45 **Abstract**

Lamivudine (LAM) is a widely used nucleoside analogue for treatment of chronic hepatitis B virus (HBV) infection. Emergence of resistant strains with amino acid substitutions in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of reverse
50 transcriptase is a serious problem in patients on LAM therapy. The amount of covalently closed circular DNA (cccDNA) in the serum is reported to be higher in patients who develop YMDD mutants than those without mutants. However, there is no useful serum marker that can predict early emergence of mutants during LAM therapy. Analysis of patients who were treated with entecavir (ETV, n=7) and LAM
55 (n=36) showed high levels of HBV RNA in sera of some patients. Serum median levels of HBV-RNA were significantly higher in patients with emergence of YMDD mutants within one year (n=6, 1.688 LOG copies/ml) than those who showed emergence after one year of treatment (n=12, 0.456, P=0.0125) or without emergence (n=18, 0.688, P=0.039). Our results suggest that HBV-RNA is a valuable predictor of
60 early occurrence of viral mutation during LAM therapy.

Word count: 168 words

Introduction

65 Hepatitis B virus (HBV) is a member of hepadnaviridae family. Worldwide, approximately 350 million people are estimated to be chronically infected with HBV.[1] Patients with chronic HBV infection develop chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, accounting for approximately one million deaths per year.[2] Recently, inhibitors of reverse transcriptase have been developed and widely used for 70 patients with chronic HBV infection. Lamivudine (LAM), a cytosine nucleoside analogue, was first developed as an anti-viral agent against human immunodeficiency virus (HIV) and later was used effectively against HBV, since HBV also uses reverse transcriptase for replication.[3, 4] Because LAM suppresses HBV replication, patients who are treated with LAM show a decrease in or disappearance of serum HBV-DNA 75 and hepatitis B e antigen, normalization of serum alanine transaminase (ALT) levels and histological improvement.[5-12] However, discontinuation of therapy often leads to reactivation of HBV.[6, 8, 13, 14] Therefore long-term therapy is necessary for many patients with chronic HBV infection. During long-term LAM therapy, drug resistant mutants with amino acid substitutions in the tyrosine-methionine-aspartate-aspartate 80 (YMDD) motif emerge, resulting in re-increase in HBV-DNA levels and worsening of hepatitis.[6, 10, 15-18] Moreover, some patients develop a severe flare-up of hepatitis, which could lead to fatal hepatic failure. Therefore, the prediction of emergence of YMDD mutants is an important issue.

In our hunt for useful serum markers to detect early emergence of YMDD 85 mutants, we noticed some patients who show a discrepancy between HBV-DNA

values measured by transcription-mediated amplification and hybridization protection assay (TMA-HPA) and Amplicor HBV Monitor test. Since the former method detects both HBV-DNA and HBV-RNA, we thought that the difference between values measured by the two methods is due to the presence of a large amount of
90 HBV-RNA.[19-21] We thus studied patients with chronic HBV infection who were treated with LAM or entecavir (ETV) for presence of HBV-RNA. We also assumed that the presence of a large amount of HBV-RNA indicates that transcription and virus particle formation is still active in such patients. We thus assessed the value of this indicator in the prediction of emergence of YMDD mutants during LAM therapy.

95

Material and Methods

Patients

We studied 36 patients with chronic hepatitis B, who were treated by LAM at
100 Hiroshima University Hospital, Kawakami Clinic and Hiroshima Red Cross Hospital and Atomic Bomb Survivors Hospital from 2001 to 2006. We also analyzed seven patients who were treated by ETV at Hiroshima University Hospital from 2004 to 2006. No patients showed clinical signs of liver cirrhosis or hepatocellular carcinoma. They were not treated by other antiviral agents, corticosteroids or immunosuppressant
105 drugs during LAM/ETV therapy. The LAM treated patients consisted of 25 men and 11 women with a median age of 52 years (27-68 years) (Table). They were divided into three groups (groups A, B and C) according to the period before the appearance of YMDD mutants. Group A (n=6) consisted of patients who showed early emergence

(within one year) of the mutants. Group B (n=12) consisted of patients who developed
110 resistance after one year of LAM therapy. Group C (n=18) consisted of patients who did not show resistance to LAM therapy. Each of the 36 patients received 100 mg of daily LAM for 4-58 months (median, 21.5 months). All patients continued LAM therapy throughout the course of the study. Patients of the ETV group were six men and one woman with a median age of 37 years (32-50 years). They received 0.01-0.5
115 mg ETV daily for 21-28 months (median, 25 months), and all patients continued ETV therapy throughout the course of the study. Blood samples were obtained from patients of both groups just before the commencement of antiviral therapy and every 4 weeks during therapy. Informed consent was obtained from each patient.

120 *Quantification of HBV-DNA*

The serum level of HBV-DNA was determined by using TMA-HPA (Fujirebio Inc., Tokyo, Japan) and Amplicor HBV monitor test (Roche Diagnostics, Tokyo). The measurement range of the former assay was $10^{3.7}$-$10^{8.7}$ genome equivalents [GE]/ml (3.7-8.7 LGE/ml)[22] while the range of the latter test was $10^{2.6}$-$10^{7.6}$ copies/ml (2.6-7.6
125 log copies/ml).[23] These quantitative assays of HBV-DNA were performed at the Special Reference Laboratory, Tokyo.

Extraction of nucleic acid of HBV and reverse transcription (RT)

Nucleic acid was extracted from 100 µL serum by SMITEST (Genome Science
130 Laboratories, Tokyo) and dissolved in 20 µL H_2O for DNA analysis or 8.8 µL ribonuclease-free H_2O for RNA analysis. The latter solution was reverse transcribed

by using Random Primer (Takara Bio Inc., Shiga, Japan) and M-MLV Reverse Transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan). In the next step, 25 pM of Random Primer was added to 8.8 μL nucleic acid extract and heated at 65°C for 5
135 min. The samples were set on ice for 5 min. Then, 4 μL of 5×RT Buffer, 2 μL of 10 mM dNTPs, 2 μL of 0.1 M dithiothreitol (DTT), 8 units of ribonuclease inhibitor and 100 units of M-MLV Reverse Transcriptase were added to each sample. The reaction mixture was incubated at 30°C for 10 min and 42°C for 60 min, followed by inactivation at 99°C for 5 min.

140

Quantitative analysis of HBV DNA by real-time polymerase chain reaction (PCR)

One μL of DNA solution or cDNA solution was amplified by real-time PCR with an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the instructions provided by the manufacturer. Amplification was performed in a 25 μL
145 reaction mixture containing SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 200 nM of forward primer (5'-TTTGGGGCATGGACATTGAC-3', nucleotides 1893-1912), 200 nM of reverse primer (5'-GGTGAACAATGGTCCGGAGAC, nucleotides 2029-2049) and 1 μL of DNA or cDNA solution. After incubation for 2 min at 50°C, the sample was heated for 10 min at 95°C for denaturing, followed by PCR cycling
150 program consisting of 40 two-step cycles of 15 s at 95°C and 60 s at 60°C. The lower detection limit of this assay was 103 copy/ml.

Confirmation of presence of HBV-RNA in serum by RNase digestion

To confirm the presence of HBV-RNA, nucleic acids extracted from the serum
155 samples by SMITEST (Genome Science Laboratories, Tokyo) were digested with 1 μg/μL of RNase A (Wako Pure Chemical Industries, Osaka, Japan) at 37 °C for 60 min, digested with proteinase K (New England Biolabs Inc., Ipswich, MA) at 37°C for 60 min, extracted with phenol/chloroform, precipitated with ethanol and dissolved in water. Treated nucleic acids with or without RNase were analyzed by real-time PCR
160 after reverse transcription with a random primer and reverse transcriptase, as described above.

Detection of YMDD mutant

Mutations in the YMDD motif of reverse transcriptase of HBV were examined by
165 PCR with peptide nucleic acid clamping, as described previously.[24]

Statistical analysis

Differences between groups were examined for statistical significance using the Student's *t*-test and correlations between parameters were examined by the
170 Spearman's rank correlation. A P value less than 0.05 denoted the presence of a statistically significant difference. All statistical analyses were performed with StatView version 5.0 (SAS Institute, Cary, NC).

Results

175

HBV-DNA levels determined by TMA-HPA and Amplicor HBV Monitor test during ETV therapy

The presence of a large amount of HBV-RNA was initially noticed by measuring HBV-nucleic acid with TMA-HPA and HBV-DNA with Amplicor HBV monitor test.
180 As shown in Fig. 1, the amount of HBV-nucleic acid was higher than HBV-DNA during the initial six months of ETV therapy. We assumed that the discrepancy in the results of these two methods is due to the presence of a large amount of HBV-RNA in the serum because TMA-HPA measures both HBV-DNA and HBV-RNA while the Amplicor HBV monitor test detects only HBV-DNA. We measured the HBV-nucleic
185 acid levels in the seven patients who received ETV therapy at three and six months from the start of therapy. The HBV-nucleic acid levels determined by TMA-HPA were higher than those determined by Amplicor HBV Monitor test by 10-100 times in all seven patients except for two patients who received a small amount (0.01 mg) of ETV (Fig. 2). The small difference in these patients is probably due to the small effect
190 of the small amount of ETV.

Comparisons of HBV-nucleic acid values and HBV-DNA determined by four measurement methods, TMA-HPA, Amplicor Monitor test, in house real-time PCR assay and RT-real-time PCR in patients treated with ETV

195 We measured HBV DNA by in house real-time PCR and HBV-nucleic acid by RT-real-time PCR using serum samples obtained from the patients at 3 and 6 months of ETV therapy and compared these values with those obtained by TMA-HPA and Amplicor monitor test. HBV-DNA determined by real-time PCR correlated well with

those values obtained by Amplicor HBV Monitor test (r = 0.968, P <0.0001) (Fig. 3a),
200 but not with HBV-nucleic acid determined by TMA-HPA (r = 0.210, P = 0.5608) (Fig. 3b). The HBV-DNA levels determined by in house real-time PCR assay were $10^{1.5}$-10^{2} higher than those by Amplicor HBV Monitor test. We confirmed the accuracy of our assay using limiting dilution and detection with nested PCR assay. When we diluted standard samples used in our in house assay to one copy/μl, we
205 detected them by nested PCR using 1 μl of such samples. Three of the 10 (30%) samples tested positive by nested PCR. We thus conclude that our assay accurately measure the amount of HBV-DNA in serum.

To examine if the measurement of TMA-HPA reflects the total amount of HBV-RNA and HBV-DNA in serum samples, we performed RT real-time PCR using
210 serum samples obtained from patients at 3 and 6 months of ETV therapy. In contrast to the values determined by real-time PCR without RT, the measurement of HBV nucleic acid determined by RT-PCR did not correlate well with values obtained by Amplicor HBV Monitor test (r = 0.359, P = 0.2790) (Fig. 3c), although they correlated well with TMA-HPA (r = 0.955, P <0.0001) (Fig. 3d). These results show that
215 TMA-HPA measures both HBV-DNA and HBV-RNA in serum. <u>To further confirm the presence of HBV-RNA, we digested three nucleic acid samples, arbitrarily picked up from serum samples obtained from patients treated by lamivudine for three months, by RNase A. As shown in Figure 4, RNase treatment reduced the amount of HBV-DNA detected by RT-real-time PCR to about 1/100.</u>

220

HBV-DNA levels determined by TMA-HPA and Amplicor HBV Monitor test during LAM therapy

We then investigated the levels of HBV-DNA in serum samples obtained from 36 patients at 3 and 6 months of LAM therapy. In some patients, HBV-DNA was already
225 negative at 3 and 6 months of therapy (Fig. 5). Similar to the results obtained from patients treated with ETV, comparisons of values obtained from patients who showed measurable HBV-DNA levels revealed that HBV nucleic acid levels determined by TMA-HPA tended to be higher than those determined by Amplicor HBV Monitor test (Fig. 4).

230

Comparisons of HBV-nucleic acid values and HBV-DNA determined by four measurement methods, TMA-HPA, Amplicor Monitor test, in house real-time PCR assay and RT-real-time PCR in patients treated with LAM

We measured HBV-nucleic acid and HBV DNA by the above four methods and
235 investigated the correlations between them at 3 and 6 months of LAM therapy (Fig. 6) HBV-DNA levels determined by real-time PCR correlated better with Amplicor HBV Monitor test ($r = 0.653$, $P = 0.0083$) (Fig. 6a) than with TMA-HPA ($r = 0.456$, $P = 0.1173$) (Fig. 6b). Similarly, measurement of HBV nucleic acid by RT PCR did not correlate well with values obtained by Amplicor HBV Monitor test (Fig. 6c), but
240 showed better correlation with those obtained by TMA-HPA (Fig. 6d) ($r = 0.452$, $P = 0.0907$ and $r = 0.675$, $P = 0.0114$, respectively). These results also show that TMA-HPA detects both HBV-RNA and HBV-DNA.

HBV-RNA in serum at three month of LAM therapy is higher in patients who showed

245 *early emergence of YMDD mutants*

In LAM-treated patients, it is assumed that a high serum level of HBV-RNA is a marker of the active transcription form of covalently closed circular DNA (cccDNA) and packaging of HBV-RNA in the liver. We assumed that YMDD mutants easily emerge under such condition. We compared HBV-RNA values (HBV nucleic acid

250 determined by RT-real-time PCR minus HBV-DNA determined by real-time PCR) in patients who showed early emergence of mutants (within 12 months) with those who showed late emergence of mutants (more than 12 months) and those without emergence of mutants (Table). As shown in Fig. 7, HBV-RNA levels were significantly higher in patients who showed early emergence of mutants than the other

255 two groups at 3 months of LAM therapy. There was no significant difference in the amount of HBV-RNA between group A (patients who showed emergence of mutants within 12 months) and the other two groups at the beginning of LAM therapy (data not shown).

260

260 **DISCUSSION**

In this study, we addressed the question of discrepancy between the two measurements of HBV nucleic acid; TMA-HPA and Amplicor. The presence of HBV-RNA in serum samples of patients with HBV infection has been reported
265 previously.[19-21] Since TMA-HPA utilizes RNA transcription and amplification of transcripts by T7 RNA polymerase,[22] we assumed that the discrepancy is due to the presence of HBV-RNA in the serum of LAM- and ETV-treated patients. The presence of HBV-RNA in a patient treated with LAM has been reported previously.[21] In their report, the authors mainly analyzed truncated HBV-RNA, which they assumed
270 transcribed from the integrated genome.[20, 21] They showed a large difference between HBV-DNA and truncated HBV-RNA, which did not decrease during LAM therapy. We also detected HBV-DNA and HBV-nucleic acid by real-time PCR and RT-real-time PCR. The values determined by these two methods only showed less than one log difference (data not shown); we assume that the effect of truncated
275 HBV-RNA in serum is only minimal in our study. As we demonstrated in this study, HBV-nucleic acid measured by RT-real-time PCR correlated with those determined by TMA-HPA. This finding suggests that the discrepancy between values measured TMA-HPA and Amplicor Monitor test is based on the presence of HBV-RNA in the serum.

280 We showed that a large amount of HBV-RNA in the serum was produced during the early stage of ETV (Fig. 1) and LAM treatments (within six months). Since ETV and LAM simply work on reverse transcription, it is difficult to conceive that the

level of transcription from the cccDNA was altered by these drugs. Thus, the slow decrease in HBV-RNA seems to reflect the fact that certain amount of cccDNA still exists in the liver and that the virus replication machinery is still actively operational. This is consistent with previous reports which showed that the amount of cccDNA in the liver tissues [25, 26] and in serum,[26] which correlated well with intrahepatic cccDNA,[27] reflected the effect LAM and is a marker for cessation of therapy without re-increase of the virus after stopping the therapy.

Whether a large amount of HBV-RNA originates from a large amount of cccDNA template in hepatocytes or active transcription (or both) is actually unknown. However, it is assumed that the probability of developing mutants is high in patients who have large amounts of HBV-RNA. We thus analyzed the amount of HBV-RNA in patients treated with LAM and compared them among patients who showed an early emergence of mutants and those who did not. As expected, the amount of HBV-RNA in the serum was significantly higher in patients who showed early emergence of mutants than those who showed late emergence and those who did not show emergence of mutants.

Using complex analysis, previous studies identified several predictive factors of emergence of YMDD mutants such as HBV genotype,[28] ALT levels,[29, 30] HBV-DNA levels before therapy,[28, 30-32] the degree of decline of HBV-DNA levels during therapy,[33, 34]~~46-48~~ presence of hepatitis B e antigen,[17, 29, 31, 32, 35] presence of core promoter mutations,[36] deletion of pre-S region[37] and HBV core-related antigen.[38] We also showed that a slow decrease in HBV-nucleic acid measured by TMA-HPA is a marker of early emergence of mutants. Our finding is important because this assay is

routinely used in daily clinical practice. However, the results did not reach statistical significance probably due to the small number of patients analyzed in our study and low sensitivity of the assay (detection limit 3.7 log copy/ml). We assume that a sensitive measurement of HBV-RNA is useful for the prediction of emergence of
310 mutants. Development of such assay is needed for proper treatment of patients using different nucleotide and nucleoside analogues. Mechanisms that control transcription of HBV from cccDNA deserve further investigation to develop more effective therapies for HBV infection.

References

1. Maddrey WC. Hepatitis B: an important public health issue. J Med Virol 2000;61:362-366
2. Beasley RP. Hepatitis B virus. The major etiology of hepatocellular carcinoma. Cancer 1988;61:1942-1956
3. Chang CN, Skalski V, Zhou JH, Cheng YC. Biochemical pharmacology of (+)-and (-)- 2', 3'-dideoxy-3'-thiacytidine as anti-hepatitis B virus agents. J Biol Chem 1992;267:22414-22420
4. Benhamou Y, Dohin E, Lunel-Fabiani F, Poynard T, Huraux JM, Katlama C, Opolon P, et al. Efficacy of lamivudine on replication of hepatitis B virus in HIV-infected patients. Lancet 1995;345:396-397
5. Dienstag JL, Perrillo RP, Schiff ER, Bartholomew M, Vicary C, Rubin M. A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med 1995;333:1657-1661
6. Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 1998;339:61-68
7. Suzuki Y, Kumada H, Ikeda K, Chayama K, Arase Y, Saitoh S, Tsubota A, et al. Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. J Hepatol 1999;30:743-748

335 8. Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, Crowther L, et al. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1999;341:1256-1263

9. Dienstag JL, Schiff ER, Mitchell M, Casey DE, Jr., Gitlin N, Lissoos T, Gelb LD, et al. Extended lamivudine retreatment for chronic hepatitis B: maintenance of
340 viral suppression after discontinuation of therapy. Hepatology 1999;30:1082-1087

10. Liaw YF, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Chien RN, et al. Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. Gastroenterology 2000; 119:172-180

11. Suzuki Y, Arase Y, Ikeda K, Saitoh S, Tsubota A, Suzuki F, Kobayashi M, et al.
345 Histological improvements after a three-year lamivudine therapy in patients with chronic hepatitis B in whom YMDD mutants did not or did develop. Intervirology 2003;46:164-170

12. Dienstag JL, Goldin RD, Heathcote EJ, Hann HW, Woessner M, Stephenson SL, Gardner S, et al. Histological outcome during long-term lamivudine therapy.
350 Gastroenterology 2003;124:105-117

13. Nevens F, Main J, Honkoop P, Tyrrell DL, Barber J, Sullivan MT, Fevery J, et al. Lamivudine therapy for chronic hepatitis B: a six-month randomized dose-ranging study. Gastroenterology 1997;113:1258-1263

14. Song BC, Suh DJ, Lee HC, Chung YH, Lee YS. Hepatitis B e antigen
355 seroconversion after lamivudine therapy is not durable in patients with chronic hepatitis B in Korea. Hepatology 2000;32:803-806

15. Ling R, Mutimer D, Ahmed M, Boxall EH, Elias E, Dusheiko GM, Harrison TJ. Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. Hepatology 1996;24:711-713

360 16. Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM, Tyrrell DL. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. Hepatology 1996;24:714-717

17. Chayama K, Suzuki Y, Kobayashi M, Tsubota A, Hashimoto M, Miyano Y, Koike H, et al. Emergence and takeover of YMDD motif mutant hepatitis B virus during 365 long-term lamivudine therapy and re-takeover by wild type after cessation of therapy. Hepatology 1998;27:1711-1716

18. Lau DT, Khokhar MF, Doo E, Ghany MG, Herion D, Park Y, Kleiner DE, et al. Long-term therapy of chronic hepatitis B with lamivudine. Hepatology 2000;32:828-834

370 19. Köck J, Theilmann L, Galle P, Schlicht HJ. Hepatitis B virus nucleic acids associated with human peripheral blood mononuclear cells do not originate from replicating virus. Hepatology 1996;23:405-413

20. Su Q, Wang SF, Chang TE, Breitkreutz R, Hennig H, Takegoshi K, Edler L, et al. Circulating hepatitis B virus nucleic acids in chronic infection: representation of 375 differently polyadenylated viral transcripts during progression to nonreplicative stages. Clin Cancer Res 2001;7:2005-2015

21. Zhang W, Hacker HJ, Tokus M, Bock T, Schröder CH. Patterns of circulating hepatitis B virus serum nucleic acids during lamivudine therapy. J Med Virol 2003;71:24-30

380 22. Kamisango K, Kamogawa C, Sumi M, Goto S, Hirao A, Gonzales F, Yasuda K, et al. Quantitative detection of hepatitis B virus by transcription-mediated amplification and hybridization protection assay. J Clin Microbiol 1999;37:310-314

23. Ranki M, Schätzl HM, Zachoval R, Uusi-Oukari M, Lehtovaara P. Quantification
385 of hepatitis B virus DNA over a wide range from serum for studying viral replicative activity in response to treatment and in recurrent infection. Hepatology 1995;21:1492-1499

24. Ohishi W, Chayama K. Rare quasispecies in the YMDD motif of hepatitis B virus detected by polymerase chain reaction with peptide nucleic acid clamping.
390 Intervirology 2003;46:355-361

25. Sung JJ, Wong ML, Bowden S, Liew CT, Hui AY, Wong VW, Leung NW, et al. Intrahepatic hepatitis B virus covalently closed circular DNA can be a predictor of sustained response to therapy. Gastroenterology 2005;128:1890-1897

26. Yuen MF, Wong DK, Sum SS, Yuan HJ, Yuen JC, Chan AO, Wong BC, et al.
395 Effect of lamivudine therapy on the serum covalently closed-circular (ccc) DNA of chronic hepatitis B infection. Am J Gastroenterol 2005;100:1099-1103

27. Wong DK, Yuen MF, Yuan H, Sum SS, Hui CK, Hall J, Lai CL. Quantification of covalently closed circular hepatitis B virus DNA in chronic hepatitis B patients. Hepatology 2004;40:727-737

400 28. Zollner B, Petersen J, Puchhammer-Stockl E, Kletzmayr J, Sterneck M, Fischer L, Schroter M, et al. Viral features of lamivudine resistant hepatitis B genotypes A and D. Hepatology 2004;39:42-50

29. Nafa S, Ahmed S, Tavan D, Pichoud C, Berby F, Stuyver L, Johnson M, et al. Early detection of viral resistance by determination of hepatitis B virus
405 polymerase mutations in patients treated by lamivudine for chronic hepatitis B. Hepatology 2000;32:1078-1088
30. Yuen MF, Sablon E, Hui CK, Yuan HJ, Decreamer H, Lai CL. Factors associated with hepatitis B virus DNA breakthrough in patients receiving prolonged lamivudine therapy. Hepatology 2001;34:785-791
410 31. Suzuki F, Tsubota A, Arase Y, Suzuki Y, Akuta N, Hosaka T, Someya T, et al. Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan, Intervirology 2003;46:182-189
32. Sun J, Wang Z, Ma S, Zeng G, Zhou Z, Luo K, Hou J. Clinical and virological
415 characteristics of lamivudine resistance in chronic hepatitis B patients: A single center experience. J Med Virol 2005;75:391-398
33. Puchhammer-Stockl E, Mandl CW, Kletzmayr J, Holzmann H, Hofmann A, Aberle SW, et al. Monitoring the virus load can predict the emergence of drug-resistant hepatitis B virus strains in renal transplantation patients during
420 lamivudine therapy. J Infect Dis 2000;181:2063-2066
34. Zollner B, Schafer P, Feucht HH, Schroter M, Petersen J, Laufs R. Correlation of hepatitis B virus load with loss of e antigen and emerging drug-resistant variants during lamivudine therapy. J Med Virol 2001;65:659-663

35. Akuta N, Suzuki F, Kobayashi M, Tsubota A, Suzuki Y, Hosaka T, Someya T, et al. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J Hepatol 2003;38:315-321

36. Lok AS, Hussain M, Cursano C, Margotti M, Gramenzi A, Grazi GL, Jovine E, et al. Evolution of hepatitis B virus polymerase gene mutations in hepatitis B e antigen-negative patients receiving lamivudine therapy. Hepatology 2000;32:1145-1153

37. Tanaka Y, Yeo AE, Orito E, Ito K, Hirashima N, Ide T, Sata M, et al. Prognostic indicators of breakthrough hepatitis during lamivudine monotherapy for chronic hepatitis B virus infection. J Gastroenterol 2004;39:769-775

38. Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, Okanoue T, et al. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance. Liver Int 2006;26:90-96

Acknowledgment

440 This work was carried out at the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University. The authors thank Kana Kunihiro, Rie Akiyama, Yoshiko Seo, Yoshiko Nakata and Kiyomi Toyota for the excellent technical assistance. This work was supported in part by Grants-in-Aid for scientific research and development from the Ministry of Education, Sports, Culture, and Technology 445 and the Ministry of Health, Labor and Welfare.