of cancer recurrence. *Cancer* 2007; **110**: 1760–7.
32. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003; **362**: 1907–17.
33. Wu TT, Coates L, Aldrich CE, Summers J, Mason WS. In hepatocytes infected with duck hepatitis B virus, the template for viral RNA synthesis is amplified by an intracellular pathway. *Virology* 1990; **175**: 255–61.
34. Newbold JE, Xin H, Tencza M, *et al.* The covalently closed duplex form of the hepadnavirus genome exists in situ as a heterogeneous population of viral minichromosomes. *J Virol* 1995; **69**: 3350–7.
35. Zoulim F. New insight on hepatitis B virus persistence from the study of intrahepatic viral cccDNA. *J Hepatol* 2005; **42**: 302–8.
36. Chan HL, Wong VW, Tse AM, *et al.* Serum hepatitis B surface antigen quantification can reflect hepatitis B virus in the liver and predict treatment response. *Clin Gastroenterol Hepatol* 2007; **5**: 1462–8.
37. Brunetto MR, Noriconi F, Bonino F, *et al.* Hepatitis B virus surface antigen levels: a guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. *Hepatology* 2007; **49**: 1141–50.

Liver INTERNATIONAL

Liver International ISSN 1478-3223

CLINICAL STUDIES

HBcrAg is a predictor of post-treatment recurrence of hepatocellular carcinoma during antiviral therapy

Tetsuya Hosaka[1], Fumitaka Suzuki[1], Masahiro Kobayashi[1], Miharu Hirakawa[1], Yusuke Kawamura[1], Hiromi Yatsuji[1], Hitomi Sezaki[1], Norio Akuta[1], Yoshiyuki Suzuki[1], Satoshi Saitoh[1], Yasuji Arase[1], Kenji Ikeda[1], Mariko Kobayashi[2] and Hiromitsu Kumada[1]

1 Department of Hepatology, Toranomon Hospital, Tokyo, Japan
2 Research Institute for Hepatology, Toranomon Hospital, Tokyo, Japan

Keywords
covalently closed circular DNA – HBcrAg – HCC recurrence – nucleot(s)ide analogue – portal vein invasion

Correspondence
Tetsuya Hosaka, Department of Hepatology, Toranomon Hospital, 1-3-1 Kajigaya, Takatsu-ku, Kawasaki City 213 8587, Tokyo, Japan
Tel: +81 44 877 5111
Fax: +81 44 860 1623
e-mail: hosa-p@toranomon.gr.jp

Received 13 February 2010
Accepted 15 August 2010

DOI:10.1111/j.1478-3231.2010.02344.x

Abstract

Background/Aims: The recurrence rate of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) is high even in patients receiving curative therapy. In this study, we analysed the risk factors for tumour recurrence after curative therapy for HBV-related HCC while under treatment with nucleot(s)ide analogues (NAs) by measuring serum HBcrAg and intrahepatic covalently closed circular DNA (cccDNA) levels to elucidate the viral status associated with HCC recurrence. *Methods:* We enrolled 55 patients who developed HCC during NA therapy and underwent either curative resection or percutaneous ablation for HCC. *Results:* Hepatocellular carcinoma recurred in 21 (38%) of the patients over a period of 2.2 (range, 0.2–7.4) years. In multivariate analysis, serum HBcrAg levels $\geq$4.8log U/ml at the time of HCC diagnosis (hazard ratio, 8.96; 95% confidential interval, 1.94–41.4) and portal vein invasion (3.94, 1.25–12.4) were independent factors for HCC recurrence. The recurrence-free survival rates of the high cccDNA group were significantly lower than those of the low cccDNA group only in patients who underwent resection ($P=0.0438$). A positive correlation ($P=0.028$; $r=0.479$) was observed between the intrahepatic cccDNA and the serum HBcrAg levels at the incidence of HCC. *Conclusion:* HBcrAg is a predictor of the post-treatment recurrence of HCC during antiviral therapy. Serum HBcrAg and intrahepatic cccDNA suppression by NAs may be important to prevent HCC recurrence.

Worldwide, an estimated 400 million people are infected with hepatitis B virus (HBV) persistently, and one million people die of decompensated cirrhosis and/or hepatocellular carcinoma (HCC) annually (1, 2). Recently, oral nucleot(s)ide analogues (NAs) have been used as the mainstay therapeutic strategy against chronic hepatitis B. Five such antiviral agents have been approved, and range in the profundity and rapidity of HBV DNA suppression, barrier to resistance and side-effect profile (3–10). Lamivudine (LAM) was the first NA to be approved for treating chronic hepatitis B, followed by adefovir dipivoxil (ADV) and entecavir (ETV), in Japan. However, a major problem with long-term LAM treatment is the potential development of drug resistance, mainly caused by mutation of the thyrosine–methionine–aspartic acid–aspartic acid (YMDD) motif of reverse transcriptase (11, 12). For preventing breakthrough hepatitis induced by LAM-resistant mutants, additional ADV administration has been recommended (13, 14).

The methods for monitoring the treatment response include measurements of the serum alanine transaminase (ALT) levels, HBV DNA levels, HBeAg and antibody levels, HBsAg and antibody levels and liver histology. Other serum markers have been reported to be useful for monitoring the effect of antiviral therapy (15, 16). Recently, a new assay was developed for detecting the HBcrAg, consisting of HBcAg, HBeAg and a 22 kDa precore protein coded with the precore/core gene (17, 18). Because NAs have no inhibiting action on the transcription and translation activities of viral mRNA, HBcAg- and HBeAg-related proteins continue to be produced for a certain period of time in spite of the achievement of adequate suppression of the viral DNA synthesis. Therefore, HBcrAg is a viral marker independent of HBV DNA for monitoring the antiviral effect of NAs (19). In addition, recent reports have indicated another interesting aspect of serum HBcrAg levels: these levels were found to be correlated with intrahepatic covalently closed circular DNA (cccDNA) levels and could be a surrogate marker of the intrahepatic cccDNA pool (20, 21). This phenomenon may be explained by the fact that the production of HBcrAg depends on the

the treatment for HCC and stored in −80 °C. Liver tissue from patients who underwent resection was collected, rapidly frozen and stored in −80 °C. Written informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in *a priori* approval by the institution's human research committee.

Antiviral therapy

Forty-seven patients received 100 mg LAM daily, and drug-resistant YMDD mutants developed in 26 (55%) of these patients, accompanied by an increase in HBV DNA ≥1log copies/ml. Seventeen of the 26 patients received 10 mg ADV in addition to LAM (100 mg) daily. The remaining nine continued to receive LAM monotherapy because of the lack of approval for ADV administration in Japan at the time, but received ADV with LAM after approval was obtained during the HCC posttreatment period. Eight NA-naïve patients received 0.5 mg ETV daily. These antiviral therapies were continued after the resection or percutaneous ablation.

Follow-up and HCC recurrence

The patients were followed for liver function and virological markers of HBV infection monthly, as well as blood counts and tumour makers including α-fetoprotein and des-γ-carboxylprothrombin. They also underwent ultrasonography or helical dynamic computed tomography every 3 months. Cirrhosis was diagnosed by laparoscopy or liver biopsy or by the clinical data, imaging modalities and portal hypertension. The median observation period after HCC treatment for the entire cohort was 2.7 years (range, 0.3–8.4 years). HCC recurrence was diagnosed by the typical hypervascular characteristics on angiography and/or histological examination with fine needle biopsy specimens, in addition to certain features on computed tomography and ultrasonography.

Markers of HBV infection

HBeAg was determined by enzyme-linked immunosorbent assay using a commercial kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). HBV DNA was quantitated using the Amplicor monitor assay (Roche Diagnostics, Tokyo, Japan) with a dynamic range over 2.6–7.6log copies/ml or COBAS TaqMan HBV v.2.0 (Roche Diagnostics) with a dynamic range over 2.1–9.0log copies/ml. Serum HBV DNA levels were measured using the Amplicor assay at both the start of NA therapy and the diagnosis of HCC and using the TaqMan assay at the diagnosis of HCC. For statistical analysis, the value of that HBV DNA was tentatively set at 2.1 if HBV DNA levels were under 2.1log copies/ml. HBV genotypes were determined serologically by the combination of epitopes expressed on the pre-S2 region product, which is specific for each of the seven major genotypes (A–G), using a commercial kit (HBV Genotype EIA; Institute of Immunology). YMDD mutants were determined by polymerase chain reaction-based enzyme-linked minisequence assay using a commercial kit (Genome Science Laboratories, Tokyo, Japan).

HBcrAg measurement

Serum HBcrAg levels were measured using a CLEIA HBcrAg assay kit (Fujirebio Inc., Tokyo, Japan) with a fully automated analyser system (Lumipulse System; Fujirebio Inc.) as described previously (21). In brief, 150 μl of serum was incubated with 150 μl of pretreatment solution containing 15% sodium dodecyl sulphate at 60 °C for 30 min. After heat treatment, 120 μl of pretreated specimen was added to a ferrite microparticle suspension in an assay cartridge. Ferrite particles were coated with monoclonal antibody mixture (HB44, HB61 and HB114) against denatured HBcAg, HBeAg and the 22 kDa precore protein. After 10 min of incubation at 37 °C and washing, further incubation was carried out for 10 min at 37 °C with alkaline phosphatase conjugated with two kinds of monoclonal antibodies (HB91 and HB110) against denatured HBcAg, HBeAg and the 22 kDa precore protein. After washing, 200 μl of substrate solution [3-(2′-spiroada-mantan)-4-methoxy-4-(3′-phosphoryloxy)pheny1-1,2-dioxetane disodium salt] (Applied Biosystems, Bedford, MA, USA) was added to the test cartridge, which was then incubated for 5 min at 37 °C. The relative chemiluminescence intensity was measured, and the HBcrAg concentration was calculated by a standard curve generated using a recombinant pro-HBeAg (amino acids −10 to 183 of the precore/core gene product). The HBcrAg concentration was expressed in U/ml, which is defined as the immunoreactivity of 10 fg/ml of recombinant pro-HBeAg. In this study, the HBcrAg values were expressed as log U/ml, and the cut-off value was set at 3.0 log U/ml. For the statistical analyses, HBcrAg-negative cases were calculated as 3.0log U/ml.

Intrahepatic cccDNA measurement

Intrahepatic cccDNA levels were analysed as described previously (21). In brief, liver specimens surrounding the tumour tissue were obtained and stored at −80 °C before DNA extraction. HBV DNA was extracted using a QIAamp DNA Mini Kit (Qiagen KK, Tokyo, Japan). The concentration of purified DNA was based on the absorbance at 260 nm. For this study, two oligonucleotide primers cccF2 (5′-cgtctgtgccttctcatctga-3′, nucleotides 1424–1444) and cccR4 (5′-gcacagcttggaggcttgaa-3′, nucleotides 1755–1737) and probe cccP2 (5′-VIC-accaatttat gcctacag-MGB-3′, nucleotides 1672–1655) were designed using PRIMER EXPRESS software (Applied Biosystems, Foster City, CA, USA) to flank the direct repeat region between the hepatitis B core and the polymerase gene. The use of cccF2 and cccR4, oligonucleotide primers spanning the direct repeat region of the HBV genome, allows the polymerase chain reaction of native viral DNA in the

was detected in the NA-naïve patients receiving ETV monotherapy.

Correlation between serum HBcrAg and serum HBV DNA levels at the incidence of HCC

The median serum HBcrAg value was 6.6log U/ml (range, 3.3 to > 6.8) at the start of NA therapy and 5.0log U/ml (range, < 3.0 to > 6.8) at the time of HCC diagnosis. We observed a positive correlation ($P < 0.001$; $r = 0.610$) between the levels of HBcrAg and HBV DNA in serum at the time of HCC diagnosis (Fig. 2A).

HBcrAg was detectable in 23 (82%) of 28 patients with undetectable HBV DNA levels using TaqMan assay and was > 4.8log U/ml in eight (29%) of 28 patients. In contrast, serum HBV DNA was detectable in spite of undetected HBcrAg in only two patients. Then, we examined the correlation between the serum HBcrAg levels at the time of HCC diagnosis and the antiviral effect. The median duration of on-treatment undetected serum HBV DNA was 1.1 years (range, 0.1–4.8) before the first diagnosis of HCC. As shown in Figure 2B, we observed a significant negative correlation between the levels of HBcrAg in serum at the time of HCC diagnosis and the duration of undetected HBV DNA in serum just before the first diagnosis of HCC ($P < 0.001$; $r = -0.568$).

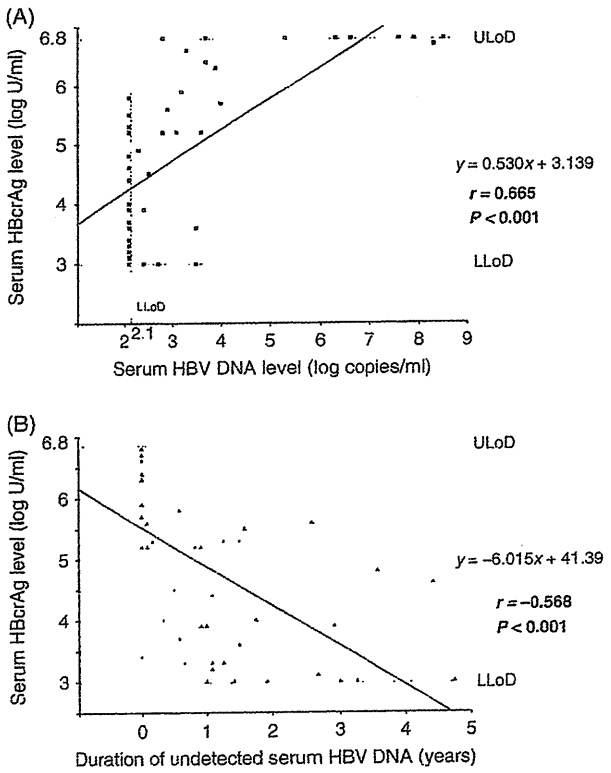


Fig. 2. (A) Correlation between serum HBcrAg and hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular carcinoma (HCC) diagnosis for each patient. (B) Correlation between serum HBcrAg levels at the time of HCC diagnosis and the duration of undetected serum HBV DNA (< 2.6log copies/ml).

Factors associated with HCC recurrence

Hepatocellular carcinoma recurred in 21 (38%) of the 55 patients, 17 (46%) of 37 patients who had undergone resection and four (22%) of 18 patients who had undergone ablation. Because a proportion of patients who had undergone resection with TNM Stage II or over (24 of 37 patients) was greater than ablation (six of 18), there were more patients who had HCC recurrence after resection than ablation. Eight factors were associated with the recurrence in univariate analysis: HBeAg positivity at the start of NA therapy, HBV DNA ≥ 2.1log copies/ml, HBcrAg level ≥ 4.8log U/ml, AST level ≥ 50 IU/L, ALT level ≥ 40 IU/L, tumour multiplicity, portal vein invasion at the time of HCC diagnosis and HCC treatment. In the multivariate analysis, HBcrAg level ≥ 4.8log U/ml and portal vein invasion were independent risk factors for the recurrence of HCC (Table 2). The cumulative recurrence-free survival rates in patients with ≥ 4.8log U/ml HBcrAg levels at the time of HCC diagnosis were 70% at 1 year, 35% at 3 years and 28% at 5 years. In contrast, the rates in patients with < 4.8log U/ml HBcrAg levels were 96% at 1 year, 89% at 3 years and 89% at 5 years. The recurrence-free survival rates of the high HBcrAg group (≥ 4.8log U/ml) were significantly lower than those of the low HBcrAg group (< 4.8log U/ml; $P < 0.001$), as shown in Figure 3A. Then, the cumulative recurrence-free survival rates in patients with ≥ 2.1log copies/ml HBV DNA levels at the time of HCC diagnosis were 70% at 1 year, 44% at 3 years and 39% at 5 years. In contrast, the rates in patients with < 2.1log copies/ml HBV DNA levels were 93% at 1 year, 76% at 3 years and 76% at 5 years. The recurrence-free survival rates of the positive HBV DNA group (≥ 2.1log copies/ml) were significantly lower than those of the negative HBV DNA group (< 2.1log copies/ml; $P = 0.007$), as shown in Figure 3B. The cumulative recurrence-free survival rates were 33% at 1 year and 33% at 2 years with portal vein invasion, and 87% at 1 year, 73% at 2 years and 64% at 3 years without invasion. Three of the six patients with portal vein invasion died of recurrent HCC.

Correlation between intrahepatic cccDNA and serum HBV DNA levels at the incidence of HCC

We measured intrahepatic cccDNA using liver specimens from 22 of 37 patients who underwent resection. The median intrahepatic cccDNA value was 4.2log copies/μg (range, 3.0–5.0). As shown in Figure 4A and B, we observed significant positive correlations between the levels of intrahepatic cccDNA and HBV DNA in serum ($P = 0.019$; $r = 0.486$) and between the levels of intrahepatic cccDNA and HBcrAg in serum at the time of HCC diagnosis ($P = 0.028$; $r = 0.479$). Twenty-eight patients who underwent resection had early- or intermediate-stage

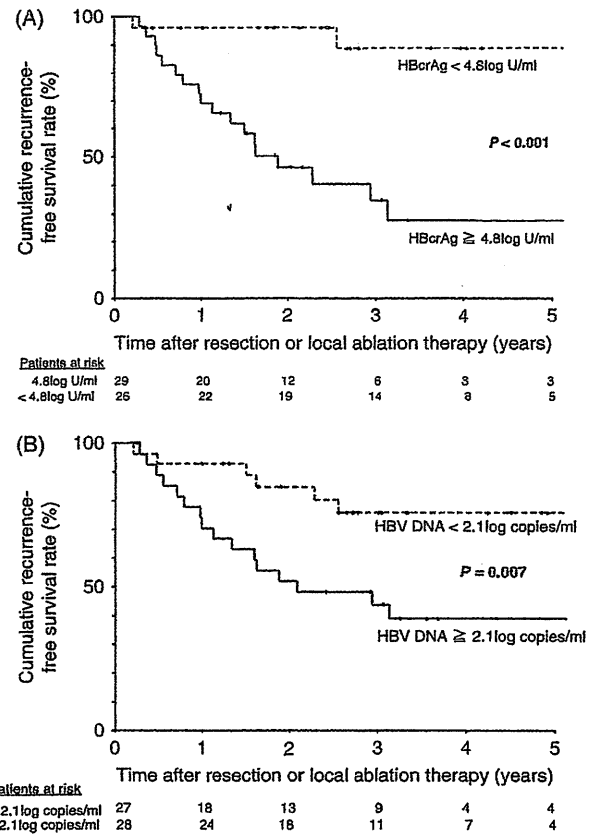


Fig. 3. (A) Kaplan–Meier life table for the cumulative recurrence-free survival rates by the serum HBcrAg levels and comparison by the log-rank test. (B) Kaplan–Meier life table for the cumulative recurrence-free survival rates by the serum hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular carcinoma (HCC) diagnosis for each patient and comparison by the log-rank test.

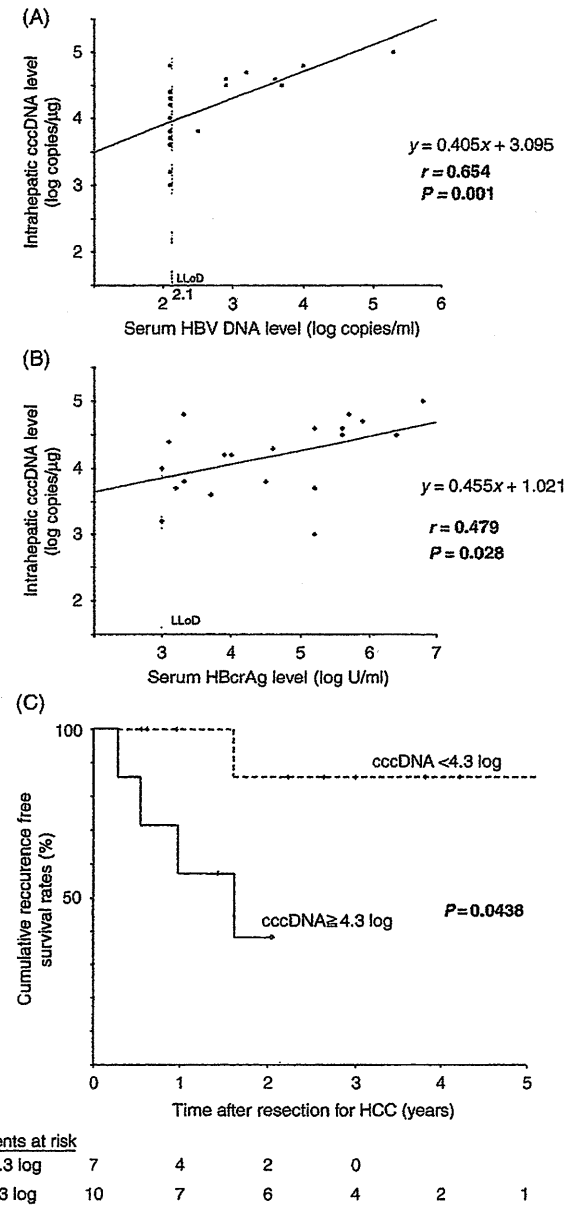


Fig. 4. (A) Correlation between intrahepatic covalently closed circular DNA (cccDNA) and serum hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular carcinoma (HCC) diagnosis for each patient who underwent resection ($n = 22$). (B) Correlation between intrahepatic cccDNA and serum HBcrAg levels at the time of HCC diagnosis. (C) Kaplan–Meier life table for the cumulative recurrence-free survival rates by the intrahepatic cccDNA levels in patients with early- or intermediate-stage HCC ($n = 17$).

developed HCC after the commencement of NA therapy and underwent radical therapy for HCC. The recurrence rates of HCC were high in patients with high levels of intrahepatic cccDNA and serum HBcrAg. In particular, HBcrAg levels were measurable by using serum samples and clinically useful.

Nucleot(s)ide analogues, including LAM, ADV and ETV, are widely used for the treatment of chronic hepatitis B, and reportedly reduce the development of HCC in such patients (22, 23). Although few events of HCC development occur during NA therapy (24–26), analysis of a large number of patients is needed to examine the risk factors for HCC. We could clarify the risk factors associated with the development of primary HCC after radical therapy by enrolling patients who underwent radical therapy for HCC in spite of their small number. High HBV loads in serum have been reported to be associated with HCC recurrence after resection or radical therapy in NA-naïve patients (27–31), but no study has demonstrated the viral risk factors of recurrence in patients receiving NAs. The novel finding of this study is that serum HBcrAg and intrahepatic cccDNA levels are predictors of HCC recurrence in patients radically treated for HCC during NA therapy.

In this study, the serum HBV DNA levels at the time of HCC diagnosis were associated with recurrence by univariate analysis. However, the serum HBcrAg level was the only viral factor associated with recurrence in multivariate analysis. There are two possible reasons for the

intrahepatic cccDNA levels is slower than that of serum HBV DNA levels during NA administration (15, 16). We found that suppression of cccDNA by NAs could prevent the development of recurrent primary HCC. Because cccDNA provides the template for pregenomic and viral messenger RNA-encoded viral proteins (33–35), the transcriptional activity of cccDNA may induce carcinogenesis. Further research is required to validate this hypothesis. Serum HBcrAg can be a surrogate marker of the intrahepatic cccDNA pool because of the viral proteins transcribed through messenger RNA from cccDNA (20, 21). Therefore, we consider that serum HBcrAg reflects the intrahepatic viral status more accurately than serum HBV DNA. Recently, Chan *et al.* (36) showed that serum HBsAg quantification could reflect intrahepatic cccDNA in patients treated with peginterferon and LAM combination therapy. They also indicated that reduction in HBsAg had good correlation with reduction in cccDNA. We tried to measure HBsAg levels at the start of NA therapy and the time of HCC diagnosis using a commercial assay (chemiluminescent immunoassay). However, HBsAg levels declined very slowly during NAs monotherapy in this study (data not shown). Brunetto *et al.* (37) showed that mean reduction for 48 weeks in HBsAg was 0.02log IU/ml in patients treated with LAM monotherapy, different from peginterferon therapy. Meanwhile, the median reduction from the start of NA to the diagnosis of HCC in HBcrAg was 1.4logU/ml in this study (Table 1). It seems that HBcrAg is a superior on-treatment risk predictor (e.g. tumour recurrence) to HBsAg during NAs monotherapy in terms of reduction of titres in each assay. HBcrAg is also more useful in terms of needless to serum sample dilution. As HBcrAg levels can be measured from serum samples, they are clinically useful, compared with the measurement of cccDNA, which requires liver specimens. It is not practical to carry out liver biopsy and the measurement of cccDNA for patients who have normal AST/ALT levels and viral suppression during antiviral therapy. Liver specimens cannot be also taken from patients who undergo ablation therapy for HCC. The measurement of serum HBcrAg levels in these patients is helpful to indirectly estimate the status of intrahepatic cccDNA. In the future, it is necessary to investigate whether HBcrAg in patients receiving NAs can be a predictor of primary carcinogenesis.

Previous studies have indicated that the rates of intrahepatic cccDNA loss and serum HBcrAg loss differ from serum HBV DNA loss under NA therapy, with the former two being much slower (15, 16, 19). In this study, the period of serum HBV DNA loss was longer, with lower intrahepatic cccDNA and serum HBcrAg levels (Fig. 2B). Therefore, these findings suggest that a long period of time is required to prevent the development of recurrent primary HCC by viral suppression under antiviral therapy. In contrast, the serum HBcrAg levels at the time of HCC diagnosis were higher in patients with emergent LAM-resistant mutants and subsequent VBT than in patients without mutants and VBT (Fig. 6). This result suggests that it is important to administer a potent NA early for drug-resistant strains and suppress viral replication to prevent subsequent carcinogenesis. Although we evaluated the relationship between the development of primary HCC and serum HBcrAg levels by a case–control study, the serum HBcrAg levels at the commencement of NA therapy and 1 year later were not associated with the development of primary HCC (unpublished data). This finding is attributable to the slow decline of the serum HBcrAg levels during antiviral therapy. The measurement of HBcrAg at intervals of 3–6 months may be helpful to predict the development of HCC. However, further studies are needed to confirm the finding.

In summary, HBcrAg is a predictor of the post-treatment recurrence of HCC during antiviral therapy. Measurement of the serum HBcrAg level is simple and useful because it reflects the intrahepatic viral status. Further, intrahepatic cccDNA and serum HBcrAg suppression by NAs is important to prevent HCC recurrence.

Acknowledgements

None of the authors received any funding from the manufacturers of the drugs or laboratory reagents used in this study. This research was partly supported by grants from the Ministry of Health, Labour and Welfare of Japan.

References

1. Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; 337: 1733–45.
2. Ganem D, Prince AM. Hepatitis B virus infection – natural history and clinical consequences. *N Engl J Med* 2004; **350**: 1118–29.
3. 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.
4. Dienstag JL, Schiff ER, Wright TL, *et al.* Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 1999; **341**: 1256–63.
5. 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.
6. 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.
7. Chang TT, Gish RG, de Man R, *et al.* A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006; **354**: 1001–10.
8. 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.

doi:10.1111/j.1440-1746.2009.06161.x

HEPATOLOGY

Efficacy of switching to entecavir monotherapy in Japanese lamivudine-pretreated patients

Fumitaka Suzuki,* Norio Akuta,* Yoshiyuki Suzuki,* Hiromi Yatsuji,* Hitomi Sezaki,* Yasuji Arase,* Miharu Hirakawa,* Yusuke Kawamura,* Tetsuya Hosaka,* Masahiro Kobayashi,* Satoshi Saitoh,* Kenji Ikeda,* Mariko Kobayashi,† Sachiyo Watahiki† and Hiromitsu Kumada*

*Department of Hepatology, Toranomon Hospital, Tokyo, and †Research Institute for Hepatology, Toranomon Branch Hospital, Kawasaki, Japan

Key words
entecavir, hepatitis B virus, lamivudine, viral resistance.

Accepted for publication 6 October 2009.

Correspondence
Dr Fumitaka Suzuki, Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan.
Email: fumitakas@toranomon.gr.jp

Abstract

Background and Aims: To assess the efficacy of switching Japanese chronic hepatitis B patients from lamivudine monotherapy to entecavir 0.5 mg/day.

Methods: A retrospective analysis was conducted on 134 patients switched to entecavir between September 2006 and February 2008 for 6 months or more. Patients were divided into three groups based on viral load at entecavir switching point (baseline < 2.6, 2.6–5.0 and > 5.0 $\log_{10}$ copies/mL).

Results: At baseline, detection of lamivudine-resistant virus was highest in patients with higher hepatitis B virus (HBV) DNA (76% *vs* 23% in ≥ 2.6 and < 2.6 $\log_{10}$ copies/mL, respectively), and in patients with longest previous exposure to lamivudine (52%, 28% and 24% for > 3 years, 1–3 years and < 1 year, respectively). Two years after entecavir switching, HBV DNA suppression to less than 2.6 $\log_{10}$ copies/mL was achieved in 100% (32/32), 92% (12/13) and 44% (4/9) of patients in the less than 2.6, 2.6–5.0 and more than 5.0 $\log_{10}$ copies/mL baseline groups, respectively. Alanine aminotransferase (ALT) normalization occurred in 76–96% and 90–100% of patients following 1 and 2 years of entecavir treatment, respectively. One patient (2.6–5.0 $\log_{10}$ copies/mL) with lamivudine-resistant mutants at baseline developed entecavir resistance at week 48 during follow up.

Conclusion: Switching to entecavir 0.5 mg/day achieves or maintains undetectable HBV DNA levels and ALT normalization over 2 years, especially in patients with viral load less than 5.0 $\log_{10}$ copies/mL.

Introduction

Hepatitis B virus (HBV) infection is a serious public health threat affecting 350–400 million people worldwide, the majority of whom live in the Asia–Pacific region.[1,2] Chronically-infected people are at risk of developing cirrhosis, liver failure and hepatocellular carcinoma. Studies have suggested that high serum HBV DNA is a key risk predictor of chronic hepatitis B (CHB) complications.[3,4] Therefore, the main purpose of CHB therapies is to permanently suppress viral replication and sustain viral suppression to prevent long-term liver damage.[2,5,6]

Lamivudine was the first nucleoside analog to be widely prescribed for CHB patients, mainly due to its antiviral efficacy and safety profile.[2] However, lamivudine's long-term efficacy is diminished by the emergence of drug-resistant substitutions, generally in the tyrosine–methionine–aspartate–aspartate (YMDD) motif of the reverse transcriptase (rt) polymerase gene.[7–9] Detection of lamivudine-resistant HBV substitutions occurs in 15–30% and 70% of patients after 1 and 5 years of treatment, respectively.[8] Continuing lamivudine monotherapy in the presence of lamivudine resistance is not recommended because it is no longer effective in suppressing viral replication.[2] Furthermore, the initial improvement in histology and clinical benefits may be reversed or decreased due to the emergence of lamivudine-resistant substitutions.

Antiviral efficacy of entecavir (0.5 mg/day) as first-line therapy was superior to lamivudine in treatment-naïve patients on all virological, biochemical and histological end-points after 48 weeks of treatment,[10–14] with very low rates of emergence of viral resistance (1.2% after 5 years of entecavir treatment).[15,16] Entecavir has a high genetic barrier to resistance,[17–19] requiring multiple substitutions (including YMDD mutations) to express viral resistance.[16–21] In agreement with this, entecavir-resistant mutants emerge more frequently in lamivudine-refractory patients.[22,23] In a study of hepatitis B e antigen (HBeAg)-positive lamivudine-refractory patients with high HBV DNA levels at baseline (mean > 9 $\log_{10}$ copies/mL), switching to entecavir 1 mg/day achieved HBV DNA suppression to undetectable levels (< 300 copies/mL; 40%, 96 weeks) and alanine aminotransferase (ALT) normalization (81%, 96 weeks) at higher proportions than continued lamivudine

monotherapy,[22] although response to therapy was less pronounced than in treatment-naïve patients with comparable baseline levels of HBV DNA.[10,13,14] The probability of achieving HBV DNA suppression to undetectable levels at 96 weeks with entecavir was 73% in patients whose baseline HBV DNA was less than 7 $\log_{10}$ copies/mL ($n = 11$), and none of these patients developed entecavir resistance.[22]

In a randomized controlled trial of lamivudine-refractory Japanese patients with mean HBV DNA at baseline of 7.6–7.7 $\log_{10}$ copies/mL, switching to entecavir (0.5 or 1 mg/day) for 48 weeks achieved HBV DNA suppression to below detectable levels in 33% of patients in the entecavir dose groups, and ALT normalization in 78–86%.[24] Switching to entecavir in patients with evidence of lamivudine-resistant substitutions and low viral load at switching point has not been prospectively investigated in Japanese patients. There are limited data concerning the efficacy of entecavir in lamivudine-pretreated patients who have not developed lamivudine resistance.

The objective of this study was to assess the efficacy of switching to entecavir 0.5 mg/day in Japanese lamivudine-pretreated patients whose HBV DNA levels at switching point (baseline) ranged from less than 2.6 to 7.6 $\log_{10}$ copies/mL, with or without lamivudine-resistant substitutions.

Methods

Design and setting

A retrospective analysis of a CHB patient population ($n = 134$) at Toranomon Hospital (Tokyo, Japan) was performed to identify patients switched from lamivudine 100 mg/day monotherapy to entecavir 0.5 mg/day between September 2006 and February 2008, and who had received entecavir for at least 6 months. Among all patients selected, only one had a history of adefovir add-on therapy prior to switching to entecavir (case report). Conserved serum from all patients was analyzed to determine baseline characteristics and study end-points.

Study end-points

Clinical efficacy of entecavir was assessed as the proportion of patients achieving HBV DNA suppression to undetectable levels (< 400 copies/mL or < 2.6 $\log_{10}$ copies/mL), and patients achieving ALT normalization (normal ALT levels: men 8–42 IU/L, women 6–27 IU/L). HBV DNA was measured using the polymerase chain reaction (PCR)-based Amplicor HBV Monitor assay (Roche Diagnostics, Indianapolis, IN, USA; lower limit of detection of < 2.6 $\log_{10}$ copies/mL).[25] HBeAg loss in patients who were HBeAg-positive at baseline was also analyzed. Measurements were made from conserved samples taken at baseline, and after 6 months, 1 and 2 years from entecavir treatment initiation.

Assessment of viral resistance

Conserved serum was used to detect the presence of viral lamivudine-resistant rtM204V/I substitutions in all patients at baseline, and following the entecavir switch in patients treated with entecavir for at least 6 months. Lamivudine-resistant virus (rtM204V/I or YMDD motif substitutions) was analyzed using a combination of the quantitative enzyme-linked immunosorbent assay standardized using a purified *Taenia solium* cysticerci fraction (PCR enzyme-linked immunosorbent assay) and the enriched PCR enzyme linked minisequence assay.[26] Direct sequencing of HBV DNA polymerase reverse transcriptase site was also performed.[27] Detection of entecavir-resistant virus was conducted using direct sequencing of HBV DNA polymerase reverse transcriptase site.[27]

Data analyses

Statistical comparisons between treatment groups were assessed using χ^2-test and Kruskal–Wallis test where appropriate. Calculations were performed using StatView software (ver. 4.5J; Abacus Concepts, Berkeley, CA, USA). A two-tailed P-value less than 0.05 was considered statistically significant.

To identify predictive factors of HBV DNA negativity (suppression to below detectable levels) after 6 months of the entecavir switch, univariate and multivariate logistic regression analyses were carried out. Potential predictive factors at baseline included: sex; age; levels of aspartate aminotransferase (AST), ALT, albumin, γ-glutamyl transpeptidase, total bilirubin and α-fetoprotein; platelet count; viral load; liver disease stage (cirrhosis or other); family history; HBV genotype; lamivudine treatment duration prior to entecavir switch; HBeAg status; and lamivudine resistance. Each variable was transformed into categorical data consisting of two simple ordinal numbers. All factors that were at least marginally associated with HBV DNA negativity ($P < 0.10$) were used in a multiple logistic regression analysis. To assess relative risk confidence, odds ratio (OR) and 95% confidence interval (CI) were calculated. All analyses were performed using SPSS II software ver. 11.0 (SPSS, Chicago, IL, USA).

Results

Patient characteristics before switching to entecavir

Lamivudine-pretreated patients switched to entecavir 0.5 mg/day ($n = 134$) were divided into three groups based on their HBV DNA level at the switching point: HBV DNA of less than 2.6 $\log_{10}$ copies/mL ($n = 92$), 2.6–5.0 $\log_{10}$ copies/mL ($n = 25$) and more than 5.0 $\log_{10}$ copies/mL ($n = 17$) (Table 1). Patients with HBV DNA levels of more than 5.0 $\log_{10}$ copies/mL had the highest AST/ALT levels and highest proportion of HBeAg-positive cases ($P < 0.05$). These patients had been treated with lamivudine for the shortest time period compared to patients from the two other groups ($P < 0.05$; Table 1).

Viral resistance to lamivudine at baseline

At baseline, lamivudine-resistant rtM204V/I mutant virus was detected in 23% of patients with HBV DNA of less than 2.6 $\log_{10}$ copies/mL, compared to 76% in each of the HBV DNA 2.6–5.0 $\log_{10}$ copies/mL and more than 5.0 $\log_{10}$ copies/mL groups (Table 2). In all treatment groups, a higher occurrence of resistant virus was observed with longer exposure to lamivudine, independent of viral DNA levels.

Table 1 Patient characteristics at point of switching to entecavir (baseline) and entecavir treatment duration

	All patients	Serum HBV DNA levels by baseline treatment group, log_{10} copies/mL			
		< 2.6	2.6–5.0	> 5.0	*P**
Patients, *n*	134	92	25	17	
Sex, *n* male/female	94/40	67/25	19/6	8/9	0.08
Age, years†	53 (23–83)	53 (27–83)	50 (32–77)	37 (23–77)	0.036
Bilirubin, mg/dL†	0.6 (0.2–3.4)	0.6 (0.2–3.4)	0.6 (0.3–1.8)	0.7 (0.3–1.2)	0.53
AST, IU/L†	24 (13–451)	23 (13–53)	23 (14–50)	37 (14–451)	0.0083
ALT, IU/L†	21 (8–1382)	21 (8–56)	20 (10–111)	46 (9–1382)	0.0002
Albumin, g/dL†	3.9 (2.7–4.8)	3.9 (2.7–4.4)	4.0 (3.3–4.8)	3.9 (3.6–4.6)	0.94
Histology, *n* CH/LC	89/45	56/36	19/6	14/3	0.11
HBeAg, *n* ±	30/104	11/81	5/20	14/3	< 0.0001
HBV DNA, log_{10} copies/mL†	< 2.6 (< 2.6–7.6)	< 2.6	3.9 (2.7–5.0)	6.5 (5.1–7.6)	–
Genotype, *n* A/B/C/unknown	3/9/115/7	2/6/78/6	1/2/22/0	0/1/15/1	0.87
Treatment duration, months†					
Lamivudine	36 (0.5–103)	36 (3–103)	70 (2–89)	17 (0.5–89)	0.009
Entecavir‡	21 (6–33)	20 (6–33)	24 (6–32)	27 (6–33)	0.034

*Comparison of the three patient subgroups using the Kruskal–Wallis test; $P < 0.05$ was considered statistically significant.
†Data are median (range).
‡Entecavir treatment duration is from point of switching.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; HBeAg, hepatitis B early antigen; HBV, hepatitis B virus; LC, liver cirrhosis.

Table 2 rtM204V/I mutant occurrence at baseline of switching to entecavir

	Duration of previous lamivudine treatment, years			All patients
	< 1	1–3	≥ 3	
Baseline treatment group				
< 2.6 log_{10} copies/mL	1/10 (10%)	4/35 (11%)	16/47 (34%)	23%
2.6–5.0 log_{10} copies/mL	1/5 (20%)	3/4 (75%)	15/16 (94%)	76%
> 5.0 log_{10} copies/mL	3/6 (50%)	6/7 (86%)	4/4 (100%)	76%
All patients	24%	28%	52%	–

Clinical efficacy of entecavir 0.5 mg/day

Switching to entecavir 0.5 mg/day for 1 year resulted in HBV DNA suppression to undetectable levels in the majority of patients with HBV DNA below 5.0 log_{10} copies/mL (100% and 96% for HBV DNA < 2.6 and 2.6–5.0 log_{10} copies/mL, respectively) (Table 3). This proportion was slightly decreased when previous lamivudine treatment duration exceeded 3 years in the 2.6–5.0 log_{10} copies/mL group. In the HBV DNA more than 5.0 log_{10} copies/mL group, approximately half (41%) of the patients achieved viral suppression after 1 year (Table 3); entecavir's efficacy seemed to decrease with prolonged previous exposure to lamivudine, with only 25% of patients having more than 3-year lamivudine treatment achieving undetectable viral load. Similarly, after 2 years, HBV DNA suppression was achieved by 100% and 92% of patients in the HBV DNA less than 2.6 and 2.6–5.0 groups, respectively, and by 44% of patients in the HBV DNA more than 5.0 log_{10} copies/mL group (Table 3).

Among those who failed to suppress viral load, only one case of virological breakthrough was found (2.6–5.0 log_{10} copies/mL group; described under case report). This patient had been previously exposed to lamivudine for more than 3 years.

Alanine aminotransferase levels were normalized in 76–96% and 90–100% of patients following 1 and 2 years of entecavir treatment, respectively (Table 3). HBeAg loss was observed in 27% (3/11), 20% (1/5) and 29% (4/14) of patients with HBV DNA of less than 2.6, 2.6–5.0 and more than 5.0 log_{10} copies/mL, respectively, in the first year.

Lamivudine-resistant substitutions in patients switched to entecavir

Of the 130 patients who received entecavir treatment for at least 1 year, 11 cases failed to suppress HBV DNA to below less than 2.6 log_{10} copies/mL and remained HBV DNA-positive in the first year (1 and 10 in the HBV DNA 2.6–5.0 and > 5.0 log_{10} copies/mL groups, respectively; Table 3). Serum HBV DNA analysis confirmed the presence of rtM204V/I substitutions in 10 of these patients, of which six were rtM204I and three were rtM204V substitutions (Table 4); the remaining patient (2.6–5.0 log_{10} copies/mL group; previous lamivudine exposure 5 years) carried a mixed type substitution, rtM204I plus rtM204V. The only HBV DNA-positive patient who did not

Table 3 Clinical efficacy of entecavir 0.5 mg/day in lamivudine-pretreated patients

End-point by baseline treatment group	Duration of entecavir treatment		
	6 months	1 year	2 years
HBV DNA suppression to undetectable levels, *n*/N (%)			
< 2.6 $\log_{10}$ copies/mL	90/92 (98%)	89/89 (100%)	32/32 (100%)
Previous lamivudine < 1 year	10/10 (100)	9/9 (100)	5/5 (100)
Previous lamivudine 1–3 years	35/35 (100)	35/35 (100)	14/14 (100)
Previous lamivudine > 3 years	45/47 (96)	45/45 (100)	13/13 (100)
2.6–5.0 $\log_{10}$ copies/mL	24/25 (96%)	23/24 (96%)	12/13 (92%)
Previous lamivudine < 1 year	5/5 (100)	5/5 (100)	3/3 (100)
Previous lamivudine 1–3 years	4/4 (100)	4/4 (100)	2/2 (100)
Previous lamivudine > 3 years	15/16 (94)	14/15 (93)	7/8 (88)
> 5.0 $\log_{10}$ copies/mL	5/17 (29%)	7/17 (41%)	4/9 (44%)
Previous lamivudine < 1 year	2/6 (33)	3/6 (50)	2/4 (50)
Previous lamivudine 1–3 years	2/7 (29)	3/7 (43)	2/4 (50)
Previous lamivudine > 3 years	1/4 (25)	1/4 (25)	0/1 (0)
ALT normalization, *n*/*n* (%)			
< 2.6 $\log_{10}$ copies/mL	88/92 (96%)	83/89 (93%)	32/32 (100%)
2.6–5.0 $\log_{10}$ copies/mL	24/25 (96%)	23/24 (96%)	12/13 (92%)
> 5.0 $\log_{10}$ copies/mL	14/17 (82%)	13/17 (76%)	9/10 (90%)

ALT, alanine aminotransferase; HBV, hepatitis B virus.

Table 4 HBV DNA positive rates in patients switched to entecavir 0.5 mg/day for at least 1 year

	HBeAg status	YMDD motif substitution	HBV DNA positive rate, *n*/N (%)	Duration of previous lamivudine treatment, years per patient
Baseline treatment group				
< 2.6 $\log_{10}$ copies/mL	Positive	Wild (or none)	0/10 (0%)	n/a
		YIDD	0/1 (0%)	n/a
	Negative	Wild (or none)	0/58 (0%)	n/a
		YIDD	0/15 (0%)	n/a
		YVDD	0/4 (0%)	n/a
		YIDD + YVDD	0/1 (0%)	n/a
2.6–5.0 $\log_{10}$ copies/mL	Positive	Wild (or none)	0/4 (0%)	
		YIDD + YVDD	1/1 (100%)†	5.0
	Negative	Wild (or none)	0/2 (0%)	n/a
		YIDD	0/10 (0%)	n/a
		YVDD	0/6 (0%)	n/a
		YIDD + YVDD	0/1 (0%)	n/a
> 5.0 $\log_{10}$ copies/mL	Positive	Wild (or none)	1/4 (25%)	0.2
		YIDD	6/9 (67%)	0.5; 1.3; 1.5; 2.7; 3.9; 7.4
		YVDD	1/1 (100%)	0.7
	Negative	YIDD	0/1 (0%)	n/a
		YVDD	2/2 (100%)	1.8; 4.5
All patients			11/130 (8%)	

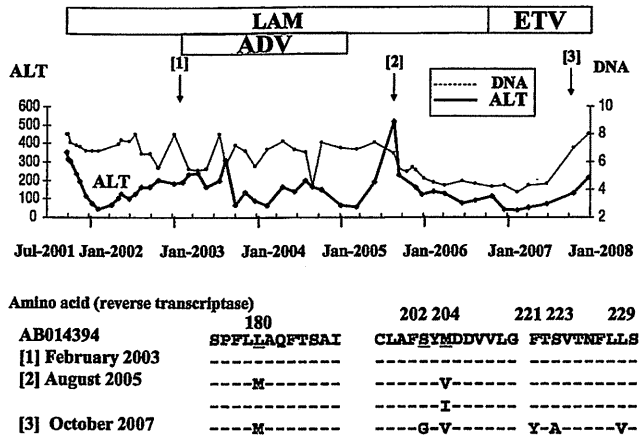
YMDD motif substitutions: wild, rt204M; YIDD, rt204I; YVDD, rt204V; YIDD + YVDD, rt204I + rt204V.
†Patient with lamivudine-resistant HBV who developed entecavir resistance.
HBeAg, hepatitis B early antigen; HBV, hepatitis B virus; n/a, not available.

carry any detectable lamivudine-resistant substitution had the shortest previous lamivudine exposure (< 6 months; Table 4).

Of the 10 patients carrying rtM204V/I substitutions, eight were HBeAg-positive; the other two patients were HBeAg-negative and carried a lamivudine-resistant rtM204V type substitution.

Emergence of entecavir-resistant mutant: case report

One patient (2.6–5.0 $\log_{10}$ copies/mL group) carrying a mixed substitution YIDD + YVDD (rtM204I + rtM204V) developed entecavir resistance with a recognized rtS202G substitution



Amino acid (reverse transcriptase)

	180	202 204	221 223	229
AB014394	SPFLLAQFTSAI	CLAFSYMDDVVLG	FTSVTNFLLS	
[1] February 2003	----------	----------	----------	
[2] August 2005	----M-------	------V------	----------	
	----------	------I------	----------	
[3] October 2007	----M-------	----G-V------	Y-A-----V-	

Figure 1 Clinical course and evolution of viral polymerase reverse transcriptase gene sequence in a patient with confirmed rtM204V/I substitutions (YIDD + YVDD) and emerging entecavir resistance substitution (rtS202G). AB014394 was a strain reported by Takahashi *et al.*[33] Two kinds of strains emerged in August 2005 (rtL180M/rtM204V and rtM204I). In October 2007, an additional amino acid substitution (rtS202G) was detected. ADV, adefovir; ALT, alanine aminotransferase; ETV, entecavir; LAM, lamivudine. — DNA; — ALT.

(Table 4). Figure 1 describes the clinical course and evolution of viral DNA sequence. This 37-year-old Japanese man was found to be seropositive for hepatitis B surface antigen with mild ALT elevation in December 1998. He was diagnosed with CHB by peritoneoscopy and liver biopsy (mild hepatitis [A1] and mild fibrosis [F1]). HBeAg was positive; serum HBV DNA was more than 7.6 $\log_{10}$ copies/mL. Treatment with lamivudine 100 mg/day was initiated in October 2001, at which time serum HBV DNA was more than 7.6 $\log_{10}$ copies/mL and ALT was 314 IU/L. In February 2003, adefovir dipivoxil 10 mg/day was added-on to lamivudine, but failed to decrease HBV DNA load. In January 2005, adefovir was withdrawn; the patient remained on lamivudine monotherapy. Amino acid substitutions of the rt gene, rtL180M, rtM204V and rtM204I were detected in August 2005. In October 2006, the patient was switched directly from lamivudine to entecavir 0.5 mg/day without treatment interruption. In February 2007, ALT levels decreased to within normal values, and serum HBV DNA was less than 4 $\log_{10}$ copies /mL. However, shortly after, both ALT levels and HBV DNA began to rise again. In October 2007, amino acid substitutions rtL180M, rtM204V and rtS202G were detected.

Predictive factors of HBV DNA negativity

Univariate analyses identified six factors that correlated with HBV DNA suppression to undetectable levels after 6 months of the entecavir switch: viral load less than 5 $\log_{10}$ copies/mL ($P < 0.001$); HBeAg-negative status ($P < 0.001$); the absence of lamivudine resistance ($P < 0.001$); normal AST level (≤ 33 IU/L; $P = 0.008$); normal ALT level (men ≤ 42 IU/L, women ≤ 27 IU/L; $P < 0.001$); and chronic hepatitis stage of liver disease ($P = 0.069$). Multivariate analyses showed that viral load below 5 $\log_{10}$ copies/mL (OR = 69.03; 95% CI = 13.23–360.09; $P < 0.001$) and the absence of lamivudine resistance (OR = 8.17; 95% CI = 1.25–53.34; $P = 0.028$) each independently influenced entecavir's efficacy to suppress HBV DNA to undetectable levels after 6 months.

Discussion

Entecavir is recommended as a first-line CHB treatment by all major guidelines, due to its antiviral potency and high genetic barrier to resistance in nucleos(t)ide-naïve patients.[2,5,6] Conversely, in lamivudine-resistant patients, switching to entecavir is not a first-choice treatment, due to increased risk of emergence of entecavir resistance on a multiple substitution background.[22,23] However, in attempts to rescue those with suboptimal antiviral response and also to avoid the emergence of viral resistance in responsive patients during their treatment course, switching to entecavir is recommended by the Japanese Ministry of Health, Welfare and Labor for lamivudine-pretreated patients with undetectable viral load (< 2.6 $\log_{10}$ copies/mL), and for patients with detectable HBV DNA but without biochemical breakthrough and lamivudine resistance.[28] This study provides a unique opportunity to evaluate the efficacy of entecavir in a lamivudine-pretreated population with low viral load at switching point.

The majority of patients with HBV DNA at baseline of less than 5 $\log_{10}$ copies/mL maintained or achieved viral suppression 1 year after switching to entecavir, despite 23–76% of them carrying lamivudine-resistant substitutions. A similar trend was maintained during the second year. Conversely, viral suppression below detection limits was reported in less than half of patients with high viral load at baseline (HBV DNA 5.1–7.6 $\log_{10}$ copies/mL) carrying rtM204V/I substitutions (76% patients), in agreement with earlier studies showing diminished entecavir efficacy in lamivudine-refractory patients with elevated viral load.[22,23,29] In addition, multivariate analyses revealed that a viral load of less than 5 $\log_{10}$ copies/mL was an independent predictive factor of HBV DNA suppression to undetectable levels, after 6 months of entecavir therapy. Taken together, these data suggest that switching to entecavir is mostly efficacious in patients with low viral load regardless of the presence of rtM204V/I substitutions. This observation adds another perspective in predicting clinical response to entecavir in lamivudine-pretreated patients.

Another predictive factor of entecavir's efficacy in this retrospective cohort is the absence of lamivudine resistance. This is consistent with previous research suggesting decreased genetic barrier of entecavir to resistance in the presence of lamivudine-resistant substitutions.[22,23] The responsiveness of lamivudine-resistant patients with low viral load reported here could be explained by the ability of entecavir to clear low loads of rtM204V/I mutants. This is suggested by *in vitro* data showing maintained sensitivity of lamivudine-resistant mutants to entecavir, although at higher EC_{50}. Assessing the kinetics of rtM204V/I mutants in response to entecavir switching in patients with undetectable viral load is worth further characterization.

Previous studies have shown that developing entecavir resistance is higher in the presence of pre-existing lamivudine-resistant substitutions.[16–21,30] Despite the presence of lamivudine-resistant virus in 23%–76% of all patient groups, the emergence of entecavir resistance was rare, with only one confirmed case from the

HBV DNA 2.6–5.0 $\log_{10}$ copies/mL group. This patient's history is suggestive of a typical refractory case, with failure of multiple regimens including the combination of lamivudine plus adefovir (Fig. 1). The low entecavir resistance rate in this study may be due to the relatively short treatment period and small sample size. Further follow up will be required to monitor for subsequent emergence of entecavir resistance in these patients.

One could argue whether it is cost-effective to switch all lamivudine-treated patients with undetectable HBV DNA to entecavir. The GLOBE study demonstrated that although fewer lamivudine-treated patients with undetectable HBV DNA at week 24 developed viral resistance, resistance could still occur after 2 years of treatment (9% and 5% of HBeAg-positive and HBeAg-negative patients, respectively).[31] Moreover, Yuen and collaborators also reported that of lamivudine-treated patients who achieved HBV DNA suppression below 200 copies/mL at week 24, 8.3% developed resistance after 5 years.[32] In countries where medicine access is an issue, further studies are needed to evaluate the cost-effectiveness of entecavir switching of all patients with undetectable viral load, versus switching only those at risk of developing viral resistance. Comparative studies integrating the efficacy and safety of standard adefovir add-on versus switching to entecavir monotherapy are also warranted in these patients.

Study limitations should be considered. This is a retrospective analysis of CHB patients which, in the absence of matching controls, may introduce confounding errors and bias. Specifically, a control arm for the HBV PCR-negative group (< 2.6 $\log_{10}$ copies/mL; $n = 92$) would be required to strengthen study conclusions. Another limitation is the small sample size of the intermediate and high HBV DNA cohorts (25 patients with 2.6–5.0 $\log_{10}$ copies/mL, and 17 patients with > 5.0 $\log_{10}$ copies/mL, respectively); adding more patients to these samples as available would add weight to describing higher number entecavir response and resistance rates in these groups.

In conclusion, this study shows that the efficacy of switching from lamivudine to entecavir 0.5 mg/day is highest for Japanese patients with no rtM204V/I substitutions and a viral load of less than 5 $\log_{10}$ copies/mL, independent of their previous exposure to lamivudine. Efficacy is decreased for patients with rtM204V/I substitutions and low viral load, and is lowest for patients with rtM204V/I substitutions and high viral load. Viral resistance to entecavir after 48 weeks is rare in these patients. Multivariate analyses showed that viral load of less than 5 $\log_{10}$ copies/mL and the absence of lamivudine resistance are independent factors predicting entecavir's efficacy to reduce HBV DNA to undetectable levels after 6 months of treatment.

Acknowledgments

This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan. Editorial support for the manuscript was provided by BioMedCom Consultants.

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 Liaw Y-F, Leung N, Kao J-H *et al.* Asian-Pacifc consensus statement on the management of chronic hepatitis B: a 2008 update. *Hepatol. Int.* 2008; **2**: 263–83.
3 Chen CJ, Yang HI, Su J *et al.* Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* 2006; **295**: 65–73.
4 Iloeje U, Yang HI, Su J, Jen CL, You SL, Chen CJ. Predicting liver cirrhosis risk based on the level of circulating hepatitis B viral load. *J. Gastroenterol.* 2006; 678–86.
5 Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507–39.
6 European Association for the Study of the Liver. EASL clinical practice guidelines: management of chronic hepatitis B. *J. Hepatol.* 2009; **50**: 227–42.
7 Locarnini S, Mason WS. Cellular and virological mechanisms of HBV drug resistance. *J. Hepatol.* 2006; **44**: 422–31.
8 Lok AS, Zoulim F, Locarnini S *et al.* Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. *Hepatology* 2007; **46**: 254–65.
9 Suzuki F, Tsubota A, Arase Y *et al.* Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. *Intervirology* 2003; **46**: 182–9.
10 Chang TT, Gish RG, de Man R *et al.* A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N. Engl. J. Med.* 2006; **354**: 1001–10.
11 Gish RG, Lok AS, Chang TT *et al.* Entecavir therapy for up to 96 weeks in patients with HBeAg-positive chronic hepatitis B. *Gastroenterology* 2007; **133**: 1437–44.
12 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.
13 Ren FY, Piao DM, Piao XX. A one-year trial of entecavir treatment in patients with HBeAg-positive chronic hepatitis B. *World J. Gastroenterol.* 2007; **13**: 4264–7.
14 Schiff E, Simsek H, Lee WM *et al.* Efficacy and safety of entecavir in patients with chronic hepatitis B and advanced hepatic fibrosis or cirrhosis. *Am. J. Gastroenterol.* 2008; **103**: 1–8.
15 Colonno RJ, Rose R, Baldick CJ *et al.* Entecavir resistance is rare in nucleoside naive patients with hepatitis B. *Hepatology* 2006; **44**: 1656–65.
16 Tenney DJ, Rose RE, Baldick CJ, Pokornowski K, Eggers BJ. Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naïve patients is rare through 5 years of therapy. *Hepatology* 2009; **49**: 1503–14.
17 Baldick CJ, Eggers BJ, Fang J *et al.* Hepatitis B virus quasispecies susceptibility to entecavir confirms the relationship between genotypic resistance and patient virologic response. *J. Hepatol.* 2008; **48**: 895–902.
18 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.
19 Tenney DJ, Levine SM, Rose RE *et al.* Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. *Antimicrob. Agents Chemother.* 2004; **48**: 3498–507.
20 Nagasaki F, Niitsuma H, Ueno Y *et al.* The high incidence of the emergence of entecavir-resistant mutants among patients infected with lamivudine-resistant hepatitis B virus. *Tohoku J. Exp. Med.* 2007; **213**: 181–6.
21 Villet S, Ollivet A, Pichoud C *et al.* Stepwise process for the development of entecavir resistance in a chronic hepatitis B virus infected patient. *J. Hepatol.* 2007; **46**: 531–8.

22 Sherman M, Yurdaydin C, Simsek H *et al.* Entecavir therapy for lamivudine-refractory chronic hepatitis B: improved virologic, biochemical, and serology outcomes through 96 weeks. *Hepatology* 2008; **48**: 99–108.
23 Sherman M, Yurdaydin C, Sollano J *et al.* Entecavir for treatment of lamivudine-refractory, HBeAg-positive chronic hepatitis B. *Gastroenterology* 2006; **130**: 2039–49.
24 Suzuki F, Toyoda J, Katano Y *et al.* Efficacy and safety of entecavir in lamivudine-refractory patients with chronic hepatitis B: randomized controlled trial in Japanese patients. *J. Gastroenterol. Hepatol.* 2008; **23**: 1320–6.
25 Matsuyama K, Hayashi K, Miura T. The quantitative assay for HBV-DNA and the detection of HBV-DNA point mutation by polymerase chain reaction—'AMPLICOR HBV MONITOR Test' and 'HBV pre Core/Core Promoter Mutation Detection kit. *Kan Tan Sui* 2000; **41**: 59–71.
26 Kobayashi S, Shimada K, Suzuki H, Tanikawa K, Sata M. 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.
27 Suzuki F, Kumada H, Nakamura H. Changes in viral loads of lamivudine-resistant mutants and evolution of HBV sequences during adefovir dipivoxil therapy. *J. Med. Virol.* 2006; **78**: 1025–34.
28 Kumada H. *Scientific Research Grant of Ministry of Health, Labour and Welfare. Research of hepatitis overcome urgent strategy. Research report of the standardization of viral hepatitis treatment including liver cirrhosis* (Japanese version). 2009.
29 Chang TT, Gish RG, Hadziyannis SJ *et al.* A dose-ranging study of the efficacy and tolerability of entecavir in Lamivudine-refractory chronic hepatitis B patients. *Gastroenterology* 2005; **129**: 1198–209.
30 Kobashi H, Fujioka S-I, Kumada H, Yokosuka O, Hayashi N, Suzuki K. Emergence of hepatitis B virus gene mutation related to entecavir-resistance in chronic hepatitis B patients participated in the phase 2 clinical studies of entecavir in Japan. *Hepatology* 2007; **46** (Suppl. 1): 666A.
31 Lai CL, Gane E, Liaw YF *et al.* Telbivudine versus lamivudine in patients with chronic hepatitis B. *Hepatology* 2006; **44**: 222A.
32 Yuen MF, Fong DY, Wong DK, Yuen JC, Fung J, Lai CL. Hepatitis B virus DNA levels at week 4 of lamivudine treatment predict the 5-year ideal response. *Hepatology* 2007; **46**: 1695–703.
33 Takahashi K, Akahane Y, Hino K, Ohta Y, Mishiro S. Hepatitis B virus genomic sequence in the circulation of hepatocellular carcinoma patients: comparative analysis of 40 full-length isolates. *Arch Virol* 1998; **143**: 2312–26.

Journal of Medical Virology 82:684–691 (2010)

Clinical and Virological Effects of Long-Term (Over 5 Years) Lamivudine Therapy

Yoshimasa Hashimoto,[1]* Fumitaka Suzuki,[1] Miharu Hirakawa,[1] Yusuke Kawamura,[1] Hiromi Yatsuji,[1] Hitomi Sezaki,[1] Tetuya Hosaka,[1] Norio Akuta,[1] Masahiro Kobayashi,[1] Satoshi Saito,[1] Yoshiyuki Suzuki,[1] Mariko Kobayashi,[2] Yasuji Arase,[1] Kenji Ikeda,[1] and Hiromitsu Kumada[1]

[1]*Department of Hepatology, Toranomon Hospital, Minato-ku, Tokyo, Japan*
[2]*Department of Liver Research Institute for Hepatology, Toranomon Hospital, Minato-ku, Tokyo, Japan*

Ideally, long-term lamivudine therapy should not induce tyrosine–methionine–aspartate–aspartate (YMDD) mutants (reverse transcription [rt]; rt M204I/V) in patients with chronic hepatitis B. There is little or no information on the clinical features of patients who do not develop such mutants. We analyzed 368 patients who received lamivudine therapy for more than 6 months between 1995 and 2003. Among them, 98 patients were negative for YMDD mutants during 5-year lamivudine therapy. Multivariate analysis identified hepatitis B e antigen (HBeAg) negativity, lack of cirrhosis, and high gamma glutamyltranspeptidase (GGTP) level as independent factors associated with lack of emergence of YMDD mutants during 5-year treatment. In these 98 patients, 21 patients developed YMDD mutants in the 5-year posttreatment follow-up. Old age was identified as the only factor associated with the emergence of YMDD mutants during that period. For all patients, 53 showed no elevation of alanine aminotransferase (ALT) or viral load after emergence of YMDD mutants during 5 years. Short latency to emergence of YMDD mutants, mixed (tyrosine–isoleucine–aspartate–aspartate (YIDD) [rtM204I]+tyrosine–valine–aspartate–aspartate (YVDD) [rtM204V]) type, and low ALT level were identified as independent factors associated with elevation ALT or viral load. HBeAg negativity, lack of cirrhosis, and high GGTP level were associated with lack of emergence of YMDD mutants during 5-year period. Young age protected against emergence of YMDD mutants over the 5-year period. Moreover, after the emergence of YMDD mutants, short latency to the emergence of YMDD mutant, mixed type mutants, and low baseline ALT level were associated with elevation of ALT or viral load. ***J. Med. Virol. 82:684–691, 2010.***

KEY WORDS: YMDD mutant; HBV; lamivudine; GGTP; ALT; long-term

INTRODUCTION

Approximately 400 million people worldwide have chronic hepatitis B (CHB) infection, and 25–40% of these will develop hepatocellular carcinoma (HCC) and/or cirrhosis [Lee, 1997]. Prevention of disease progression is the primary target of treatment. To date, the nucleoside analogs, lamivudine, adefovir, dipivoxil, and entecavir, have been approved for the treatment of CHB [Zoulim and Perrillo, 2008]. Lamivudine markedly reduces viral load and hepatic necroinflammatory activity [Lai et al., 1998; Dienstag et al., 1999], and improves liver fibrosis [Dienstag et al., 2003a], and function. Unfortunately, failure of antiviral therapy is associated with the appearance of new viral variants, allowing hepatitis B virus (HBV) to become resistant. Lamivudine has the highest rate of drug resistance emergence. The number of patients with tyrosine–methionine–aspartate–aspartate (YMDD) mutation is higher with prolonged use of lamivudine. The cumulative rate of YMDD mutant reaches 60–70% after 4–5 years of treatment [Nafa et al., 2003; Suzuki et al., 2003]. On the other hand, 20–30% of patients continue long-term lamivudine therapy without YMDD mutations. There is little information at this stage about the

Abbreviations used: AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHB, chronic hepatitis B; DNA, deoxyribonucleic acid; GGTP, gamma glutamyltranspeptidase; HBeAg, hepatitis B e antigen; LC, liver cirrhosis; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; PCR, polymerase chain reaction; YIDD, tyrosine–isoleucine–aspartate–aspartate; YMDD, tyrosine–methionine–aspartate–aspartate; YVDD, tyrosine–valine–aspartate–aspartate.

Grant sponsor: Ministry of Health, Labor and Welfare, Japan (partial support).

*Correspondence to: Yoshimasa Hashimoto, MD, Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105 8470, Japan. E-mail: y_hashimoto5327@yahoo.co.jp

Accepted 3 September 2009
DOI 10.1002/jmv.21681
Published online in Wiley InterScience (www.interscience.wiley.com)

clinical differences between patients with and without YMDD mutants on long-term lamivudine therapy.

After the emergence of YMDD mutant, breakthrough hepatitis occurs at a high frequency. This is important because breakthrough hepatitis can occasionally cause liver decompensation [Liaw et al., 2000]. However, alanine aminotransferase (ALT) and viral load are not elevated at least in some patients with YMDD mutant. The difference between these groups remains poorly defined. The aims of the present investigation were the following: (1) characterize the clinical and virological features of patients who do not show emergence of YMDD mutants during 5 years of lamivudine therapy. (2) Identify the factor(s) associated with the emergence of YMDD mutants in patients on >5 years of lamivudine therapy. (3) Determine the factors associated with elevation of ALT (>50 IU/L) and viral load (>5.0 log copies/ml) after the emergence of YMDD mutant.

PATIENTS AND METHODS

Patients

The study subjects were 368 patients (66 females and 302 males, median age 43 years [range 19–76]) who commenced treatment with lamivudine at the Department of Hepatology, Toranomon Hospital, Tokyo, between September 1995 and June 2003 and adhered to treatment for more than 6 months (Table I). All patients were followed from commencement of therapy at our hospital. Some of these patients have been reported previously [Chayama et al., 1998; Suzuki et al., 2003]. All patients were negative for hepatitis C serologic markers, but all had detectable hepatitis B virus surface antigen (HBsAg) for at least 6 months prior to commencement of lamivudine therapy. Lamivudine was administered orally at 100 mg/day. Chronic hepatitis or cirrhosis was confirmed by needle biopsy, peritoneoscopy, or clinically before treatment. The clinical criteria for chronic hepatitis included elevated ALT levels over 6 months and absence of clinical evidence of portal hypertension, such as esophageal varices, ascites, hepatic encephalopathy, and imaging features suggestive of cirrhosis on ultrasonography. Chronic hepatitis and cirrhosis were diagnosed in 309 and 57, respectively. Informed consent was obtained from each patient enrolled in the study; and the study protocol conformed to the ethical guidelines of Declaration of Helsinki and was approved by the human research committee of our institution.

Blood Tests, Serum Viral Markers, and Assessment of Response to Therapy

Routine biochemical tests were performed before and during therapy at least once every 2 months, using standard procedures. Serial blood samples were taken before and during therapy and stored at −80°C until used for HBV molecular analysis. Viral load was measured by polymerase chain reaction (PCR)-based method (Amplicor HBV monitor; Roche Diagnostics, Tokyo, Japan). Mutation of the HBV deoxyribonucleic acid (DNA) polymerase gene (rtM204I/V) was determined using PCR and restriction fragment length polymorphism, as described previously [Chayama et al., 1998] or PCR-ELMA method [Kobayashi et al., 2000]. The presence of YMDD mutation was determined at baseline and at yearly intervals. Resistance to lamivudine was determined annually before the development of mutations, and, if a mutation appeared, the time of appearance of resistance was confirmed by monthly measurement.

Statistical Analysis

Differences between groups were examined for statistical significance using the χ^2 test for categorical variables and Mann–Whitney U-test for continuous variables. The association of mutations with specific

TABLE I. Characteristics of Patients at Commencement of Lamivudine Therapy

Demography	
Total patients	368
Sex: female/male	302/66
Age (years)	43 (19–76)
Family history of HBV	245 (66.6%)
Cirrhosis	57 (15.5%)
Median duration of treatment, years (range)	5 (0.5–12.8)
Laboratory data	
Aspartate aminotransferase (IU/L)	80 (19–2,593)
Alanine aminotransferase (IU/L)	120.5 (12–2,274)
Bilirubin (mg/dl)	0.7 (0.2–16.5)
Gamma glutamyltranspeptidase (IU/L)	64 (13–475)
Albumin (g/dl)	3.9 (2.1–4.8)
Viral load (log copies/ml)	7.1 (<2.7 to >7.6)
HBeAg positive	187 (50.8%)
HBV genotypes: A/B/C/D/F/unknown	12/25/317/1/2/11

HBV, hepatitis B virus; HBeAg, hepatitis B envelope antigen.
The family history of six patients was not clear. Viral load was measured by PCR. All viral load values below the lower limit of detection (<2.7 log copies) were set to 2 while those over the upper limit of detection (>7.6) ware set to 8 for calculation purposes.
Data are median and range values except for the last two parameters.

predictive variables was assessed by Cox proportional hazard model. To determine the factors that affect YMDD mutation, multiple logistic regression analysis was carried out. Spearman correlation coefficient (two-tailed) was used to evaluate the correlation between gamma-glutamyltranspeptidase (GGTP) and other factors. Two-tailed P-value <0.05 was considered statistically significant. All data were analyzed using the statistical package SPSS (version 11.0, SPSS, Inc., Chicago, IL).

RESULTS

Clinical and Virological Features of Patients Free of YMDD Mutations

Lamivudine therapy was provided for a median duration of 5 years [range 0.5–12.8 years]. Forty patients discontinued lamivudine therapy due to pregnancy, expectation of a change to another therapy, or loss to follow-up. Among the remaining 328 patients, YMDD mutants were identified in 230 patients during the 5-year treatment. Table II summarizes the characteristics of patients without and with YMDD mutant during the 5-year treatment. There were more patients with genotype B, and fewer patients with genotype A in the former than in the latter group ($P<0.001$). Furthermore, a high proportion of hepatitis B e antigen (HBeAg)-negative patients were noted in the former group than in the latter group ($P=0.001$). In the latter group, the emergence of YMDD mutant was associated with elevated ALT and/or viral load in 177 patients while it was not in 53 patients. On the other hand, 98 patients showed no emergence of YMDD mutants during the 5-year treatment (Fig. 1).

Figure 2 shows the cumulative rate of patients who showed emergence of YMDD mutations duringlamivudine therapy [129, 74, and 48 patients developed tyrosine–isoleucine–aspartate–aspartate (YIDD), tyrosine–valine–aspartate–aspartate (YVDD), and mixed (YIDD + YVDD) mutants, respectively]. YMDD mutants were registered in 11 (92%) of 12 patients with genotype A, 13 (52%) of 25 patients with genotype B, 219 (69%) of 317 patients with genotype C, 0 (100%) of 1 patients with genotype D, 2 (100%) of 2 patients with genotype F, and 6 (55%) of 11 patients with unidentified genotype.

We then explored the factors associated without emergence of YMDD mutants. Patients free of YMDD mutants were considered to have ideal response to lamivudine therapy. The following significant independent factors for the lack of YMDD mutations during the 5-year treatment were identified in univariate analysis: HBV genotype B, lack of cirrhosis, HBeAg negativity, free family history of liver disease, high aspartate aminotransferase (AST) level (>75 IU/L), high ALT level (>180 IU/L), high GGTP level (>110 IU/L), high albumin level (3.7 g/dl), and low viral load (<5.9 log copies/ml). Multivariate analysis identified HBeAg negativity, high GGTP level (>110 IU/L), and lack of liver cirrhosis (LC) as significant determinants for the lack of YMDD mutations during the 5-year treatment (Table III).

GGTP is regarded as a marker of fatty liver and alcoholic liver disease [Patton et al., 2008]. Fatty liver disease correlates with liver fibrosis and carcinogenesis [Yuan et al., 2004; Yu et al., 2008]. However, the influence of treatment with nucleos(t)ide analog is not clear. Next, we investigated the correlation between GGTP and other factors (Table IV). GGTP correlated significantly with ALT ($r=0.562$, $n=355$, $P<0.001$), AST ($r=0.562$, $n=355$, $P<0.001$), α-fetoprotein (AFP) ($r=0.430$, $n=319$, $P<0.001$), total bilirubin ($r=0.264$, $n=354$, $P<0.001$), and platelet count ($r=-0.129$, $n=330$, $P=0.019$). GGTP did not correlate with liver fibrosis ($r=-0.28$, $n=276$, $P=0.641$), total cholesterol ($r=-0.77$, $n=132$, $P=0.379$), or blood glucose ($r=0.118$, $n=115$, $P=0.355$) was. Based on the above results, GGTP correlated with ALT, AST, and other liver function-related parameters and does not seem to be related to other metabolic factors.

Among 163 patients who were positive for HBeAg at the commencement of lamivudine therapy, 35 (21%) did not show emergence of YMDD mutants during the 5-year treatment. Of these, 31 (89%) achieved HBeAg

TABLE II. Comparison of Patients With and Without YMDD Mutants During 5-Year Lamivudine Therapy

Category	Without YMDD mutation (n = 98)	With YMDD mutation (n = 230)	P-value
Age (years)[a]	43 (24–76)	44 (23–71)	0.783
Sex: male/female	77/21	194/36	0.206
Genotype: A/B/C/others	1/12/81/4	11/9/203/7	<0.001
Histology: chronic hepatitis/cirrhosis	88/10	185/43	0.052
Bilirubin (mg/dl)[a]	0.7 (0.2–12.2)	0.7 (0.2–16.5)	0.898
Alanine aminotransferase (IU/L)[a]	136 (16–2,077)	118.5 (14–2,274)	0.237
Gamma glutamyltranspeptidase (IU/L)[a]	72 (13–442)	58 (16–402)	0.197
Viral load (log copies/ml)[a]	7.1 (<2.7 to >7.6)	7.2 (<2.7 to >7.6)	0.136
HBeAg: positive/negative	35/63	128/102	0.001
Latency to emergence of YMDD mutation		2 (0–4.9)	

YMDD, tyrosine–methionine–aspartate–aspartate; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen.
Viral load was measured by PCR. All viral load values below the lower limit of detection (<2.7 log copies) were set to 2 and those over the upper limit of detection (>7.6) ware set to 8 for calculation purposes.
[a]Data are median (range) values.

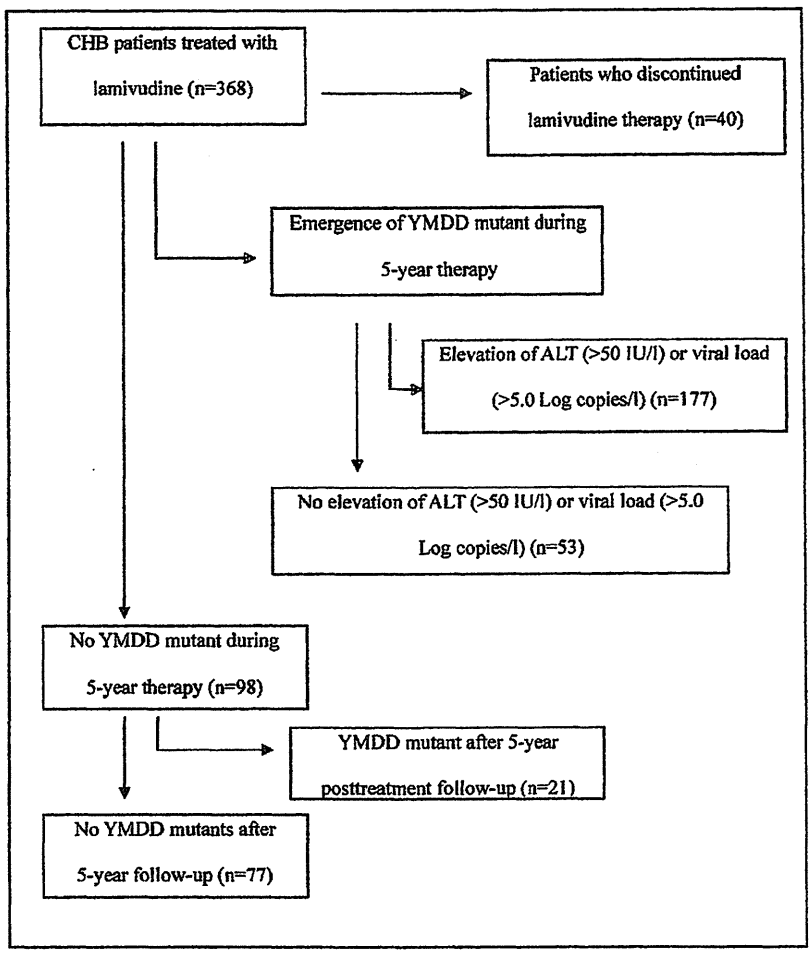


Fig. 1. Outcome of patients with lamivudine therapy. CHB, chronic hepatitis B; YMDD, tyrosine–methionine–aspartate–aspartate; ALT, alanine aminotransferase.

loss during 5-year treatment. On the other hand, in 128 patients who showed emergence of YMDD mutants, 42 (33%) achieved HBeAg loss. Analysis of various parameters showed that only the platelet count was different between the two HBeAg-positive groups; that is, in HBeAg-positive patients, those with high platelet counts were less likely to develop YMDD mutations ($P = 0.051$).

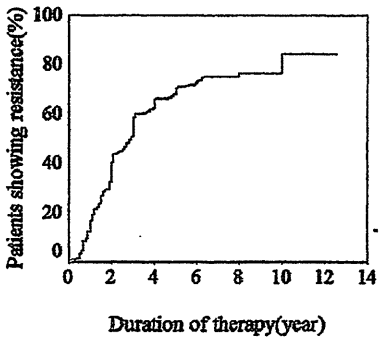


Fig. 2. Cumulative rate of patients who showed emergence of YMDD mutants during lamivudine therapy (Kaplan–Meier method). YMDD, tyrosine–methionine–aspartate–aspartate.

Emergence of YMDD Mutant After 5 Years of Lamivudine Therapy

As described above, 98 patients showed no emergence of YMDD mutants during the 5-year treatment. We investigated in this group the emergence of YMDD mutants after the 5-year treatment period. Twenty-one (21%) patients showed emergence of YMDD mutants following the completion of the 5-year treatment period (Table V). Univariate analysis showed only age (>50 years) influenced the emergence of the YMDD mutants after the 5-year treatment ($P = 0.012$). At time 5 years, 94 (96%) patients were negative for HBeAg. Therefore, the status of HBeAg at 5 years did not influence the emergence of YMDD mutant. After the emergence of YMDD mutant, 4 of the 21 patients had elevated ALT and viral load; they were further treated

TABLE III. Results of Multivariate Analysis of Factors Associated With Lack of Appearance of YMDD Mutants During 5-Year Lamivudine Therapy

Factors	Risk ratio (95% confidence interval)	*P*-value
Pretreatment HBeAg		
0: Positive	1	
1: Negative	2.492 (1.440–4.311)	0.001
Pretreatment GGTP (IU/L)		
0: <110	1	
1: ≥110	2.226 (1.296–4.900)	0.004
Pretreatment histology		
0: LC	1	
1: Not cirrhosis	2.254 (1.037–4.900)	0.04

YMDD, tyrosine–methionine–aspartate–aspartate; HBeAg, hepatitis B envelope antigen; GGTP, gamma glutamyltranspeptidase; LC, liver cirrhosis.

with a combination of adefovir and lamivudine. The remaining 17 patients showed no elevation of ALT or viral load, 3 of the 17 patients were treated with a combination of adefovir and lamivudine, treatment was switched in 6 of the 17 patients from lamivudine to entecavir, while 8 of the 17 patients continued lamivudine treatment. No emergence of YMDD mutant after the 5-year treatment period was noted in 77 patients, but 4 of 77 patients discontinued lamivudine therapy due to pregnancy, or loss to follow-up. Furthermore, treatment in 14 of the 77 patients was changed from lamivudine to entecavir while the remaining 59 patients continued lamivudine therapy.

Characteristics of Patients With Elevated ALT or Viral Load After Emergence of YMDD Mutant

As mentioned above, 230 (62.5%) of the 368 patients developed YMDD mutations during the 5-year treatment period, and 177 had elevated ALT or viral load level after the emergence of YMDD mutants, while 53 patients had neither ALT elevation (>50 IU/L) nor HBV DNA elevation (>5.0 log copies/ml) during the treatment period. We then explored the risk factors for the elevation of viral load and ALT level in these patients. In univariate analysis, the following seven factors correlated significantly with elevation of viral load or ALT level: HBeAg ($P < 0.001$), latency to emergence of YMDD mutant ($P < 0.001$), mixed type YMDD mutant (YIDD + YVDD) ($P < 0.001$), ALT level ($P = 0.003$), viral load ($P = 0.007$), and AFP level ($P = 0.021$). These variables were entered into multivariate analysis. In the last step of the analysis, the following three variables were identified as significant determinants of elevation of viral load or ALT level: latency to emergence of YMDD mutant ($P < 0.001$), mixed type YMDD mutant ($P < 0.001$), and ALT level ($P = 0.016$) (Table VI).

TABLE IV. Correlation Between GGTP and Laboratory Tests

Factors	r	n	*P*-value
ALT	0.562	355	<0.001
AST	0.562	355	<0.001
AFP	0.43	319	<0.001
Total bilirubin	0.264	354	<0.001
Platelet count	−0.219	330	0.019
Liver fibrosis	−0.28	276	0.641
Total cholesterol	−0.77	132	0.379
Blood glucose	0.118	115	0.355

ALT, alanine aminotransferase; AST, aspartate aminotransferase; AFP, α-fetoprotein.

Characteristics of Patients With YMDD Mutant During and After 5-Year Treatment

Throughout the present study, YMDD mutant developed in 251 patients. As described above, 230 of these 251 patients developed YMDD mutant during the 5-year period. The remaining 21 patients developed YMDD mutant after the 5-year period. Table VII summarizes the characteristics of patients with YMDD mutant during and after the 5-year treatment. HBV genotype and HBeAg negativity were found to correlate with the development of YMDD mutants after 5-year treatment.

DISCUSSION

The aim of the present study was to identify the factors associated with the lack of emergence of YMDD mutant during long-term lamivudine therapy. Our analysis showed that negativity for HBeAg, high GGTP level (≥110 IU/L), and lack of LC protected against the appearance of YMDD mutants during the 5-year lamivudine therapy. Since positivity for HBeAg is a well-known factor associated with emergence of YMDD mutant [Yuen et al., 2001; Suzuki et al., 2003], we focused on the correlation between GGTP and other factors (Table IV). The results showed that GGTP correlated with ALT, AST, and other liver function-related parameters. Previous studies identified high pretreatment ALT level as an independent factor associated with no appearance of YMDD mutant [Tsubota et al., 2004; Chang et al., 2005]. In this regard, GGTP is regarded as a marker of fatty liver and alcoholic liver disease [Patton et al., 2008]. Fatty liver disease correlates with liver fibrosis and carcinogenesis [Yuan et al., 2004; Yu et al., 2008]. However, the influence of treatment with nucleos(t)ide analog is not clear. Based on the above results, GGTP does not seem to be related to other metabolic factors (e.g., total cholesterol and blood glucose). However, further investigation of other

TABLE V. Comparison of Clinicopathological Features of Patients With and Without Emergence of YMDD Mutants After 5-Year Posttreatment Follow-Up

Category	Without YMDD mutation (n=77)	With YMDD mutation (n=21)	*P*-value
Age (years)[a]	42 (24–76)	50 (33–69)	0.032
Sex: male/female	63/14	14/7	0.134
Genotype: A/B/C/others	1/8/65/3	0/4/16/1	0.275
Histology: no cirrhosis/cirrhosis[b]	69/8	19/2	0.636
Bilirubin (mg/dl)[a]	0.7 (0.2–12.2)	0.7 (0.4–4.4)	0.644
ALT (IU/L)[a]	135 (16–1,975)	142 (25–2,077)	0.997
GGTP (IU/L)[a]	69 (13–442)	82 (24–264)	0.878
Viral load (log copies/ml)[a]	7 (<2.7 to >7.6)	7.4 (<2.7 to >7.6)	0.394
HBeAg: positive/negative	28/49	7/14	0.797
Status of HBeAg at 5 years: positive/negative	3/74	1/20	0.860
Latency to emergence of YMDD mutation		5.6 (5–10)	

YMDD, tyrosine–methionine–aspartate–aspartate; ALT, alanine aminotransferase; GGTP, gamma glutamyltranspeptidase; HBV, hepatitis B virus; CHB, chronic hepatitis B; HBeAg, hepatitis B envelope antigen.
[a]Data are median (range) values.
[b]Chronic hepatitis and cirrhosis were confirmed by needle biopsy, peritoneoscopy, or clinically before treatment. Diagnosis of chronic hepatitis was based on elevated ALT levels over 6 months and absence of clinical evidence of portal hypertension, such as esophageal varices, ascites, hepatic encephalopathy, and imaging features suggestive of cirrhosis on ultrasonography. Viral load was measured by PCR. All viral loads below the lower limit of detection (<2.7 log copies) were set to 2 and those over upper limit of detection (>7.6) were set to 8 for calculation purposes.

metabolic factors is needed, such as body mass index, HOMA-IR, and alcohol intake. The third factor, lack of liver fibrosis and cirrhosis based on histopathological examination, was associated with lack of YMDD mutants. Previous study reported that the presence of cirrhosis correlated with emergence of YMDD mutant [Ooga et al., 2004]. Moreover, among patients with LC, those who develop YMDD mutants are more likely to have high Child-Pugh scores than those without such mutants [Liaw et al., 2004]. On the other hand, viral load has been reported to promote the emergence of YMDD mutants [Yuen et al., 2001]. In the present study, although viral load was identified as a factor in univariate analysis, it was not identified as such in multivariate analysis.

We performed additional investigation on elevation of ALT or viral load after the emergence of YMDD mutants. In this analysis, 77% of these patients (n=177) had elevated ALT or viral load, while 23% (n=53) had not. We found several common characteristics among patients of the high ALT/viral load group. The latency to emergence of YMDD mutants and mixed type mutants (YIDD+YVDD) were significant factors in this group. Early emergence of YMDD mutant could reflect a rapid increase of HBV DNA. The mixed type was reported as a risk factor of HBV DNA breakthrough and breakthrough hepatitis [Akuta et al., 2003; Suzuki et al., 2006]. Previous studies reported that a low pretreatment ALT was an independent factor associated with appearance of YMDD mutants [Tsubota et al., 2004; Chang et al., 2005]. Based on the above findings, patients with low baseline ALT level and during treatment emergence of YMDD mutants seem to be at high risk of breakthrough hepatitis.

Younger patients had less opportunity to develop YMDD mutations after the 5-year treatment. We reported previously that age was not associated with emergence of YMDD mutant [Kawaoka et al., 2007]. However, the duration of treatment in our previous study was <5 years. Patients free of YMDD mutants during the 5-year treatment might have adequate immune response to suppress the development of YMDD mutants. The immune response is lower in elderly patients [Adler and Nagel, 1994; Marcus and Tur-Kaspa, 1997]. Considered together, younger patients seem to be more immune against the emergence

TABLE VI. Factors Associated With Elevation of ALT or Viral Load After Emergence of YMDD Mutant

Factors	Hazard ratio (95% confidence interval)	*P*-value
Latency to emergence of YMDD mutant		
0: ≥1 year	1	
1: <1 year	7.429 (4.769–11.572)	<0.001
YMDD mutant type		
0: YIDD or YVDD	1	
1: Mixed (YIDD+YVDD) type	2.939 (1.834–4.677)	<0.001
Pretreatment ALT level (IU/L)		
0: >160	1	
1: ≤159	1.583 (1.089–2.301)	0.016

YMDD, tyrosine–methionine–aspartate–aspartate; ALT, alanine aminotransferase; HBV, hepatitis B virus; YIDD, tyrosine–isoleucine–aspartate–aspartate; YVDD, tyrosine–valine–aspartate–aspartate.

TABLE VII. Comparison of Clinicopathological Features of Patients With YMDD Mutants During and After 5-Year Treatment Period

Category	With YMDD mutation during 5-year (n = 230)	With YMDD mutation after 5-year (n = 21)	*P*-value
Age (years)[a]	44 (23–71)	50 (33–69)	0.109
Sex: male/female	194/36	14/7	0.063
Genotype: A/B/C/others	11/9/203/7	0/4/16/1	0.0184
Histology: no cirrhosis/cirrhosis[b]	185/43	19/2	0.384
Bilirubin (mg/dl)[a]	0.7 (0.2–16.5)	0.7 (0.4–4.4)	0.570
ALT (IU/L)[a]	118.5 (14–2,274)	142 (25–2,077)	0.527
GGTP (IU/L)[a]	58 (16–402)	82 (24–264)	0.382
Viral load (log copies/ml)[a]	7.2 (<2.7 to >7.6)	7.4 (<2.7 to >7.6)	0.936
HBeAg: positive/negative	128/102	7/14	0.0496

YMDD, tyrosine–methionine–aspartate–aspartate; ALT, alanine aminotransferase; GGTP, gamma glutamyltranspeptidase; HBV, hepatitis B virus; CHB, chronic hepatitis B; HBeAg, hepatitis B envelope antigen.
[a]Data are median (range) values.
[b]Chronic hepatitis and cirrhosis were confirmed by needle biopsy, peritoneoscopy, or clinically before treatment. Diagnosis of chronic hepatitis was based on elevated ALT levels over 6 months and absence of clinical evidence of portal hypertension, such as esophageal varices, ascites, hepatic encephalopathy, and imaging features suggestive of cirrhosis on ultrasonography. Viral load was measured by PCR. All viral loads below the lower limit of detection (<2.7 log copies) were set to 2 and those over upper limit of detection (>7.6) were set to 8 for calculation purposes.

of YMDD mutant in long-term lamivudine treatment than elderly patients.

Several new nucleos(t)ide analogs, for example, adefovir and entecavir, are available at present [Gish et al., 2007; Marcellin et al., 2008]. These new drugs have greater inhibitory effects on HBV replication and their use is associated with a lower incidence of drug resistance. However, resistant to the new drugs has already been reported [Suzuki et al., 2007; Baldick et al., 2008]. Lamivudine was the first nucleoside analog and has been used over a long time worldwide. Based on the result of our study, younger patients (<50 years) who continued lamivudine monotherapy without emergence of YMDD mutant during 5-year period showed less opportunity to develop mutants after a 5-year follow-up and were able to continue lamivudine monotherapy. After the cessation of lamivudine therapy, flare up of ALT accompanied with elevation of HBV DNA was observed at a high frequency [Song et al., 2000; Dienstag et al., 2003a; Akuta et al., 2005]. Moreover, we reported previously HBsAg clearance from the serum in some patients who received long-term lamivudine therapy [Kobayashi et al., 2007]. Taken together, it seems that before any treatment, one can predict a less likelihood of development of YMDD mutants during long-term lamivudine therapy in young patients with genotype C who are HBeAg negative, have no cirrhosis, and no elevated GGTP level. Tables II and VII suggest that patients with genotype B are also less likely to develop YMDD mutant, but their numbers are too small to make a firm conclusion. Further studies of larger number of patients with genotype B, A, and others are needed to clarify this issue.

In conclusion, factors associated with lack of appearance of YMDD mutants during 5-year lamivudine therapy in patients with HBV infection are HBeAg negativity, lack of cirrhosis, and high GGTP level. Patients who do not show the emergence of YMDD mutants during 5-year lamivudine therapy, younger age protected against the emergence of such mutants during the following 5 years of follow-up. On the other hand, in those who show emergence of YMDD mutant, elevation of ALT or viral load correlate with a short latency to emergence of YMDD mutants, presence of mixed (YIDD + YVDD) type, and low baseline ALT level.

REFERENCES

Adler WH, Nagel JE. 1994. Clinical immunology and aging. In: Hazzard WR, Bierman EL, Blass JP, Ettinger WH, Halter JB, editors. Principles of geriatric medicine and gerontology. 3rd edition. New York: McGraw-Hill, Inc. pp 61–75.

Akuta N, Suzuki F, Kobayashi M, Matsuda M, Sato J, Takagi K, Tsubota A, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2003. Virological and biochemical relapse according to YMDD motif mutant type during long-term lamivudine monotherapy. J Med Virol 71:504–510.

Akuta N, Suzuki F, Kobayashi M, Matsuda M, Sato J, Suzuki Y, Sezaki H, Hosaka T, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2005. Virological and biochemical relapse after discontinuation of lamivudine monotherapy for chronic hepatitis B in Japan: Comparison with breakthrough hepatitis during long-term treatment. Intervirology 48:174–182.

Baldick CJ, Tenney DJ, Mazzucco CE, Eggers BJ, Rose RE, Pokornowski KA, Yu CF, Colonno RJ. 2008. Comprehensive evaluation of hepatitis B virus reverse transcriptase substitutions associated with entecavir resistance. Hepatology 47:1473–1482.

Chang ML, Chien RN, Yeh CT, Liaw YF. 2005. Virus and transaminase levels determine the emergence of drug resistance during long-term lamivudine therapy in chronic hepatitis B. J Hepatol 43:72–77.

Chayama K, Suzuki Y, Kobayashi M, Kobayashi M, Tsubota A, Hashimoto M, Miyano Y, Koike H, Kobayashi M, Koida I, Arase Y, Saitoh S, Murashima N, Ikeda K, Kumada H. 1998. Emergence and takeover of YMDD motif mutant hepatitis B virus during longterm lamivudine therapy and re-takeover by wild type after cessation of therapy. Hepatology 27:1711–1716.

Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HWL, Goodman Z, Crowther L, Condreay LD, Woessner M, Rubin M, Brown MA, for the US Lamivudine Investigator Group. 1999. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 341:1256–1263.

Dienstag JL, Goldin RD, Heathcote EJ, Hann HWL, Woessner M, Stephenson SL, Gardner S, Gray DF, Schiff ER. 2003a. Histological outcome during long-term lamivudine therapy. Gastroenterology 124:105–117.

Dienstag JL, Cianciara J, Karayalcin S, Kowdley KV, Willems B, Plisek S, Woessner M, Gardner S, Schiff E. 2003b. Durability of serologic response after lamivudine treatment of chronic hepatitis B. Hepatology 37:748–755.

Gish RG, Lok AS, Chang TT, de Man RA, Gadano A, Sollano J, Han KH, Chao YC, Lee SD, Harris M, Yang J, Colonno R, Brett-Smith H. 2007. Entecavir therapy for up to 96 weeks in patients with HBeAg-positive chronic hepatitis B. Gastroenterology 133:1437–1444.