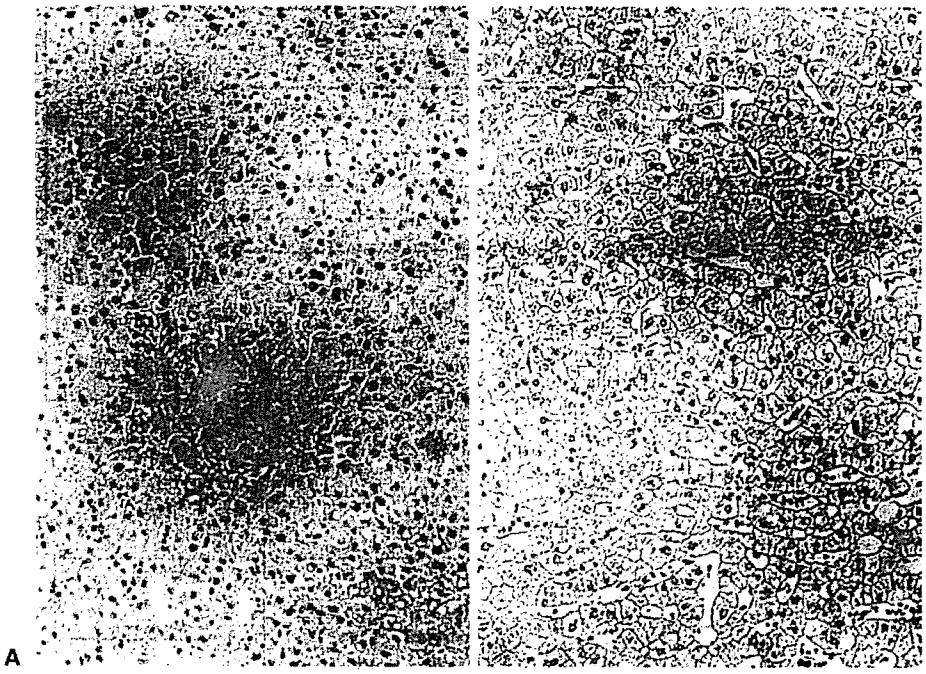

Fig. 2A,B. Immunohistochemical studies: **A** liver with fulminant hepatitis (removed for liver transplantation), in a patient who showed a serum level of 8600 pg/ml cytochrome c. TdT mediated dUTP nick end labeling (TUNEL)-positive hepatocytes were seen. **B** Transplanted liver was normal; it showed no TUNEL-positive hepatocytes, **A**, **B** ×100

actually occurred in the hepatocytes of this patient with FH.

Clinical courses in three patients with FH

The clinical courses of three patients with FH are shown in Fig. 3. Case 1 had a high level of serum cytochrome c at the time of hospitalization but, as a result of therapy, the patient recovered. Cases 2 and 3 were patients who did not recover from FH.

The most significant difference between the patient who survived and the patients who died was that the serum cytochrome c level increased before their deaths (although there was no increase of serum ALT or AST).

Discussion

It has been reported that FH in humans, including patients with hepatitis B, causes apoptosis of hepatocytes.[11–13] In our patient who had a living-donor transplantation, the liver tissue clearly had TUNEL-positive hepatocytes, indicating the occurrence of apoptosis with FH caused by the type B virus.

Cytochrome c is also involved in apoptosis. Cytochrome c is known as a mitochondrial substrate that finally activates caspase-3, resulting in DNA fragmentation in cells, and leading to apoptosis, as described previously.[5–9] A recent report[14] indicates that enzymes, e.g., caspases, will leak out of not only necrotic cells but also apoptotic cells into the blood. Additionally, Ben-Ari et al.[15] have reported that the circulating cytochrome c concentration increases in subjects with a variety of hepatic disorders, and that the level correlates with the apoptotic index in the liver, indicating that serum cytochrome c is derived from apoptotic cells. However, further examination to determine the mechanism of cytochrome c leakage from apoptotic cells is necessary.

In their study, Ben-Ari et al.[15] reported that the serum cytochrome c level in patients with liver diseases showed an increased level compared with that in healthy controls. In their healthy controls the level of serum cytochrome c was 39.9 ± 35.1 ng/ml, while patients with primary sclerosing cholangitis had the highest level (1041.0 ± 2844.8 ng/ml). However, our data indicated a level of 112 ± 57 pg/ml for healthy controls. Differences between rat and human cytochrome c as antigens for establishing the assay systems may have resulted in these different data. Nor did their data show a correlation between cytochrome c and AST, and, in their report, the cytochrome c level of patients with FH was almost the same as the value for patients with hepatitis B or C and patients with HCC. However, our data showed strong positive correlations between cytochrome c and AST, m-GOT, ALP, LDH, and HGF; and negative correlations with AFP and T.Bil. Our histochemical study indicated apoptosis in the fulminant hepatic liver (Fig. 2A). Thus, in our study, serum cyto-

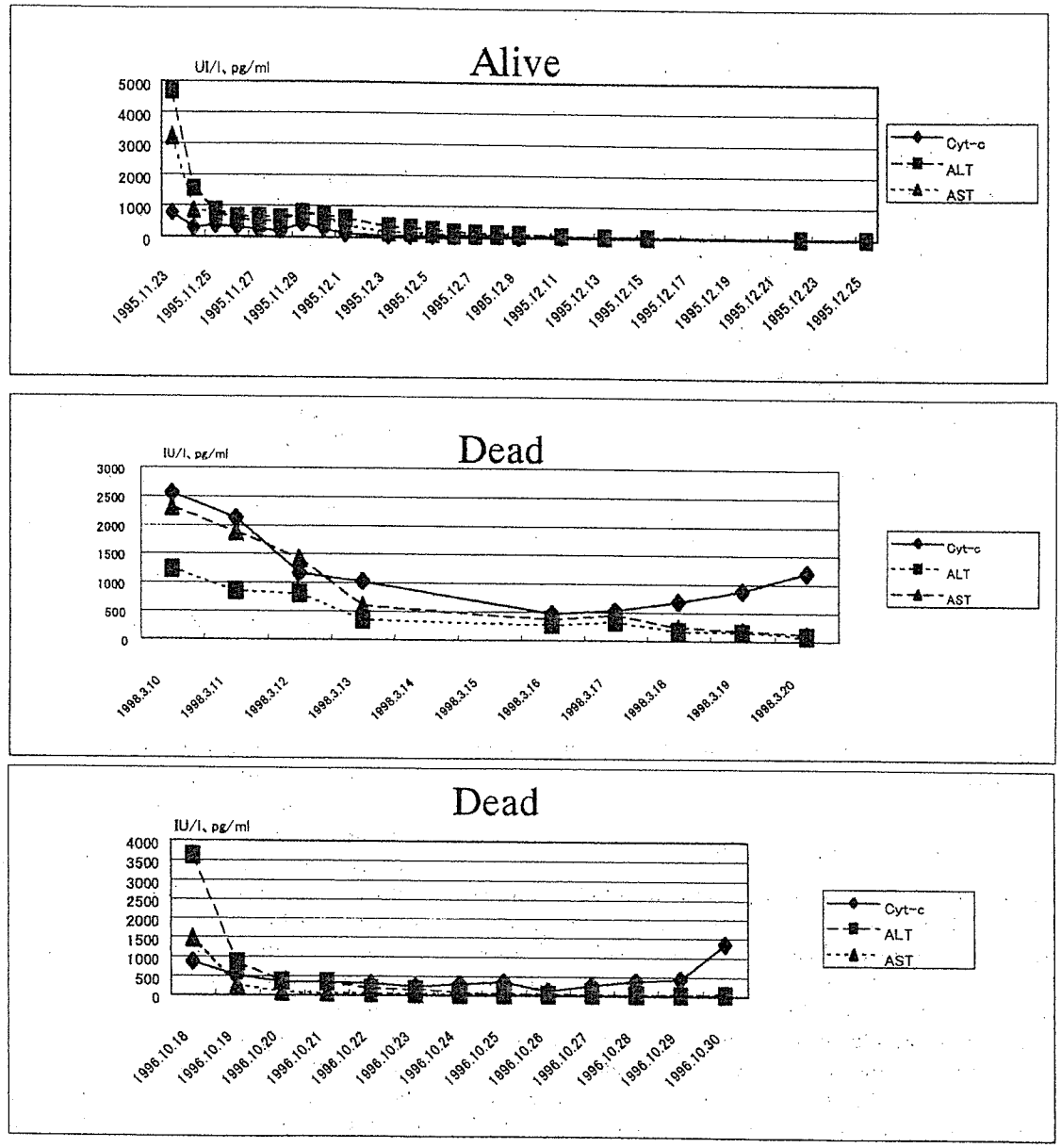

Fig. 3. Clinical courses of serum cytochrome c (*Cyt-c*), ALT, and AST in three patients with fulminant hepatitis. One patient (case 1) recovered and showed no re-increase of serum markers during the clinical course. However, two patients (cases 2 and 3) showed re-increases of serum cytochrome c, without increases of ALT and AST, before death

chrome c seemed mainly to reflect the extent of liver damage.

One interesting finding of our report was that serum cytochrome c may also reflect the grade of hepatic coma, possibly indicating brain damage. Adachi and Endo[10] recently reported that the serum cytochrome c level was increased in patients with the systemic inflammatory response syndrome, serving as a prognostic indicator. Thus, the re-increase of serum cytochrome c before death in FH (Fig. 3) may be attributable to cytochrome c release from the injured brain, due to edema.

It has been shown that cytochrome c is released from the injured brain,[16,17] but the mechanism of its release, presumably caused by brain edema, and direct evidence of the relationship between cytochrome c and brain damage should be examined in detail in the future.

Our findings, together with those in other studies,[16,17] indicate that serum cytochrome c may be a possible new marker not only for liver damage but also for brain

damage and prognosis. TNF-alpha, TNF receptor, and interleukin (IL)-10 have been reported to be possible markers of the severity of FH.[18,19] However, so far, no serum marker has been reported to predict brain damage, and only imaging analysis, e.g., computed tomography (CT) or magnetic resonance imaging (MRI), can detect brain edema with safety. Measuring intracranial pressure to detect brain edema in patients with FH suffering from a bleeding tendency seems unsuitable, for safety reasons. Although more detailed studies are necessary in future, our recent examination showed that the cytochrome c concentration in cerebrospinal fluid obtained from the autopsy material of a patient with FH was higher than that of a patient with HCC (1934 vs 588 pg/ml).

Thus, our system for measuring serum cytochrome c may become a new predictive marker not only for the severity of liver damage but also for brain damage, and it will be useful to determine the timing of liver transplantation.

In summary, the present study indicated that the assay of serum cytochrome c is very sensitive for the detection of liver damage, and such assays may also be applicable for predicting the prognosis of patients with FH, in those including the prediction of prognosis the complication of brain edema.

References

1. Gores JG, Rust C. Apoptosis and liver disease. Am J Med 2000; 108:567–74.
2. Kaplowitz N. Cell death at the millennium. Clin Liver Dis 2000; 4:1–23.
3. Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement of dATP and cytochrome c. Cell 1996;86:147–57.
4. Meier P, Evan G. Dying like flies. Cell 1998;95:295–8.
5. Green D. Apoptotic pathways: the roads to ruin. Cell 1998;94:95–8.
6. Bernardi P. Mitochondrial transport of cations: channels, exchangers, and permeability transition. Physiol Rev 1999;79:1127–55.
7. Petronilli V, Nicolli A, Costantini P, Colonna R, Bernardi P. Regulation of the permeability transition pore, a voltage-dependent mitochondrial channel inhibited by cyclosporine A. Biochim Biophys Acta 1994;1187:255–9.
8. Petronilli V, Penzo D, Scorrano L, Bernardi P, DiLisa F. The mitochondrial permeability transition, release of cytochrome C and cell death. J Biol Chem 2001;276:12030–4.
9. Martinou JC, Green D. Breaking the mitochondrial barrier. Nat Rev Mol Cell Biol 2001;2:63–7.
10. Adachi N, Endo F. Serum cytochrome C level as a prognostic indicator in patients with systemic inflammatory response syndrome. Clin Chim Acta 2004;342:127–36.
11. Kasahara I, Saitoh K, Nakamura K. Apoptosis in acute hepatic failure: histopathological study of human liver tissue using the TUNEL method and immunohistochemistry. J Med Dent Sci 2000;47:167–75.
12. Rivero M, Crespo J, Fabrega E, Casafont F, Mayorga M, Gomez-Fleitas M, et al. Apoptosis mediated by the Fas system in the fulminant hepatitis by hepatitis B virus. J Viral Hepat 2002;9:107–13.
13. Kanzler S, Galle PR. Apoptosis and the liver. Semin Cancer Biol 2000 Jun;10:173–84.
14. Hentze H, Schwoebel F, Lund S, Keel M, Ertel W, Wendel A, et al. In vivo and in vitro evidence for extracellular caspase activity released from apoptotic cells. Biochem Biophys Res Commun 2001;283:1111–7.
15. Ben-Ari Z, Schmilovotz-Weiss H, Belinki A, Pappo O, Sulkes J, Neuman MG, et al. Circulating soluble cytochrome C in liver disease as a marker of apoptosis. J Intern Med 2003;254:168–75.
16. Zhu C, Wang X, Cheng X, Qiu L, Xu F, Simbruner G, et al. Post-ischemic hypothermia-induced tissue protection and diminished apoptosis after neonatal cerebral hypoxia-ischemia. Brain Res 2004;996:67–75.
17. Morita-Fujimura Y, Fujimura M, Kawase M, Chen SF, Chan PH. Release of mitochondrial cytochrome C and DNA fragmentation after cold injury-induced brain trauma in mice: possible role in neuronal apoptosis. Neurosci Lett 1999;267:201–5.
18. Tokushige K, Yamaguchi N, Ikeda I, Hashimoto E, Yamauchi K, Hayashi N. Significance of soluble TNF receptor-I in acute-type fulminant hepatitis. Am J Gastroenterol 2000;95:2040–6.
19. Nagaki M, Iwai H, Naiki T, Ohnishi H, Muto Y, Moriwaki H. High levels of serum interleukin-10 and tumor necrosis factor-alpha are associated with fatality in fulminant hepatitis. J Infect Dis 2000;182:1103–8.

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HEPATOLOGY

Hepatitis B virus core and core-related antigen quantitation in Chinese patients with chronic genotype B and C hepatitis B virus infection

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Abstract

Background and Aims: Hepatitis B virus (HBV) core-related antigen (HBcrAg) and HBV core antigen (HBcAg) assays were developed for the measurement of serum HBV load. The aim of this study was to assess the clinical utility of these assays in Chinese patients with chronic genotype B and C HBV infection.

Methods: One hundred and ninety-three chronic hepatitis B patients were enrolled. Serum HBcrAg and HBcAg were measured by chemiluminescence enzyme immunoassay, and HBV-DNA was measured by using a sensitive polymerase chain reaction assay. The data were analyzed in patients with HBV genotype B (HBV/B) and genotype C (HBV/C). The HBcrAg/HBcAg ratio was calculated and compared between patients with and without hepatitis B e antigen (HBeAg).

Results: The concentrations of HBcrAg and HBcAg showed significant positive correlation with the HBV-DNA concentration in both HBV/B ($r = 0.79$, $P < 0.001$, and $r = 0.77$, $P < 0.001$, respectively) and HBV/C ($r = 0.87$, $P < 0.001$, and $r = 0.90$, $P < 0.001$, respectively). The cut-off for a positive HBcAg corresponded to approximately 4.5 log copies/mL, and that for a positive HBcrAg result corresponded to 3–4 log copies/mL. The HBcrAg/HBcAg ratio was higher in patients with HBeAg than in those without HBeAg.

Conclusions: The HBcrAg assay and HBcAg assay are clinically useful in viral quantitation of HBV/B and HBV/C. A combination of these assays would be a valuable tool for analyzing the clinical status of HBV infection.

Key words: hepatitis B e antigens (HBeAg), hepatitis B antigens, hepatitis B core antigens (HBcAg), hepatitis B virus, viral proteins.

INTRODUCTION

Infection with hepatitis B virus (HBV) remains one of the major human infectious diseases and involves approximately 350 million people.[1] In a significant proportion of cases, infection progresses to cirrhosis and liver failure as well as hepatocellular carcinoma (HCC).[2] As therapeutic advances have emerged, detailed information is required to assess HBV replication in individual patients in clinical management.

Recently, two sensitive chemiluminescence enzyme immunoassays (CLEIA) specific for HBV were developed in our laboratory.[3,4] One is an HBV core-related antigen (HBcrAg) assay that measures the serum levels of hepatitis B e antigen (HBeAg) and hepatitis B core antigen (HBcAg) simultaneously using monoclonal antibodies, and the other is an assay that measures the serum level of HBcAg. Although assessments of clinical performance relating to the HBcAg and HBcrAg assays have already been reported in Japanese patients,[3–5] an

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evaluation of these two antigen assays was not performed in patients with HBV genotype B. The aim of this study is to assess the clinical utility of the HBcAg and HBcrAg assays for measurement of HBV load in Chinese patients who are infected with genotype B or C.

METHODS

Patients

Patients attending the Second Hospital of Hebei Medical University, Shijiazhuang, in northern China, between June and August 2001, who had carried hepatitis B surface antigen (HBsAg) for at least 6 months, were enrolled for the study. Serum samples obtained from 193 patients (125 male and 68 female, median age 27 years, range 5–73 years) were examined. One hundred and eighty-two patients were diagnosed as chronic HBV carriers according to the consensus diagnostic criteria of HBV infection.[6] The remaining 11 patients had persistently normal alanine aminotransferase (ALT) levels, suggesting an inactive HBsAg carrier stage.[6] None of the 193 patients were treated with antiviral agents such as interferon or lamivudine. All were non-reactive for antibody to hepatitis C virus infection. All sera were stored at −20°C until use. The study design conformed to the 1995 Declaration of Helsinki, and was approved by ethics committees of our institutions. Informed consent was obtained from each patient.

HBcAg CLEIA and HBcrAg CLEIA

Concentrations of HBcAg and HBcrAg were measured in serum using the CLEIA reported previously.[3,4] Briefly, 100 μL serum was mixed with 50 μL pretreatment solution containing 15% sodium dodecyl sulfate. After incubation at 70°C for 30 min, 50 μL pretreated serum was added to wells coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and filled with 100 μL assay buffer. The mixture was incubated for 2 h at room temperature and the wells were washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies were added to the wells and incubated for 1 h at room temperature. After washing, substrate solution was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBcAg or HBcrAg concentration was read by comparison with a standard curve. Recombinant HBcAg (rHBcAg: amino acids 1–183 of precore/core gene product) and recombinant ProHBeAg (rProHBeAg: amino acids −10 to 183) were expressed in *Escherichia coli* and purified to single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Recombinant HBcAg and rProHBeAg were used as the standard for the HBcAg assay and the HBcrAg assay, respectively. The HBcrAg immunoreactivity for rProHBeAg at 10 fg/mL was defined as 1 U/mL[3] The cut-off for a positive HBcAg result was 4.0 pg/mL and that for HBcrAg was 1.0×10^3 U/mL (=immunoreactivity of rProHBeAg at 10 pg/mL), which were determined based on the mean +4 SD values of healthy control sera ($n = 160$ or 108) and sera of hepatitis C patients ($n = 55$ or 59).[3,4]

The HBcrAg/HBcAg immunoreactivity ratio was calculated in order to assess the relative amounts of HBeAg and HBcAg in sera. The immunoreactivity of HBcrAg (pg/mL) was divided by that of HBcAg (pg/mL) in each sample.

Conventional HBV markers and genotyping of HBV

Using commercially available enzyme immunoassay kits, HBsAg, HBeAg, and anti-HBe were measured (Dinabbott, Tokyo, Japan). The levels of HBV-DNA in the serum samples were measured using an Amplicor HBV Monitor test (Roche Molecular Systems, Branchburg, NJ, USA) with a detection range between 4×10^2 and 4×10^7 copies/mL. Samples with an HBV-DNA level greater than 10^8 copies/mL were measured after dilution in HBV-negative serum. Nucleic acids were extracted from 100 μL of sera using a Smitest Ex R&D kit (Genome Science Laboratories, Tokyo, Japan). HBV genotype was determined using restriction fragment length polymorphism.[7]

Statistical analysis

The Mann–Whitney *U*-test was used for analysis of the quantitative data, and Fisher's exact test was used analysis of the qualitative data. The Spearman rank correlation was also employed where appropriate. Statistical analyses were done using the StatView software package (version 5.0; SAS Institute, Cary, NC, USA). A *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Genotypic distribution

Among the 193 patients studied, 169 (87.6%) patients were infected with HBV of genotype C (HBV/C), 21 (10.9%) patients were infected with HBV/B, and three (1.5%) were infected with HBV/A. The clinical backgrounds of the patients who were infected with HBV/B and HBV/C are compared in Table 1. There were no statistical differences in clinical backgrounds, serum HBV-DNA levels, serum concentrations of HBcAg, or serum concentrations of HBcrAg between the patients infected with HBV/B and HBV/C.

Correlation between HBcAg/HBcrAg and HBV-DNA concentrations

The correlation between the concentrations of HBcAg and HBV-DNA, and that of the concentrations of

Table 1 Background characteristics of patients infected with hepatitis B virus (HBV) of genotype B and genotype C

Features	Genotype B ($n = 21$)	Genotype C ($n = 169$)	P-value
Age (years)†	22 (9–65)	27 (5–73)	NS
No. males‡	12 (57.1%)	111 (65.7%)	NS
HBeAg positivity‡	16 (76.2%)	102 (60.4%)	NS
ALT (U/L)†	50 (21–105)	47 (10–2100)	NS
HBV-DNA (log copies/mL)†§	8.7 (4.4–9.4)	7.5 (3.0–9.4)	NS
HBcAg (log U/mL)†	6.3 (2.2–7.4)	5.7 (1.9–7.5)	NS
HBcrAg (log U/mL)†	8.3 (2.9–8.9)	8.0 (2.5–9.0)	NS

ALT, alanine aminotransferase; HBcAg, HBV core antigen; HBcrAg, hepatitis B virus core-related antigen; HBeAg, hepatitis B e antigen; NS, not significant. †Data are expressed as median (range). ‡Data are expressed as positive number (%). §HBV-DNA was measured by using an Amplicor HBV Monitor test (Roche Molecular Systems, Branchburg, NJ, USA).

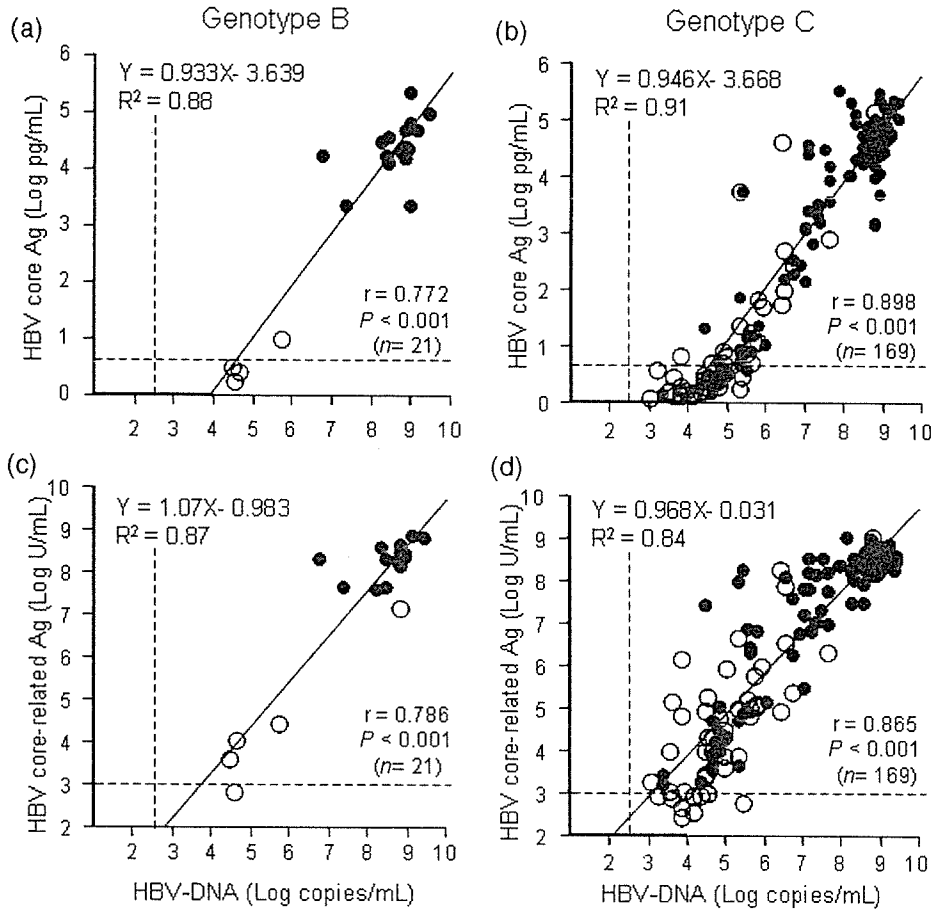

Figure 1 Degree of correlation between the concentrations of hepatitis B virus (HBV) core antigen (HBcAg) and HBV-DNA, and those of hepatitis B virus core-related antigen (HBcrAg) and HBV-DNA. Correlations between the concentrations of HBcAg and HBV-DNA in the sera from patients infected with (a) HBV genotype B (HBV/B) and (b) HBV genotype C (HBV/C). Correlation between the concentrations of HBcrAg and HBV-DNA in the sera from patients infected with (c) HBV/B and (d) HBV/C, respectively. (●), Data from HBeAg-positive sera; (○), data from hepatitis B e antigen (HBeAg)-negative sera. HBV-DNA levels were determined by using the Amplicor HBV Monitor test (Roche Molecular Systems, Branchburg, NJ, USA). (- - -), Lower cut-off of the assays.

HBcrAg and HBV-DNA are shown in Figure 1. The serum concentrations of HBcAg and HBV-DNA correlated significantly in the patient group infected with HBV/B ($r = 0.772$, $P < 0.001$), as well as in the patient group infected with HBV/C ($r = 0.898$, $P < 0.001$). The serum concentrations of HBcrAg and HBV-DNA also correlated significantly in the patient group infected with HBV/B ($r = 0.786$, $P < 0.001$), as well as in the patient group infected with HBV/C ($r = 0.865$, $P < 0.001$). The cut-off for a positive HBcAg result was 4 pg/mL, which corresponded to approximately 4.5 log copies/mL (Fig. 1). The cut-off for a positive HBcrAg result corresponded to 3–4 log copies/mL (Fig. 1).

HBcrAG/HBcAg ratio

The HBcrAg/HBcAg immunoreactivity ratio was calculated in each patient and was compared between the patients with and without HBeAg (Fig. 2). The data are represented in log scale. The median value of the

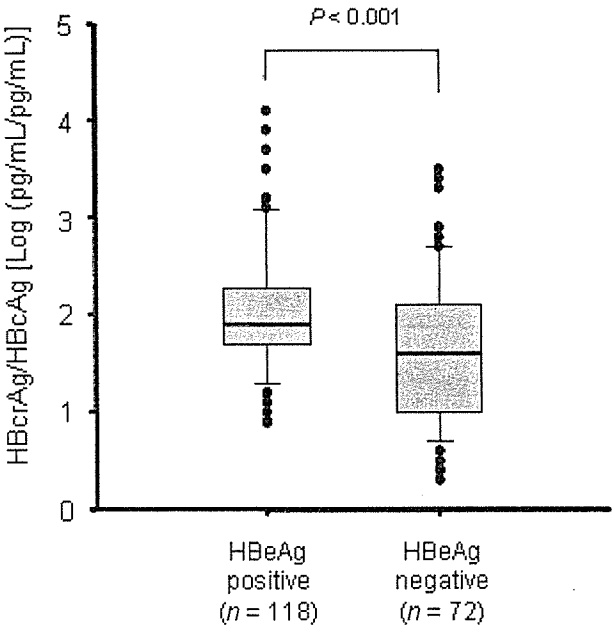

Figure 2 Hepatitis B virus core-related antigen/HBV core antigen ratios in relation to hepatitis B e antigen (HBeAg) status. Data are represented by a box-plot showing the 25th percentile, median, and 75th percentile as vertical box. Tick marks above and below the box indicate the 90th and 10th percentiles (log representation). (●), Outlier data points.

HBcrAg/HBcAg immunoreactivity ratio was significantly higher in patients with HBeAg (median 1.9, range 0.9–4.1) than in patients without HBeAg (median 1.6, range 0.3–3.5; $P < 0.001$).

DISCUSSION

In this report, an attempt was made to assess the clinical utility of the HBcAg and HBcrAg assays for the measurement of HBV load in the sera from Chinese patients who were infected with HBV/B or HBV/C. In a previous study, a good quality estimation of the accuracy of the HBcrAg assay in HBV/B-infected patients could not be obtained because of the small number of patients who were infected with HBV/B.[5] Twenty-one patients with HBV/B were enrolled in the present study. As a result, a significant positive correlation was observed between the serum concentrations of HBcAg and HBV-DNA, as well as between HBcrAg and HBV-DNA in both HBV/B- and HBV/C-infected Chinese patients. The HBcrAg assay has a high level of sensitivity, which was comparable with the real-time detection polymerase chain reaction.[5] The cut-off for a positive HBcAg result corresponded to a range of 4–5 log copies/mL. Because an HBV level less than 4 log copies/mL indicates inactive liver disease,[8,9] and an HBV level greater than 5 log copies/mL is associated with active liver disease,[10,11] the HBcAg assay could be valuable to postulate chronic active hepatitis B.

If all Dane particles contain one copy of HBV-DNA and 240 molecules of HBcAg, 9.0 log copies of HBV-DNA would correspond to 3.9 log pg (=8.26×10^3 pg) of core protein. But in our experiment, approximately 4.5 log pg/mL of HBcAg was measured in sera containing 9.0 log copies/mL of HBV-DNA (Fig. 1), which is fourfold (0.6 logs) the calculated value. Although the HBV-DNA and HBcAg assays have some inaccuracies, this gap between 3.9 and 4.5 log pg/mL might indicate that the DNA-negative "empty" Dane particles were predominant in sera, as has been suggested by electron microscopy and radiolabeling studies.[12–14]

The HBcrAg assay detects HBcAg and HBeAg simultaneously, using monoclonal antibodies that recognize both denatured HBcAg and HBeAg, even in anti-HBe antibody-positive samples.[3] Current commercial HBeAg assays do not detect the HBeAg/anti-HBe complex, because the epitopes of HBeAg are masked by the anti-HBe antibody.[15] For capturing HBcAg, we used HB44, HB61, and HB114 immobilized monoclonal antibodies, which were the same as in the HBcrAg assay.[4] The HBcAg assay differs from the HBcrAg assay in the detection antibody, which recognizes core-specific SRRRR repeats in the C-terminal protamine-like nucleic acid binding domain, and is therefore specific for HBcAg. In the present report, the HBcrAg/HBcAg ratio was significantly higher in patients with HBeAg than in patients without HBeAg. Because the HBcrAg assay mainly reflects the levels of HBeAg and HBeAg/anti-HBe complex,[3] the HBcrAg/HBcAg ratio would represent the relative amounts of HBeAg and HBcAg. If this is true, this ratio could be used as a marker that indicates a balance of HBeAg production and HBV load at some points. As HBeAg states in sera largely depend on the HBeAg production from HBV, the mechanism of this result could be explained by the reduction of HBeAg in the sera, via mechanisms such as mutations in the precore and core promoter regions.[16–18] HBV viral load and the concentration of HBeAg vary widely in individual patients during the course of HBV infection. This variation and the immunological reaction of the host result in various pathological manifestations of HBV infection. It would therefore be more useful for diagnostic purposes to measure the HBcAg and HBcrAg levels simultaneously, instead of checking only the HBeAg state. Clearly, further analysis in longitudinal studies is required, and the mechanisms associated with these results remain to be explored.

In conclusion, we assessed the utility of the HBcAg and HBcrAg assays in Chinese patients with HBV/B and HBV/C. These results showed that these two HBV antigen assays are clinically useful in viral quantitation as well as HBV-DNA quantitation. Using a combination of these two assays could be more useful for analyzing clinical status in patients with HBV infection.

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REFERENCES

1 Lee WM. Hepatitis B virus infection. *N. Engl. J. Med.* 1997; **337**: 1733–45.
2 Beasley RP. Hepatitis B virus. The major etiology of hepatocellular carcinoma. *Cancer* 1988; **61**: 1942–56.
3 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.
4 Kimura T, Rokuhara A, Matsumoto A *et al.* New enzyme immunoassay for detection of hepatitis B virus core antigen (HBcAg) and relation between levels of HBcAg and HBV-DNA. *J. Clin. Microbiol.* 2003; **41**: 1901–6.
5 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.
6 Lok AS, McMahon BJ. Practice Guidelines Committee, American Association for the Study of Liver Diseases. Chronic hepatitis B. *Hepatology* 2001; **34**: 1225–41.
7 Mizokami M, Nakano T, Orito E *et al.* Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett.* 1999; **450**: 66–71.
8 Lindh M, Horal P, Dhillon AP, Norkrans G. Hepatitis B virus DNA levels, precore mutations, genotypes and histological activity in chronic hepatitis B. *J. Viral Hepat.* 2000; **7**: 258–67.
9 Manesis EK, Papatheodoridis GV, Hadziyannis SJ. Serum HBV-DNA levels in inactive hepatitis B virus carriers. *Gastroenterology* 2002; **122**: 2092–3.
10 Lok AS, Heathcote EJ, Hoofnagle JH. Management of hepatitis B: 2000. Summary of a workshop. *Gastroenterology* 2001; **120**: 1828–53.
11 Martinot-Peignoux M, Boyer N, Colombat M *et al.* Serum hepatitis B virus DNA levels and liver histology in inactive HBsAg carriers. *J. Hepatol.* 2002; **36**: 543–6.
12 Alberti A, Diana S, Scullard GH, Eddleston WF, Williams R. Full and empty Dane particles in chronic hepatitis B antigen and presence of liver damage. *Gastroenterology* 1978; **75**: 869–74.
13 Gerin JL, Ford EC, Purcell RH. Biochemical characterization of Australia antigen: evidence for defective particles of hepatitis B virus. *Am. J. Pathol.* 1975; **81**: 651–68.
14 Sakamoto Y, Yamada G, Mizuno M *et al.* Full and empty particles of hepatitis B virus in hepatocytes from patients with HBsAg-positive chronic active hepatitis. *Lab. Invest.* 1983; **48**: 678–82.
15 Raimondo G, Recchia S, Lavarini C, Crivelli O, Rizzetto M. Dane particle-associated hepatitis B e antigen in patients with chronic hepatitis B virus infection and hepatitis B e antibody. *Hepatology* 1982; **2**: 449–54.
16 Carman WF, Jacyna MR, Hadziyannis S *et al.* Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 1989; **2**: 588–91.
17 Okamoto H, Yotsumoto S, Akahane Y *et al.* Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. *J. Virol.* 1990; **64**: 1298–303.
18 Okamoto H, Tsuda F, Akahane Y *et al.* Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. *J. Virol.* 1994; **68**: 8102–10.

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Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: A multicenter retrospective study of 2795 patients

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Abstract

A retrospective survey of Japanese patients histologically diagnosed with chronic hepatitis B was conducted to determine the effectiveness of lamivudine in preventing hepatocellular carcinoma (HCC). Of the 2795 patients who satisfied criteria for analysis after treatment from any of 30 medical institutions, 657 had received lamivudine and the remaining 2138 had not. A Cox regression model with liver biopsy as the starting point revealed seven factors related to HCC: lamivudine therapy, gender, family clustering of hepatitis B, age at liver biopsy, hepatic fibrosis stage, serum albumin level, and platelet count. In a matched case-controlled study, 377 patients in a lamivudine-treated group and 377 matched patients in a non-treated group were selected based on their propensity scores. The mean follow-up period was 2.7 years in the lamivudine group and 5.3 years in the control group. In the lamivudine group, HCC occurred in four patients (1.1%) with an annual incidence rate of 0.4%/(patient/year), whereas in the control group HCC occurred in 50 patients (13.3%) for a rate of 2.5%/(patient/year). A comparison of the cumulative HCC incidence between the two groups by the Kaplan–Meier method showed a significantly lower incidence of HCC in the lamivudine group ($p < 0.001$). These findings suggest that lamivudine effectively reduces the incidence of HCC in patients with chronic hepatitis B.

Keywords: Chronic hepatitis B; Hepatocellular carcinoma; Anti-viral treatment; Lamivudine

1. Introduction

An estimated 350 million people worldwide are chronically infected with the hepatitis B virus (HBV), most in southeast Asia [1,2]. In this region, infection occurs during infancy, including that through mother–child transmission. Infected persons with HBV are initially asymptomatic, and

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active hepatitis emerges years later. In most patients, seroconversion from hepatitis Be antigen (HBeAg) to antibody to HBeAg (HBeAb) occurs spontaneously with age. At the same time, the virus levels decrease and hepatitis abates. Some patients, however, remain positive for HBeAg, and in those patients the hepatitis virus persists at high levels, resulting in the progression to hepatic cirrhosis, and the onset of hepatocellular carcinoma (HCC) in a high percentage of such patients [3–5]. The number of HBV carriers is decreasing in Japan and some other countries as a result of the prevention of mother–child transmission through the use of HBV vaccines and/or high-potency antibody to hepatitis B surface antigen (HBsAb) human immunoglobulin (HBIG) [6]. Even in these countries, however, only persons born after 1986 are protected by vaccination, and many chronic hepatitis B patients still need treatment. In the past, it was not easy to manage chronic hepatitis B using anti-viral agents such as interferon. In recent years, however, the development of lamivudine, a nucleoside analogue that inhibits reverse transcriptase, has drastically changed the treatment of hepatitis B [7–9]. By virtue of this inhibitory ability, lamivudine was developed as an anti-viral agent against human immuno-deficiency virus (HIV). It was later also found to be effective against HBV because HBV is a member of the Hepadnaviridae family, which utilizes reverse transcriptase in its replication process [10]. Lamivudine was found to inhibit the replication of HBV, reduce hepatitis, and improve liver histological findings in long-term treatment [11]. It is also useful when hepatitis B becomes severe due to acute exacerbation, as well as in the treatment of liver cirrhosis associated with symptoms of hepatic failure, such as ascites and edema [12–16]. However, a number of problems are associated with lamivudine therapy, such as relapse of hepatitis due to the appearance of YMDD mutant viruses and the difficulty of estimating the optimal time to discontinue the treatment [17,18]. In addition, until recently no adequate studies had been conducted to determine whether or not lamivudine inhibits the onset of hepatic cancer, even though it is known to slow the progression of histological changes in the liver. This lack of research is attributable partly to the need for long-term follow-up of a large number of patients and partly to the difficulty of conducting clinical trials. We conducted a multicenter study of a large number of registered patients to evaluate the effects of lamivudine on the course of hepatitis B and the onset of HCC. The data obtained were analyzed in a matched case-controlled study.

2. Materials and methods

2.1. Study design

The Inuyama Hepatitis Study Group designed this multicenter retrospective study to determine whether or not lamivudine is effective in preventing HCC. The subjects were Japanese patients with hepatitis B who were diagnosed with chronic liver disease by liver biopsy after 1980 and were followed up until March 2002. Each patient completed a questionnaire containing 16 items in four categories: background factors: date of birth, sex, family clustering of hepatitis B, and alcohol consumption during follow-up (80 g or more per day as ethanol); examination and test items: date of liver biopsy, grade and stage of histological findings of the liver, hepatitis Be antigen (HBeAg), antibody to HBeAg (HBeAb), albumin, asparate aminotransferase (AST), alanine aminotransferase (ALT), and platelet counts; clinical outcomes: the presence or absence of HCC during the follow-up period and the date of onset if present; lamivudine therapy: the presence or absence of lamivudine therapy during the follow-up period, and the date of initiation and duration of therapy if provided. The study was allowed by the review board of each participating institution. The names, ID numbers, and all other information that would directly identify individual patients were deleted to protect their privacy.

2.2. Patients

The present study included 3022 patients with chronic hepatitis B who underwent liver biopsy at any of 30 medical institutions after 1980. No patient had superinfection with hepatitis C virus and HIV. Two hundred and twenty-seven patients who had not answered the question about lamivudine treatment were excluded from the study. This left a total of 2795 patients for analysis. Among them, 657 patients had received lamivudine therapy and 2138 patients had not.

Histological findings of the liver were scored with respect to the grade of inflammation and stage of hepatic fibrosis according to the New Inuyama Histological Criteria [19] by a pathologist at each institution.

2.3. Lamivudine treatment

The lamivudine treatment group consisted of 657 patients who had received lamivudine therapy (100 mg/day). The median lamivudine treatment period was 18.9 months. Lamivudine therapy was continued until the end of the follow-up period in 45% of the patients.

2.4. Matched case-controlled study

In our analysis of the relationship between lamivudine therapy and hepatic carcinogenicity, the starting point was the day of liver biopsy. However, many patients in the lamivudine group (279 patients or 41.4%) initiated lamivudine therapy more than 2 years after liver biopsy, making them inappropriate subjects for the evaluation of the effects of lamivudine on hepatic carcinogenicity. For this reason, 377 patients who started lamivudine therapy within 2 years after liver biopsy were selected for analysis from the 657 patients in the lamivudine group. The interval from liver biopsy to lamivudine therapy was 5.8 ± 9.0 months, and the treatment

period was 23.1 ± 19.0 months (range 3–96 months). For the control group, seven factors were selected on the basis of the propensity scores from the 2138 patients who had not received lamivudine: age at the time of liver biopsy, gender, family clustering of hepatitis B, stage of hepatic fibrosis, serum albumin level, and platelet count. On that basis, 377 matching patients were selected for the control group [20].

2.5. Statistical analyses

A series of analyses was conducted using the day of liver biopsy as the starting point. Background factors at the time of liver biopsy were compared by the Student's *t*-test (numerical data) or the χ^2 test (categorical data), and differences were regarded as significant if $p < 0.05$ on both sides. Factors related to HCC were analyzed using a Cox regression model. The incidence of HCC was reported as an annual incidence rate (%/(patient/year)).

Because of the large differences in background factors between the lamivudine and control groups, the groups were matched for further analysis of HCC-related factors. For this analysis, all patients who had started lamivudine therapy within 2 years after liver biopsy were selected. The propensity score method was used to select patients from the control group [20]. Matching was done with respect to the HCC-related factors selected using the Cox regression model. After the matching, the incidence of HCC was shown by the Kaplan–Meier method and compared between the groups by the log-rank test. Differences were regarded as significant if $p < 0.05$ on both sides.

3. Results

3.1. Comparison of background factors

Table 1 demonstrates the comparison of background factors at the time of liver biopsy between the lamivudine and control groups. Significant differences were found in the mean age ($p < 0.001$), duration of follow-up ($p < 0.001$), history of IFN therapy ($p < 0.001$), inflammation of the liver ($p < 0.001$), HBeAg ($p < 0.001$), HBeAb ($p = 0.001$), serum albumin level ($p < 0.001$), AST level ($p = 0.011$), and platelet count ($p < 0.001$).

3.2. Evaluation of factors related to hepatic carcinogenicity by univariate analyses

HCC occurred in 31 of the 657 patients (4.7%) in the lamivudine group and in 239 of the 2138 patients (11.2%) in the control group. The mean follow-up periods after liver biopsy were 4.9 and 6.2 years in the lamivudine and control groups, respectively. Thus, the crude incidence of HCC determined was 1.0 and 1.8%/(patient/year) in the lamivudine and control groups, respectively.

Table 2 shows the incidences of HCC in the lamivudine and control groups in an analysis stratified with respect to background factors. In the lamivudine group, HCC did not occur in patients whose histological findings were grade 0 in inflammation and stage 0 in fibrosis, and significant intergroup differences were noted in this respect. No significant differences were observed other than in the histological findings.

3.3. Evaluation of factors related to hepatic carcinogenicity using a multivariate Cox regression model

Factors contributing to the incidence of HCC were analyzed using a Cox regression model (Table 3). The following variables were selected by the forward–backward stepwise selection method: lamivudine therapy (no therapy, $p = 0.002$), gender (male, $p < 0.001$), family history of hepatitis B (present, $p = 0.015$), age at the time of liver biopsy (older than 40 years, $p < 0.001$), stage of liver fibrosis (more than F2, $p < 0.001$), serum albumin level (less than 4.0 g/dL, $p = 0.001$), and platelet count (less than 150,000/μL, $p < 0.001$)). This analysis showed that lamivudine reduces the risk of HCC.

3.4. Evaluation of factors related to hepatic carcinogenicity by a six-factor matched case-controlled study

Matched case-control analyses were performed for six factors (sex, family history of hepatitis B, age at the time of liver biopsy, stage of liver fibrosis, serum albumin level, and platelet count). There were no significant differences in background factors between the groups, as shown in Table 4. The mean follow-up period in the control group (5.3 years) was about twice that in the lamivudine group (2.7 years). In the lamivudine group, HCC occurred in 4 of 377 patients (1.1%), with an annual incidence rate of 0.4%/(patient/year), compared to 50 of 377 patients (13.3%) and 2.5%/(patient/year), respectively, in the control group. A comparison of the cumulative HCC incidence between the two groups by the Kaplan–Meier method showed a significantly lower incidence in the lamivudine group ($p < 0.001$) (Fig. 1).

Next, the background factors were compared between patients with HCC and those without it in the lamivudine and control groups. In the lamivudine group (Table 5), the mean age was significantly higher in patients with HCC than in those without it (55.0 years versus 41.3 years, $p = 0.024$), but there were no significant differences in the other factors. In the control group (Table 6), the mean age was significantly higher in patients with HCC than in those without it (50.6 years versus 40.0 years, $p < 0.001$). Significant differences were also noted in the stage of liver fibrosis ($p < 0.001$), serum albumin level ($p < 0.001$), and platelet count ($p < 0.001$), suggesting that underlying liver disease was more advanced in patients who developed HCC.

Table 1
Comparison of background factors between lamivudine group and control group assessed at the time of liver biopsy

Parameter	Lamivudine group ($n=657$)	Control group ($n=2138$)	p-Value
Gender[a]			
Male	503 (76.6%)	1583 (74.0%)	0.194
Female	154 (23.4%)	555 (26.0%)	
Age (years)[b]	40.9 ± 11.0	37.3 ± 12.4	<0.001
Follow-up period (years)[b]	4.9 ± 4.4	6.2 ± 5.5	<0.001
Family clustering of hepatitis B[a]			
Yes	376 (57.2%)	1085 (50.7%)	0.011
No	242 (36.8%)	924 (43.2%)	
Unknown	39 (5.9%)	129 (6.0%)	
Drinking during the course of the study (>ethanol 80 g/day)			
Yes	69 (10.5%)	359 (16.8%)	<0.001
No	557 (84.8%)	1708 (79.9%)	
Unknown	31 (4.7%)	71 (3.3%)	
IFN therapy[a]			
Yes	269 (40.9%)	812 (38.0%)	<0.001
No	369 (56.2%)	1306 (61.1%)	
Unknown	19 (2.9%)	20 (0.9%)	
Liver histology			
Grade of inflammation[a]			
A0	15 (2.3%)	84 (3.9%)	<0.001
A1	194 (29.5%)	642 (30.0%)	
A2	283 (43.1%)	996 (46.6%)	
A3	142 (21.6%)	389 (18.2%)	
Unknown	23 (3.5%)	27 (1.3%)	
Stage of fibrosis[a]			
F0	12 (1.8%)	49 (2.3%)	0.491
F1	201 (30.6%)	721 (33.7%)	
F2	167 (25.4%)	524 (24.5%)	
F3	171 (26.0%)	491 (23.0%)	
F4	98 (14.9%)	331 (15.5%)	
Unknown	8 (1.2%)	22 (1.0%)	
HBeAg[a]			
+	355 (54.0%)	1272 (59.5%)	<0.001
−	280 (42.6%)	723 (33.8%)	
Unknown	22 (3.3%)	143 (6.7%)	
HBeAb[a]			
+	215 (32.7%)	642 (30.0%)	0.001
−	418 (63.6%)	1330 (62.2%)	
Unknown	24 (3.7%)	166 (7.8%)	
Albumin (g/dL)[b]	4.01 ± 0.49 ($n=629$)	4.14 ± 0.49 ($n=1941$)	<0.001
AST (IU/L)[b]	110.2 ± 131.8 ($n=593$)	94.5 ± 131.5 ($n=2023$)	0.011
ALT (IU/L)[b]	183.4 ± 211.1 ($n=641$)	163.5 ± 234.3 ($n=2022$)	0.056
Platelet count ($\times 1000/mm^3$)[b]	165.4 ± 54.9 ($n=629$)	176.9 ± 59.6 ($n=1931$)	<0.001

[a] Data are expressed as positive numbers (%).
[b] Data are expressed as means ± S.D.

4. Discussion

It is clear that this study has several limitations: it is not prospective, it is not randomized, there is no single regimen of lamivudine, and there is a lack of virological analysis (including that of the HBV genotype and that of YMDD mutations). It would be desirable to conduct a well-designed prospective study using controls. However, because lamivudine has been used in general practice under the insurance system in Japan, it is difficult to conduct a prospective and randomized control study of lamivudine therapy for chronic hepatitis B. In addition, it is ethically unacceptable to leave patients untreated for a long period of time in a control group, because lamivudine has been shown to abate hepatitis and improve histological findings of the liver [12–16].

Table 2
Comparison of the incidence of HCC in relation to each background factor between lamivudine group and control group

Parameter	Category	Group	Total number of patients (number)	No. of patients with HCC (number)	Average follow-up period (year)	Adjusted incidence of HCC (%/year)
Gender	Male	Lamivudine group	503	27	5.0	1.07
		Control group	1583	191	6.4	1.89
	Female	Lamivudine group	154	4	4.3	0.60
		Control group	555	48	5.6	1.54
Age (years)	<30	Lamivudine group	110	2	4.7	0.39
		Control group	642	8	5.9	0.21
	30≦ and <40	Lamivudine group	192	9	5.7	0.82
		Control group	646	52	6.8	1.18
	40≦ and <50	Lamivudine group	206	9	5.3	0.82
		Control group	491	75	6.7	2.28
	50≦	Lamivudine group	149	11	3.3	2.24
		Control group	359	104	5.3	5.47
Duration of lamivudine treatment (years)	<1	Lamivudine group	178	7	5.0	0.79
		Control group	–	–	–	–
	1≦ and <2	Lamivudine group	215	13	4.4	1.37
		Control group	–	–	–	–
	2≦ and <3	Lamivudine group	145	7	4.6	1.05
		Control group	–	–	–	–
	3≦	Lamivudine group	107	4	5.9	0.63
		Control group	–	–	–	–
Family clustering of hepatitis B	No	Lamivudine group	242	10	4.8	0.86
		Control group	924	100	6.4	1.69
	Yes	Lamivudine group	376	20	5.0	1.06
		Control group	1085	128	5.9	2.00
	Unknown	Lamivudine group	39	1	4.4	0.58
		Control group	129	11	8.2	1.04
Drinking during the course of the study (>ethanol 80 g/day)	No	Lamivudine group	557	23	4.8	0.86
		Control group	1708	158	5.8	1.59
	Yes	Lamivudine group	69	7	5.6	1.81
		Control group	359	76	7.8	2.71
	Unknown	Lamivudine group	31	1	3.8	0.85
		Control group	71	5	7.7	0.91
IFN therapy	No	Lamivudine group	369	19	4.2	1.23
		Control group	1306	167	6.0	2.13
	Yes	Lamivudine group	269	12	6.0	0.74
		Control group	812	70	6.5	1.33
	Unknown	Lamivudine group	19	0	2.6	0.00
		Control group	20	2	7.9	1.27
Liver histology Grade of inflammation	A0	Lamivudine group	15	0	9.3	0.00
		Control group	84	8	6.6	1.44
	A1	Lamivudine group	194	4	5.4	0.38
		Control group	642	59	6.4	1.44
	A2	Lamivudine group	283	15	4.9	1.08
		Control group	996	109	6.3	1.74
	A3	Lamivudine group	142	10	3.4	2.07
		Control group	389	52	5.5	2.43
	Unknown	Lamivudine group	23	2	6.1	1.43
		Control group	27	11	8.7	4.68

Table 2 (*Continued*)

Parameter	Category	Group	Total number of patients (number)	No. of patients with HCC (number)	Average follow-up period (year)	Adjusted incidence of HCC (%/year)
Stage of fibrosis	F0	Lamivudine group	12	0	7.2	0.00
		Control group	49	3	5.7	1.07
	F1	Lamivudine group	201	6	6.0	0.50
		Control group	721	29	6.7	0.60
	F2	Lamivudine group	167	8	4.7	1.02
		Control group	524	38	5.8	1.25
	F3	Lamivudine group	171	11	4.0	1.61
		Control group	491	61	6.0	2.07
	F4	Lamivudine group	98	6	3.6	1.70
		Control group	331	99	6.2	4.82
	Unknown	Lamivudine group	8	0	6.7	0.00
		Control group	22	9	8.3	4.93
HBeAg	–	Lamivudine group	280	10	4.2	0.85
		Control group	723	83	6.4	1.79
	+	Lamivudine group	355	19	5.3	1.01
		Control group	1272	134	6.0	1.76
	Unknown	Lamivudine group	22	2	6.2	1.47
		Control group	143	22	7.4	2.08
HBeAb	–	Lamivudine group	418	19	4.9	0.93
		Control group	1330	137	6.0	1.72
	+	Lamivudine group	215	10	4.7	0.99
		Control group	642	75	6.3	1.85
	Unknown	Lamivudine group	24	2	6.1	1.37
		Control group	166	27	7.4	2.20
Albumin (g/dL)	<4.0	Lamivudine group	257	19	4.5	1.64
		Control group	619	113	5.7	3.20
	4.0≦	Lamivudine group	372	9	4.9	0.49
		Control group	1322	90	6.1	1.12
AST (IU/L)	<50	Lamivudine group	187	7	5.7	0.66
		Control group	905	82	6.1	1.49
	50≦ and <100	Lamivudine group	200	14	4.7	1.49
		Control group	572	81	5.9	2.40
	100≦ and <200	Lamivudine group	142	7	5.1	0.97
		Control group	367	31	6.2	1.36
	200≦	Lamivudine group	64	2	4.4	0.71
		Control group	179	15	6.0	1.40
ALT (IU/L)	<50	Lamivudine group	117	5	4.7	0.91
		Control group	570	69	6.1	1.98
	50≦ and <100	Lamivudine group	155	7	4.9	0.92
		Control group	506	60	5.8	2.04
	100≦ and <150	Lamivudine group	109	9	4.7	1.76
		Control group	297	36	5.9	2.05
	150≦	Lamivudine group	260	9	4.8	0.72
		Control group	649	44	6.2	1.09
Platelet count ($\times 1000/mm^3$)	<150	Lamivudine group	254	18	3.8	1.86
		Control group	629	125	5.8	3.43
	150≦	Lamivudine group	375	11	5.3	0.55
		Control group	1302	67	6.1	0.84

Table 3
Estimation of effects of covariates following selection of regressor in Cox regression model

Category	Hazard ratio	95% Confidence interval (CI)	*p*-Value
Lamivudine therapy			
No	1		
Yes	0.49	0.31–0.77	0.002
Gender			
Male	1		
Female	0.42	0.28–0.62	<0.001
Family clustering of hepatitis B			
No	1		
Yes	1.44	1.08–1.94	0.015
Age at liver biopsy			
<40 y.o.	1		
≧40 y.o.	2.09	1.77–2.48	<0.001
Stage of liver fibrosis			
F0 or F1	1		
F2, F3, or F4	1.43	1.24–1.64	<0.001
Serum albumin level			
<4.0 g/dL	1		
≧4.0 g/dL	0.58	0.43–0.79	0.001
Platelet count			
<150 × 1000/μL	1		
≧150 × 1000/μL	0.53	0.38–0.73	<0.001

In the analysis of retrospective studies, great precautions are required in order to eliminate any bias between lamivudine-treated and non-treated groups. To minimize inter-group bias, we conducted with the cooperation of multiple medical institutions and a large number of patients ($n=2795$). The effect of lamivudine on HCC was ultimately analyzed in a matched case-controlled study. Because the time of liver biopsy was used as the starting point in our analysis, the analytical results were not expected to appropriately reflect lamivudine's effect if the therapy was started a long time after the biopsy. Therefore, from among the 657 patients who received lamivudine therapy, we selected 377 patients who started lamivudine therapy within 2 years after biopsy. For a control group, the same number of patients ($n=377$) without lamivudine therapy was selected from the 2138 subjects.

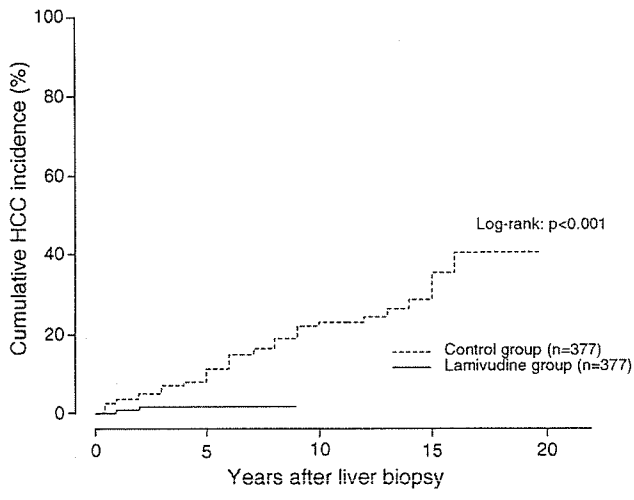

Fig. 1. Comparison of the cumulative HCC incidence between the lamivudine group (solid line) and the control group (broken line) by the Kaplan–Meier method in a case-matched control study. A significant difference was seen between the two groups ($p<0.001$, log-rank test).

The regimen was not the same in all patients who have been treated by lamivudine. It was transiently discontinued before being recommenced later in some patients, whereas it was uninterrupted throughout the follow-up period in the majority (63%) of subjects in the matched case-controlled study. The duration of lamivudine regimen was not taken into account in the design of our study. Some patients received lamivudine for relatively short periods to improve acute exacerbation of their clinical course in chronic hepatitis B. On the other hand, some patients received lamivudine for the long-term to suppress the development of HCC. In the analysis by a multivariate Cox regression model in all unmatched patients, lamivudine therapy was selected as one of the factors inhibiting the occurrence of HCC. In the matched case-controlled study, the annual occurrence rate of HCC was significantly lower (0.4%/(patient/year)) in the lamivudine group than in the control group (1.8%/(patient/year)), suggesting that lamivudine treatment is effective for inhibiting the occurrence of HCC.

Recently, Liaw et al. conducted a multicenter, centrally randomized, double-blind, placebo-controlled, parallel group study to evaluate the effects of lamivudine on the progression of chronic hepatitis B to hepatic cancer [21]. They randomized 651 patients with histologically confirmed (F3 and F4), compensated hepatic cirrhosis to receive either lamivudine or a placebo at a ratio of 2:1 and continued the treatment for up to 5 years. The study was terminated after a median treatment duration of 32.4 months (range 0–42) owing to a significant difference between the groups in the number of end points reached. The end points were reached by 7.8% of the patients receiving lamivudine and 17.7% of those receiving placebo (hazard ratio for disease progression, 0.45; $p=0.001$). The Child–Pugh score increased in 3.4% of the patients receiving lamivudine and in 8.8% of those receiving placebo (hazard ratio, 0.45; $p=0.02$), whereas HCC occurred in 3.9% of those in the lamivudine group and in 7.4% of those in the placebo group (hazard ratio, 0.49: $p=0.047$). The results of our analysis, which included patients with F0 through F2 hepatic fibrosis, were similar to those of Liaw et al. [21]. Thus, two studies demonstrated that the use of potent anti-viral agents such as lamivudine represents a major advance in the treatment of chronic hepatitis B and slows the progression of severe liver disease to liver cirrhosis as well as HCC.

Both hepatitis B and C are caused by persistent infection with hepatitis viruses, and both have a high probability of resulting in HCC. For this reason, these two diseases have a number of common traits, but some differences have been noted in their relationships with HCC. Among both

Table 4
Comparison of background factors between lamivudine group and control group assessed at the time of liver biopsy (matched case-controlled study)

Parameter	Lamivudine group ($n=377$)	Control group ($n=377$)	p-Value
Gender[a]			
Male	276 (73.2%)	273 (72.4%)	0.806
Female	101 (26.8%)	104 (27.6%)	
Age (years)[b]	41.5 ± 12.0	41.4 ± 12.2	0.950
Follow-up period (years)[b]	2.7 ± 2.1	5.3 ± 4.7	<0.001
Family clustering of hepatitis B[a]			
Yes	238 (63.1%)	242 (64.2%)	0.762
No	139 (36.9%)	135 (35.8%)	
Drinking during the course of the study (>ethanol 80 g/day)[a]			
Yes	38 (10.1%)	62 (16.4%)	0.007
No	333 (88.3%)	314 (83.3%)	
Unknown	6 (1.6%)	1 (0.3%)	
IFN therapy[a]			
Yes	129 (34.2%)	143 (37.9%)	0.046
No	236 (62.6%)	231 (61.3%)	
Unknown	12 (3.2%)	3 (0.8%)	
Liver histology			
Grade of inflammation[a]			
A0	6 (1.6%)	18 (4.8%)	0.001
A1	110 (29.2%)	101 (26.8%)	
A2	157 (41.6%)	186 (49.3%)	
A3	98 (26.0%)	72 (19.1%)	
Unknown	6 (1.6%)	0 (0.0%)	
Stage of fibrosis[a]			
F0	7 (1.9%)	6 (1.6%)	0.647
F1	103 (27.3%)	117 (31.0%)	
F2	95 (25.2%)	97 (25.7%)	
F3	107 (28.4%)	90 (23.9%)	
F4	65 (17.2%)	67 (17.8%)	
HBeAg[a]			
+	193 (51.2%)	220 (58.4%)	0.005
−	178 (47.2%)	141 (37.4%)	
Unknown	6 (1.6%)	16 (4.2%)	
HBeAb[a]			
+	126 (33.4%)	121 (32.1%)	0.030
−	245 (65.0%)	237 (62.9%)	
Unknown	6 (1.6%)	19 (5.0%)	
Albumin (g/dL)[b]	4.00 ± 0.51	4.00 ± 0.52	0.989
AST (IU/L)[b]	118.5 ± 155.4	95.5 ± 126.4	0.031
ALT (IU/L)[b]	191.7 ± 234.8	151.5 ± 180.5	0.009
Platelet count ($\times 1000/mm^3$)[b]	161.7 ± 52.7	164.3 ± 59.5	0.523

[a] Data are expressed as positive numbers (%).
[b] Data are expressed as means ± S.D.

hepatitis B patients and hepatitis C patients, HCC occurs mainly in those with advanced hepatic fibrosis, but the incidence of liver cirrhosis as a background of liver disease is lower in patients with B than in those with C. Furthermore, among hepatitis C patients HCC occurs mainly in those 60 years or older, while among hepatitis B patients it occurs mainly in those under 60 [22–24]. Studies on the cumulative incidence of HCC in hepatitis B patients showed that the HCC incidence increases linearly during the initial 12 years, plateaus, and then increases again in the 17th or 18th year [24,25]. In hepatitis C patients, on the other hand, the HCC incidence shows a continuous, linear increase [26,27]. Various findings obtained to date suggest that these clinical differences are related not only to differences in the hepatitis viral infection route and the timing of infection but also to differences in the mechanisms underlying cancer associated with hepatitis B and C. HCV is an RNA virus, and viral genes are not integrated into the host's genes, whereas HBV is a DNA virus with reverse-transcriptase activity. Thus, HBV genes are often integrated into the host's chromosomes

Table 5
Comparison of distribution of background factors between patients who developed HCC and those who did not in the lamivudine group (matched case-controlled study)

Parameter	Patients with HCC ($n=4$)	Patients without HCC ($n=373$)	p-Value
Gender[a]			
Male	3 (75.0%)	273 (73.2%)	1.000[c]
Female	1 (25.0%)	100 (26.8%)	
Age (years)[b]	55.0 ± 19.5 ($n=4$)	41.3 ± 11.9 ($n=373$)	0.024
Follow-up period (years)[b]	1.5 ± 0.6 ($n=4$)	2.7 ± 2.1 ($n=373$)	0.236
Family clustering of hepatitis B[a]			
Yes	2 (50.0%)	236 (63.3%)	0.628[c]
No	2 (50.0%)	137 (36.7%)	
Drinking during the course of the study (>ethanol 80 g/day)[a]			
Yes	1 (25.0%)	37 (9.9%)	0.393[c]
No	3 (75.0%)	330 (88.5%)	
Unknown	0 (0.0%)	6 (1.6%)	
IFN therapy[a]			
Yes	0 (0.0%)	129 (34.6%)	0.387[c]
No	4 (100.0%)	232 (62.2%)	
Unknown	0 (0.0%)	12 (3.2%)	
Liver histology			
Grade of inflammation[a]			
A0	0 (0.0%)	6 (1.6%)	0.458[c]
A1	0 (0.0%)	110 (29.5%)	
A2	3 (75.0%)	154 (41.3%)	
A3	1 (25.0%)	97 (26.0%)	
Unknown	0 (0.0%)	6 (1.6%)	
Stage of fibrosis[a]			
F0	0 (0.0%)	7 (1.9%)	0.918[c]
F1	1 (25.0%)	102 (27.3%)	
F2	1 (25.0%)	94 (25.2%)	
F3	2 (50.0%)	105 (28.2%)	
F4	0 (0.0%)	65 (17.4%)	
HBeAg[a]			
+	3 (75.0%)	190 (50.9%)	0.648[c]
−	1 (25.0%)	177 (47.5%)	
Unknown	0 (0.0%)	6 (1.6%)	
HBeAb[a]			
+	2 (50.0%)	124 (33.2%)	0.632[c]
−	2 (50.0%)	243 (65.1%)	
Unknown	0 (0.0%)	6 (1.6%)	
Albumin (g/dL)[b]	4.23 ± 0.45 ($n=4$)	4.00 ± 0.51 ($n=373$)	0.384
AST (IU/L)[b]	47.0 ± 22.8 ($n=4$)	119.4 ± 156.2 ($n=326$)	0.356
ALT (IU/L)[b]	46.3 ± 24.2 ($n=4$)	193.2 ± 235.5 ($n=372$)	0.213
Platelet count ($\times 1000/mm^3$)[b]	141.0 ± 27.0 ($n=4$)	161.9 ± 52.9 ($n=373$)	0.431

[a] Data are expressed as positive numbers (%).
[b] Data are expressed as means ± S.D.
[c] Fisher's exact test.

and play an important role in hepatic carcinogenesis [28,29]. It is known that the repeat of necrosis and regeneration of liver might accelerate the mutation of oncogenes. In addition, de novo carcinogenesis is thought to be promoted in hepatitis B patients as a result of the increased genetic instability caused by the integration of the HBV genome into the host's chromosomes. When administered to patients with hepatitis B, lamivudine decreases the blood HBV-DNA concentration and markedly improves ALT levels, with consequent improvement of liver histological findings [7,11,13,14]. An early in vitro study showed that lamivudine decreases the amount of free HBV-DNA in hepatocytes but does not affect integrated HBV genes [30]. Therefore, lamivudine is thought to inhibit HCC by abating hepatitis and not by inhibiting viral gene integration. In fact, as shown in the matched case control study, all four patients who developed HCC in the lamivudine group had non-cirrhotic liver disease, whereas 23 (46%) of 50 patients who developed HCC had liver cirrhosis. Due to the small number of patients included, however, further studies are necessary to confirm this finding.

Table 6
Comparison of distribution of background factors between patients who developed HCC and those who did not in the control group (matched case-controlled study)

Parameter	Patients with HCC ($n=50$)	Patients without HCC ($n=327$)	p-Value
Gender[a]			
Male	40 (80.0%)	233 (71.3%)	0.236[c]
Female	10 (20.0%)	94 (28.7%)	
Age (years)[b]	50.6 ± 10.1	40.0 ± 11.9	<0.001
Follow-up period (years)[b]	5.3 ± 4.3	5.2 ± 4.8	0.951
Family clustering of hepatitis B[a]			
Yes	29 (58.0%)	213 (65.1%)	0.345[c]
No	21 (42.0%)	114 (34.9%)	
Drinking during the course of the study (>ethanol 80 g/day)[a]			
Yes	14 (28.0%)	48 (14.7%)	0.050[c]
No	36 (72.0%)	278 (85.0%)	
Unknown	0 (0.0%)	1 (0.3%)	
IFN therapy[a]			
Yes	16 (32.0%)	127 (38.8%)	0.578[c]
No	34 (68.0%)	197 (60.2%)	
Unknown	0 (0.0%)	3 (0.9%)	
Liver histology			
Grade of inflammation[a]			
A0	2 (4.0%)	16 (4.9%)	0.026[c]
A1	6 (12.0%)	95 (29.1%)	
A2	27 (54.0%)	159 (48.6%)	
A3	15 (30.0%)	57 (17.4%)	
Stage of fibrosis[a]			
F0	0 (0.0%)	6 (1.8%)	<0.001[c]
F1	7 (14.0%)	110 (33.6%)	
F2	8 (16.0%)	89 (27.2%)	
F3	12 (24.0%)	78 (23.9%)	
F4	23 (46.0%)	44 (13.5%)	
HBeAg[a]			
+	26 (52.0%)	194 (59.3%)	0.564[c]
−	22 (44.0%)	119 (36.4%)	
Unknown	2 (4.0%)	14 (4.3%)	
HBeAb[a]			
+	20 (40.0%)	101 (30.9%)	0.319[c]
−	27 (54.0%)	210 (64.2%)	
Unknown	3 (6.0%)	16 (4.9%)	
Albumin (g/dL)[b]	3.63 ± 0.59	4.06 ± 0.49	<0.001
AST (IU/L)[b]	96.9 ± 100.8	95.3 ± 130.0	0.934
ALT (IU/L)[b]	132.8 ± 165.5	154.4 ± 182.7	0.431
Platelet count ($\times 1000/mm^3$)[b]	126.8 ± 50.7	170.0 ± 58.7	<0.001

[a] Data are expressed as positive numbers (%).
[b] Data are expressed as means ± S.D.
[c] Fisher's exact test.

Seven HBV genotypes (A–G) have been identified to date, and their distribution shows regional variations [31–36]. In Japan, genotypes C, B, and the other five account for 85, 12, and 3% of hepatitis B patients [36]. The virological differences between HBV genotype B and genotype C might influence not only on the natural course of hepatitis B but also the efficacy by lamivudine. The patients with HBV genotype B are frequently negative for HBeAg, have lower ALT levels and a better prognosis. In contrast, the patients with HBV genotype C tend to remain HBeAg-positive for a longer duration and tend to have elevated ALT levels and more advanced liver disease, such as liver cirrhosis and HCC. This indicates that the analysis of HBV genotypes will be needed in this study.

In conclusion, our multicenter, retrospective, matched case study indicated that lamivudine treatment might suppress the risk of HCC in patients with chronic hepatitis B. However, the study has several limitations, such as the relatively short duration of treatment and the lack of virological analyses (HBV genotype, YMDD mutation, and HBV-DNA volume). To relief these limitations, further long-term observation should be continued to clarify the conclusion.

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Appendix A

The Inuyama Hepatitis Study Group consists of the following 30 institutions and members: Dr. Sumio Watanabe (Akita University School of Medicine, Akita, Yamagata), Dr. Sumio Kawada (Yamagata University School of Medicine, Yamagata), Dr. Osamu Yokosuka (Chiba University, Graduate School of Medicine, Chiba), Dr. Kunihiko Hino (Delta Clinic, Tokorozawa), Dr. Hiromasa Ishii (Keio University, School of Medicine, Tokyo), Dr. Hiromitu Kumada (Toranomon Hospital, Tokyo), Dr. Gotaro Toda (Jikei University School of Medicine, Tokyo), Dr. Yasuyuki Arakawa (Nihon University School of Medicine, Tokyo), Dr. Nobuyuki Enomoto (Yamanashi University, School of Medicine, Kofu), Dr. Kendo Kiyosawa (Shinshu University School of Medicine, Matsumoto), Dr. Takafumi Ichida (Niigata University, Graduate School of Medical and Dental Science, Niigata), Dr. Tomoteru Kamimura (Niigata Saiseikai Hospital Dai-2, Niigata), Dr. Masashi Mizogami (Nagoya City University Graduate School of Medical Science, Nagoya), Dr. Shinichi Kakumu (Aichi Medical University, Nagoya), Dr. Hisataka Moriwaki (Gifu University School of Medicine, Gifu), Dr. Shuichi Kaneko (Kanazawa University, Graduate School of Medical Science, Kanazawa), Dr. Takeshi Okanoue (Kyoto Prefectural University, Graduate School of Medical Science, Kyoto), Dr. Norio Hayashi (Osaka University Graduate School of Medicine, Osaka), Dr. Masatoshi Kudo (Kinki University School of Medicine, Sayama), Dr. Yasushi Shiratori (Okayama University, Graduate School of Medicine and Dentist[r]y, Okayama), Dr. Gotaro Yamada (Kawasaki Hospital, Kawasaki Medical School, Okayama), Dr. Kazuaki Chayama (Hiroshima University, Graduate School of Biomedical Science, Hiroshima), Dr. Kiwamu Okita (Yamaguchi University, School of Medicine, Ube), Dr. Shigeki Kuriyama (Kagawa Medical University, Takamatsu), Dr. Morikazu Onji (Ehime University School of Medicine, Juushin-cho), Dr. Saburo Ohnishi (Kochi University School of Medicine, Nangoku), Dr. Michio Sata (Kurume University School of Medicine, Kurume), Dr. Shigetoshi Fujiyama, and Dr. Hiroshi Sasaki (Kumamoto University, Faculty of Medical and Pharmaceutical Science, Kumamoto), Dr. Hirohito Tsubouchi (Miyazaki University School of Medicine, Miyazaki), and Dr. Hiromi Ishibashi and Dr. Hiroshi Yatsuhashi (Nagasaki Medical Center, Omura).

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] Chen CJ, Wang LY, Wheiyu M. Epidemiology of hepatitis B virus infection in the Asia-Pacific region. J Gastroenterol Hepatol 2000;15(Suppl):E3–6.
[3] Lok SF. Chronic hepatitis B. N Engl J Med 2002;346:1682–3.
[4] Chu CM. Natural history of chronic hepatitis B virus infection in adults with emphasis on the occurrence of cirrhosis and hepatocellular carcinoma. J Gastroenterol Hepatol 2000;15(Suppl):E25–30.
[5] Tang B, Kruger WD, Chen G, et al. Hepatitis B viremia is associated with increased risk of hepatocellular carcinoma in chronic carriers. J Med Virol 2004;72:35–40.
[6] Oda T. Viral hepatitis and hepatocellular carcinoma prevention strategy in Japan. Jpn J Cancer Res 1999;90:1051–60.
[7] Dienstag JL, Perrillo RP, Schiff ER, et al. A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med 1995;333:1657–61.
[8] Nevens F, Main J, Honkoop P, et al. Lamivudine therapy for chronic hepatitis B: a six-month randomized dose-ranging study. Gastroenterology 1997;113:1258–63.
[9] Lai CL, Chien RN, Leung NW, et al. A one-year trial of lamivudine for chronic hepatitis B. N Engl J Med 1998;339:61–8.
[10] Severini A, Liu XY, Wilson JS, et al. Mechanism of inhibition of duck hepatitis B virus polymerase by (−)-beta-L-2′,3′-dideoxy-3′-thiacytidine. Antimicrob Agents Chemother 1995;39:1430–5.
[11] Ohkoshi S, Norio O, Ichida T. The long-term clinical outcome of 1-year treatment of chronic hepatitis B with lamivudine—5 years observation. Hepatol Res 2003;27:13–7.
[12] Leung NW, Lai CL, Chang TT, et al. Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis Be antigen seroconversion rates: results after 3 years of therapy. Hepatology 2001;33:1527–32.
[13] Lee HC, Suh DJ. Lamivudine therapy for decompensated liver cirrhosis related to hepatitis B virus infection. Intervirology 2003;46:388–93.
[14] Malekzadeh R, Mohamadhejad M. Reversibility of cirrhosis in chronic hepatitis B. Clin Gastroenterol Hepatol 2004;2:344–7.
[15] Fontana RJ, Hann HL, Perrillo RP, et al. Determinants of early mortality in patients with decompensated chronic hepatitis B treated with antiviral therapy. Gastroenterology 2002;123:719–27.
[16] Villeneuve JP, Condreay LD, Willems B, et al. Lamivudine treatment for decompensated cirrhosis resulting from chronic hepatitis B. Hepatology 2000;31:207–10.
[17] Lok AS, Hussain M, Cursano C, et al. Evolution of hepatitis B virus polymerase gene mutations in hepatitis E antigen-negative patients receiving lamivudine therapy. Hepatology 2000;32:1145–53.
[18] Fujioka S, Shimomura H. Two cases of chronic hepatitis B with emergence of lamivudine-resistant virus during long-term therapy. Hepatol Res 1999;12:97–104.
[19] Ichida F, Tsuji T, Omata M, et al. New Inuyama classification: new criteria for histological assessment of chronic hepatitis. Int Hepatol Com (Hepatol Res) 1996;6:112–9.
[20] D'agostino RB. Tutorial in biostatistics: propensity score methods for bias reduction in the comparison of a treatment to a non-randomized control group. Stat Med 1998;17:2265–88.
[21] Liaw YF, Sung JJY, Chow WC, et al. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med 2004;351:1521–31.
[22] Hamada H, Yatsuhashi H, Yano K, et al. Impact of aging on the development of hepatocellular carcinoma in patients with posttransfusion chronic hepatitis C. Cancer 2002;95:331–9.
[23] Ohnishi W, Kitamoto M, Aikata H, et al. Impact of aging on the development of hepatocellular carcinoma in patients with hepatitis C virus infection in Japan. Scand J Gastroenterol 2003;38:894–900.
[24] Kiyosawa K, Umemura T, Ichijo T, et al. Hepatocellular carcinoma: recent trends in Japan. Gastroenterology 2004;127:S17–26.
[25] Ikeda K, Saitoh S, Koida I, et al. A multivariate analysis of risk factors for hepatocellular carcinogenesis: a prospective observa-

tion of 795 patients with viral and alcoholic cirrhosis. Hepatology 2003;18:47–53.
[26] Ikeda K, Saitoh S, Suzuki Y, et al. Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. J Hepatol 1998;28:930–8.
[27] Takano S, Yokosuka O, Imazeki F, et al. Incidence of hepatocellular carcinoma in chronic hepatitis B and C, a prospective study of 251 patients. Hepatology 1995;21:650–5.
[28] Buendia MA. Hepatitis B viruses and hepatocellular carcinoma. Adv Cancer Res 1992;59:167–226.
[29] Robinson WS. Molecular events in the pathogenesis of hepadnavirus-associated hepatocellular carcinoma. Ann Rev Med 1994; 45:297–323.
[30] Doong SL, Tsai CH, Schinazi kF, et al. Inhibition of the replication of hepatitis B virus in vitro by 2′,3′-dideoxy-3′-thiacytidine and related analogues. Proc Natl Acad Sci USA 1991;88:8495–9.
[31] Miyakawa Y, Mizokami M. Classifying hepatitis B virus genotype. Intervirology 2003;46:329–38.
[32] Orito E, Ichida T, Sakugawa H, et al. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology 2001;34:590–4.
[33] Kao JH, Chen PJ, Lai MY, et al. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology 2000;118:554–9.
[34] Sugauchi F, Kumada H, Acharya SA, et al. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. J Gen Virol 2004;85:811–20.
[35] Orito E, Mizokami M. Hepatitis B virus genotypes and hepatocellular carcinoma in Japan. Intervirology 2003;46:408–12.
[36] Orito E, Ichida T, Sakugawa H, et al. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology 2001;34:590–4.