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Hepatology Research 2010; **40**: 432–437 doi: 10.1111/j.1872-034X.2009.00612.x

Short Communication

Expression of the RNA-binding protein Musashi1 in adult liver stem-like cells

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Aim: Musashi1 is an RNA-binding protein that regulates the Notch signaling pathway in stem cells. Our previous study revealed that Musashi1 is expressed in early hepatocytes during liver development in the mouse. However, whether this unique protein is expressed with Notch signaling markers in adult liver stem-like cells remains unknown.

Methods: Established hepatic stem-like cells (HSLC), which were derived from adult Sprague–Dawley rats, were used for experiments *in vitro*. HSLC were differentiated into mature cells in terms of producing albumin when co-cultured with epidermal growth factor (EGF). The mRNA expression of *Musashi1*, *Notch* family (*Notch1* and *Notch2*), *Jagged1* and *Hes1* was examined in HSLC before and after cell differentiation using polymerase chain reaction-based techniques. Protein expression of Musashi1 was examined in the HSLC and normal mature hepatocytes by immunofluorescence staining.

Results: The mRNA expression of *Musashi1*, *Notch1*, *Jagged1* and *Hes1* was detected in the original HSLC before culturing with EGF but not in primary cultured mature hepatocytes. The mRNA expression of *Musashi1* and *Hes1* was found to be downregulated in differentiated HSLC that produce albumin. Protein expression of Musashi1 was detectable in the original HSLC but not in both differentiated HSLC and mature hepatocytes.

Conclusion: These findings demonstrate that the RNA-binding protein Musashi1 is expressed with Notch signaling markers in adult liver stem-like cells.

Key words: hepatic stem cell, Musashi1, Notch, liver, RNA-binding protein

INTRODUCTION

IT HAS BEEN demonstrated that liver cell regeneration originates from epithelial cells through two mechanisms. First, mature hepatocytes can proliferate independently by division after the loss of liver cells, as is often observed after a partial hepatectomy.[1] An alternative mechanism, in which liver stem/progenitor cells that subsequently differentiate into hepatocytes, cholangiocytes or other liver components are produced, is involved in reconstruction of the liver after severe liver damage.[2] The signal transduction in liver stem cell differentiation has not been fully investigated.

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Received 25 February 2009; revision 2 September 2009; accepted 11 September 2009.

Musashi1, a neural RNA-binding protein was first isolated as a mammalian homolog of the *Drosophila* protein, which is required for the asymmetric division of sensory neural precursor cells.[3] It is also known that Musashi1 is a positive regulator of the Notch signaling pathway,[4,5] which is essential for the determination of cell fate,[6] thereby maintaining the self-renewing ability of stem cells. Thus, Musashi1 is closely involved in the regulation of asymmetric cell division of stem-like cells, which generates differentiated cells.

In our previous study, we have shown that Musashi1 is expressed in early hepatocytes during liver development in the mouse.[7] Whether this unique RNA-binding protein has any association with the process of liver stem cell differentiation in adults is of considerable interest. In this study, we investigated the expression of Musashi1 in adult liver stem-like cells that regulates the Notch signaling. Our results suggest a possible association of Musashi1 in liver stem-like cell differentiation.

METHODS

Liver stem cell line and culture

AN ESTABLISHED HEPATIC epithelial stem-like cell (HSLC) line derived from the healthy liver of adult male Sprague–Dawley rats[8] was used for experiments *in vitro*. This cell line has an immature liver cell phenotype with positive expression only for α-fetoprotein and negative for both albumin and cytokeratin (CK)19, and exhibits the potential to differentiate into cells of the hepatocytic lineage and serve as stem-like cells for differentiated hepatocytes.[8] The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C. Spheroidal aggregates of hepatocytes are known to exhibit higher functions than hepatocytes produced by monolayer culture.[9,10] In order to demonstrate the differentiation of HSLC into cells of the hepatocytic lineage *in vitro*, the cells were co-cultured for 24 h with epidermal growth factor (EGF) at a concentration of 10 ng/mL. These cells formed spheroids in culture. The expression of albumin was examined as a marker of cell differentiation in culture cells. The mRNA expression profile for both CK19 and tyrosine aminotransferase (TAT) was also examined in HSLC before and after culturing with EGF. Primary cultured, normal adult hepatocytes were used as control cells.[11]

Western blot analysis of albumin expression in HSLC

Expression of albumin was analyzed in HSLC cultured with or without EGF. The proteins were prepared by treating the cells with cell lysis buffer, followed by centrifugation. A 15-μg sample of proteins was subjected to a 10% sodium dodecylsulfate polyacrylamide ready gel (Bio-Rad Laboratories, Richmond, CA, USA). Resolved proteins were transferred electrophoretically to an Immunobilon-P membrane (Millipore, Bedford, MA, USA) at 4°C and processed for immunodetection. After blocking with 5% nonfat milk for 1 h at room temperature, the membrane was incubated with rabbit antirat albumin antibody (1:200 dilution; Cappel, Aurora, OH, USA) at 37°C for 2.5 h. The membrane was then incubated with alkaline phosphatase-labeled goat antirabbit immunoglobulin (Ig)G antibody (1:1000 dilution; KPL, Gaithersburg, MD, USA) for 1.5 h at room temperature. Detection of the immunoreaction was performed with the BCIP/NBT phosphate substrate system (KPL), according to the manufacturer's protocol.

Reverse transcription polymerase chain reaction (RT–PCR)

The mRNA expression of *CK19*, *TAT*, *Notch* family (*Notch1* and *Notch2*) and its ligand *Jagged1*, and *Hes1* in both HSLC cultured with or without EGF and in primary cultured mature hepatocytes were examined by RT–PCR according to the procedure we previously described.[7] The PCR consisted of 35 cycles at a denaturation temperature of 94°C for 30 s, an annealing temperature of 58°C for 2 min and an extension temperature of 72°C for 1 min using a Perkin-Elmer 9600 thermal cycler platform (Perkin-Elmer, Norwalk, CT, USA). The primers for PCR to detect mRNA expression were: *Musashi1*, 5′-GGC TTCGTCACTTTCATGGACCAGGCG-3′ and 5′-GGGACC TGGTAGGTGTAAC-3′ (PCR product; 542 bp); *Hes1*, 5′-CCACTGCTACCCGTAAAGTC-3′ and 5′-GGCCTGAG GCTCTCAGTTCC-3′ (228 bp); *Notch1*, 5′-GACTATGCC TGCAGCTGTGCC-3′ and 5′-GGCTGCAGGGCACGTA GG-3′ (421 bp); *Notch2*, 5′-ATGTGTGTTACCTACCA CA-3′ and 5′-CCACAGTGGTACAGGTACTT-3′ (371 bp); *Jagged1*, 5′-CATCATAGCCTGTGAGCCTTC-3′ and 5′-ATATCATCCTCTTCCACTTCC-3′ (492 bp); CK19, 5′-TT GCGCGACAAGATTCTTGG-3′ and 5′-CATCTCACTCAG GATCTTGG-3′ (361 bp); and TAT, 5′-TGAACAGCAC TACCACTGTG-3′ and 5′-AGGCATCCTCCGTCTTCT GC-3′ (380 bp). The PCR reaction for β-actin was performed as an internal control (191 bp).[7]

Quantitation of Musashi1 mRNA levels in HSLC before and after differentiation

The total cellular RNA extracted from Hep3B cells positive for *Musashi1* mRNA expression[7] was used as a standard. The methods for RNA isolation and cDNA amplification were performed as previously.[7] To quantitate *Musashi1* mRNA levels in HSLC before and after differentiation, real-time PCR was performed using a LightCycler quick system 350S (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. The primers for detection of *Musashi1* mRNA in the real-time PCR were 5′-GGCTTCGTCACTTTCATGGA CCAGGCG-3′ and 5′-GGGACCTGGTAGGTGTAAC-3′. Quantitation test was performed in quadruplicate and the results were expressed as mean ± standard error (SE). Differences at $P < 0.05$ by Mann–Whitney *U*-test were considered significant.

Immunofluorescence staining for Musashi1 in HSLC

Expression of Musashi1 was analyzed by indirect immunofluorescence staining in HSLC cultured with or

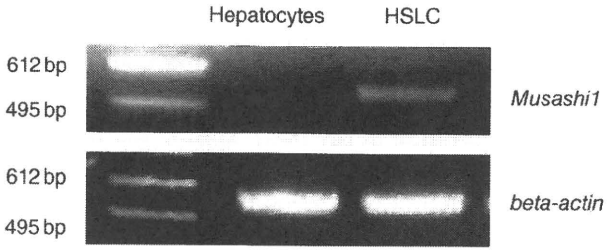

Figure 1 Reverse transcription polymerase chain reaction (RT-PCR) analysis of *Musashi1* mRNA expression in hepatic stem-like cells (HSLC) and primary cultured mature hepatocytes. The mRNA expression of *Musashi1* was detected in HSLC but not in hepatocytes. The predicted size of the PCR-amplified *Musashi1* product was 542 bp.

without EGF. A polyclonal rabbit anti-Musashi peptide antibody (Chemicon International, Temecula, CA, USA) that recognizes human and rodent Musashi1 was used as a primary antibody. Fluorescein isothiocyanate-labeled F(ab')$_2$ fragments of goat antirabbit IgG (Dako-Cytomation, Kyoto, Japan) were used as a secondary antibody. The cells were examined with the aid of a fluorescence microscope.

RESULTS

Expression of Musashi1 mRNA and Musashi1 protein in HSLC

THE MRNA EXPRESSION of *Musashi1* was detected in HSLC but not in primary cultured mature hepatocytes by RT-PCR (Fig. 1). The RT-PCR product of the *Musashi1* mRNA amplified using specific primers predicted a band of 542 bp. Protein expression of Musashi1 was detected in HSLC by immunofluorescence staining (Fig. 2), but it was not detected in primary cultured hepatocytes.

Downregulated expression of Musashi1 mRNA and Musashi1 protein in differentiated HSLC producing albumin

Hepatic stem-like cells were cultured with 10 ng/mL EGF for 24 h and harvested when they formed spheroids. Albumin expression in these cells was examined as a marker of cell differentiation from an immature to a mature state. Albumin expression was not detected in HSLC before culturing with EGF, but was detectable by western blot analysis after culturing with EGF. Quantitative analysis revealed that the level of *Musashi1* mRNA in the differentiated HSLC was significantly lower than that of original HSLC ($1.06 \pm 1.34 \times 10^{10}$ copies/mL vs $4.33 \pm 2.68 \times 10^{13}$ copies/mL, mean ± SE, $P < 0.05$) (Fig. 3). Expression of Musashi1 protein was not detected in differentiated HSLC producing albumin by immunofluorescence staining.

Changes in mRNA expression of the Notch signaling markers in HSLC differentiation

Because the expression of *Musashi1* mRNA was found to be downregulated in the cell differentiation process, the expression of the *Notch* family mRNA was investigated in HSLC before and after culturing with EGF by RT-PCR analysis. *Notch1* mRNA expression was detected in HSLC before and after culturing with EGF, but its expression was not detected in primary cultured mature hepatocytes. *Notch2* mRNA expression was found in HSLC before and after culturing with EGF as well as in primary cultured mature hepatocytes. The notch ligand *Jagged1* mRNA expression was detected in HSLC before and after culturing with EGF, but its expression was not detected in primary cultured mature hepatocytes. *Hes1* mRNA expression was detected in the original HSLC, but not in those producing albumin after culturing with EGF, and nor was it detected in primary cultured mature hepatocytes. The biliary cell marker, CK19 mRNA expression was not detected in any cells examined. The hepatocyte marker, TAT mRNA expression was not detected in the original HSLC, but its expression was detectable in those

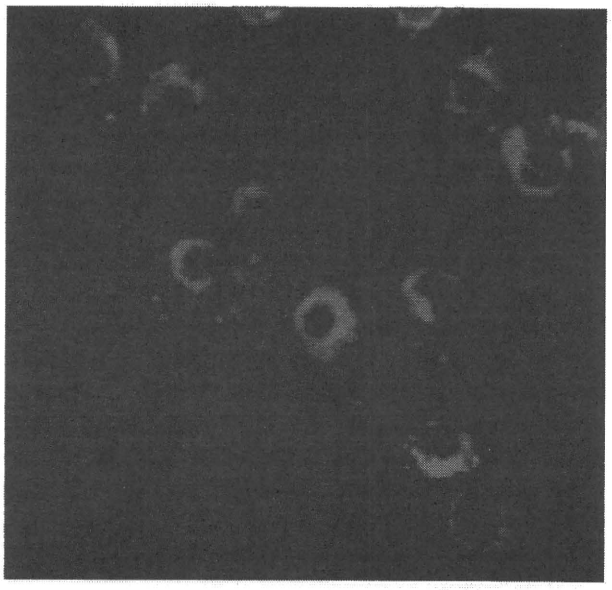

Figure 2 Immunofluorescence staining for Musashi1 protein in the cytoplasm of hepatic stem-like cells (original magnification ×400).

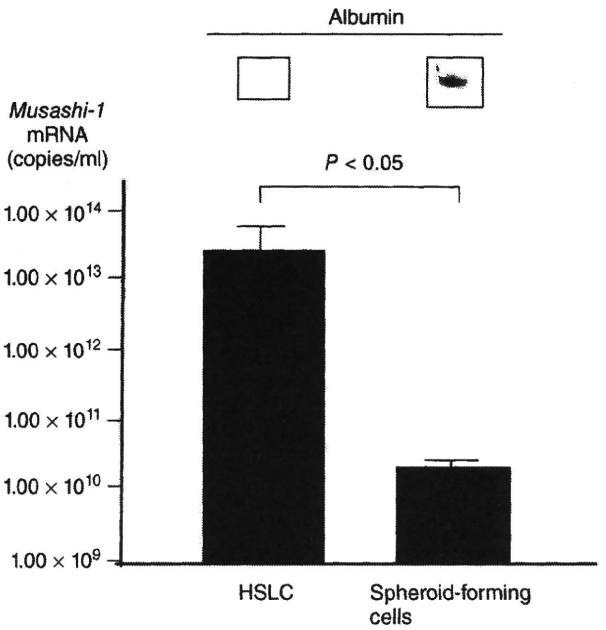

Figure 3 Western blot analysis of albumin expression in hepatic stem-like cells (HSLC). Albumin expression was not detected in the original HSLC, but was detected in spheroid-forming cells after culturing with epidermal growth factor (EGF). Real-time polymerase chain reaction analysis of *Musashi1* mRNA expression in HSLC before and after culturing with EGF. The level of *Musashi1* mRNA in the spheroid-forming differentiated cells was significantly lower than that of original HSLC. Quantitation test was performed in quadruplicate.

producing albumin after culturing with EGF as well as in primary cultured mature hepatocytes (Fig. 4).

DISCUSSION

ALTHOUGH A CLOSE association has been shown to exist between Musashi1 and Notch signaling in neural stem cell differentiation,[4] the involvement of such a mechanism in the differentiation of stem cells in digestive organs has not been fully elucidated. Recently, it was shown that Musashi1 is expressed in putative intestinal stem cells [12] and can be used as a marker of stem cells and early-lineage progenitor cells in murine intestinal tissue.

In this study, we have demonstrated that Musashi1 is expressed in putative rat liver stem-like cells at the mRNA and protein level. Interestingly, the mRNA expression of *Hes1* was downregulated along with *Musashi1* mRNA expression in the differentiated cells that produced albumin. Notch proteins were initially identified in *Drosophila* and *Caenorhabditis elegans*, but have subsequently been identified in vertebrate species.[13] It has been reported that there is an association between expression of the *Notch* family, and bile duct formation,[14] liver cell regeneration after partial hepatectomy[15] and neovascularization in the human diseased liver,[16] although no such association has been demon-

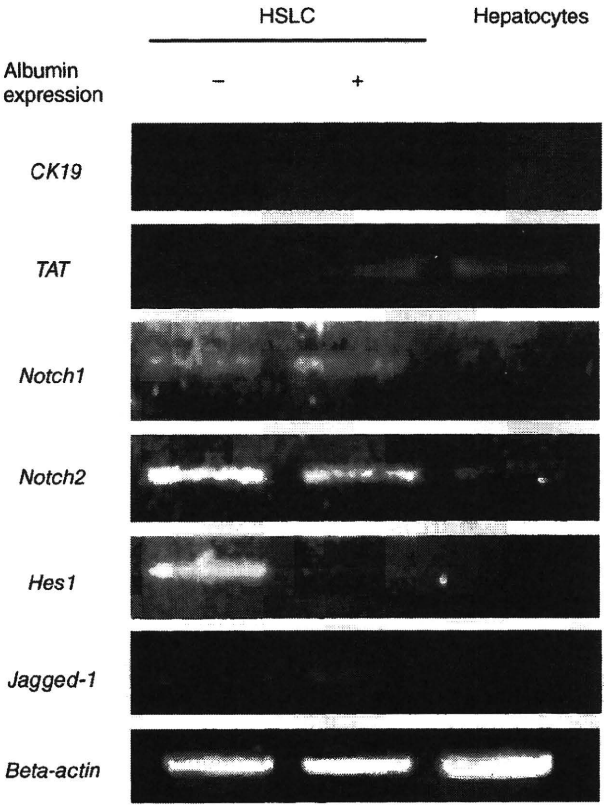

Figure 4 The mRNA expression for cytokeratin (CK)19, tyrosine aminotransferase (TAT) and *Notch* family in hepatic stem-like cells (HSLC), as revealed by reverse transcription polymerase chain reaction (RT–PCR) analysis. CK19 mRNA expression was not detected in all cells. TAT mRNA expression was not detected in the original HSLC, but it was detectable in differentiated cells producing albumin as well as in mature hepatocytes. The mRNA expression for *Notch1* and *Jagged1* was detected in HSLC, but it was not detected in mature hepatocytes. *Notch2* mRNA expression was detected in all cells. The mRNA expression of *Hes1* was detected in HSLC, but was undetectable in differentiated cells producing albumin as well as in mature hepatocytes. The predicted size of the PCR-amplified product was 361 bp for CK19, 380 bp for TAT, 421 bp for *Notch1*, 371 bp for *Notch2*, 492 bp for *Jagged-1* and 228 bp for *Hes1*.

strated in liver stem cells. Both Notch-1 and its ligand Jagged-1 have been detected in the hepatic progenitor cells, referred to as oval cells, in the liver of the 2-acetylaminofluorene 70% hepatectomy models.[17] In this study, *Notch1* and *Jagged1* mRNA expressions were detectable in HSLC , but not in mature rat hepatocytes. The absence of Notch1 expression in mature hepatocytes has also been demonstrated in humans.[17] *Hes1* mRNA expression is activated by a nuclear translocation of the Notch intracellular domain.[11] In the present study, we could show that *Hes1* mRNA expression was also detectable in HSLC at a location downstream of this signaling. The mRNA expression of *Notch1–Hes1* signaling was upregulated in Musashi1-positive HSLC and was undetectable in the differentiated cells producing albumin. To confirm the association of Musashi1 with an activation of the Notch signaling, it would be important to see if changes in Musashi1 expression level by the gene knockdown influence of liver stem-like cell differentiation. In addition, expression of Musashi1 in the liver tissue remains unknown. Further studies are needed to elucidate these issues.

The roles of Musashi1 in the development of liver morphology and function remain unknown. A report on the *Musashi1* gene disruption model revealed that homozygous newborn mice are not prone to immediate death, but frequently develop obstructive hydrocephalus with aberrant proliferation of ependymal cells.[5] As Musashi2, another member of the RNA-binding protein family,[18] is co-expressed in this model, gene compensation of *Musashi2* in the *Musashi1* disruption model might contribute to organ development, and hence improve the chances of survival. Analyses of alteration of liver-specific mRNA expression as well as liver morphology in such a model would provide information that could extend our understanding of the role of Musashi1 in the development of liver morphology and function.

In conclusion, the results of this study suggest that Musashi1 is expressed with Notch signaling markers in liver stem-like cells as well as in neural stem cells in adults. The role of Musashi1 in liver regeneration warrants further investigation.

ACKNOWLEDGEMENTS

THE AUTHORS THANK Dr Toshihiro Sugiyama for the gift of HSLC and Dr Hideyuki Okano for critical reading of the manuscript. This paper was supported by a Grant-in-Aid from the Global COE program of the Japan Society for the Promotion of Science, and in part by a grant from the Ministry of Health, Labor and Welfare of Japan.

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Hepatology Research 2010; 40: 125–134 doi: 10.1111/j.1872-034X.2009.00565.x

Original Article

Correlation of YMDD mutation and breakthrough hepatitis with hepatitis B virus DNA and serum ALT during lamivudine treatment

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Aim: Continuous lamivudine treatment is associated with high frequency of drug resistance. We analyzed the incidence of tyrosine-methionine-aspartate-aspartate (YMDD) motif mutant and breakthrough hepatitis (BTH) in hepatitis B virus (HBV) DNA positive patients receiving lamivudine for > 1 year and correlated it with HBV DNA and alanine aminotransferase (ALT) levels to evaluate if these measurements can provide a practical option for monitoring patients in clinical practice and define early switch from lamivudine therapy.

Methods: Of the 929 patients receiving lamivudine for > 1 year, 359 patients who maintained an ALT level of ≤ 40 IU/L during the course of lamivudine treatment were stratified into two groups based on the duration of lamivudine treatment – one receiving lamivudine for < 3 years and the other for ≥ 3 years.

Results: The incidence of YMDD motif in patients receiving lamivudine for < 3 years was 27% in patients with ALT ≤ 20 IU/L, 58% with ALT ≤ 30 IU/L, and 63% with ALT ≤ 40 IU/L, ($P = 0.002$). The corresponding incidence of BTH was 2%, 7%, and 48% ($P < 0.001$). The incidence of YMDD motif and BTH in these patients was 7% and 2% with HBV DNA < 2.6 (log copies/mL) and ALT ≤ 20 IU/L, while with ALT at 21–30, the YMDD motif mutant was 16% and BTH was 0%.

Conclusion: Correlation of ALT and HBV DNA levels with YMDD motif mutant and BTH indicates that these measurements can be used in clinical practice for deciding early switch from lamivudine to other suitable antiviral therapies.

Key words: alanine transaminase, breakthrough hepatitis, hepatitis B virus, lamivudine, mutation, viral DNA

INTRODUCTION

LAMIVUDINE HAS GAINED increasing popularity since its approval in 1998 for the treatment of chronic hepatitis B virus (CHBV).[1–4] Lamivudine blocks HBV replication, reduces HBV DNA levels, normalizes alanine aminotransferase (ALT) levels, thereby resulting in histological improvement of the liver.[5] It is a reverse transcriptase inhibitor that acts by competing with the natural polymerase substrate deoxycytidine triphosphate (dCTP) and thus inhibits the elongation of HBV DNA minus strand. It incorporates into the nascent DNA strand and thereby acts as a chain terminator. Although lamivudine is very effective in inhibiting viral replication, the incidence of resistance is high, with an estimated 14–32% of patients developing resistance after 1 year of treatment, 38% after 2 years of treatment, and 53–76% after 3 years of treatment.

Resistance to lamivudine, which increases over years is due to development of mutations in the tyrosine-methionine-aspartate-aspartate (YMDD) motif in the DNA polymerase/reverse transcriptase, which is the main target of lamivudine.[4,6–9] This amino acid sequence in YMDD motif is predominantly involved in deoxynucleoside triphosphate (dNTP) binding in the catalytic site of the HBV DNA polymerase.

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Grant sponsor: Ministry of Health, Labour and Welfare of Japan.
Received 10 March 2009; revision 25 May 2009; accepted 26 May 2009.

Table 1 2007 Ministry of Health, Labour and Welfare of Japan guidelines for hepatits B virus (HBV)-positive patients for nucleoside analogue treatment for patients with chronic HBV receiving lamivudine therapy

Lamivudine therapy HBV DNA		< 3 years	≥ 3 years
Keep < 2.6 log copies/mL		Switch to entecavir 0.5 mg/day	Continue lamivudine 100 mg/day
≥ 2.6 log copies/mL	No BTH†	Switch to entecavir 0.5 mg/day	
	With BTH	Adefovir 10mg/day (duo therapy with lamivudine)	Adefovir 10 mg/day (duo therapy with lamivudine)

†After checking for absence of tyrosine-methionine-aspartate-aspartate (YMDD) motif mutation. BTH, breakthrough hepatitis.

Long-term lamivudine therapy is associated with amino acid substitutions mainly in the YMDD motif and also in the proximal FLLAQ (phenylalanine, leucine, alanine, glutamine) motif.[10] Common mutation may occur in the YMDD motif where the methionine residue is replaced either by valine (rtM204V) or isoleucine (rtM204I).[11] These amino acid substitutions form the basis of emergence of lamivudine-resistant strains of HBV and when these occur, the clinical condition may worsen, which is usually accompanied by increase in viral load and serum aminotransferase levels. YMDD mutants cause breakthrough hepatitis (BTH) and, therefore, require withdrawal or switch-over from lamivudine treatment. The American Association for the Study of Liver Diseases (AASLD) and the United States Algorithm for Management of Patients with Drug Resistance recommend either switching over to entecavir or adding adefovir in the event of lamivudine resistance.[12] The 2007 Japanese guidelines of the study group (Ministry of Health, Labour and Welfare of Japan)[13] on standardization of treatment for HBV positive patients for nucleoside analogue treatment for patients with CHBV receiving lamivudine therapy are explained below and also summarized in Table 1.

According to the 2007 guidelines for patients on lamivudine therapy, switching over criteria from lamivudine therapy has been changed from BTH to HBeAg status in patients maintaining HBV DNA copies ≥ 2.6 log copies/mL. Patients on lamivudine for < 3 years and maintaining HBV DNA copies ≥ 2.6 log copies/mL can be switched over to entecavir 0.5 mg/day if they are also HBeAg negative, whereas HBeAg-positive patients can be co-administered adefovir 10 mg/day in both the treatment duration groups (> 3 years or < 3 years).

Unfortunately, the cost of measuring HBV resistance to lamivudine by molecular methods is high and is not presently covered by Japanese reimbursement system in clinical practice. Development of HBV resistance to lamivudine is typically indicated by an increase in HBV DNA followed by an increase in serum ALT levels. Increase in HBV DNA represents active viral replication whereas serum ALT levels provide an indirect assessment of the degree of liver injury.[14]

Hence, in this study, we analyzed the correlation of the incidence of YMDD motif mutant and BTH with HBV DNA and serum ALT levels, either separately or together, in HBV DNA-positive patients who are treated with lamivudine for ≥ 1 year and who had maintained an ALT level of ≤ 40 IU/L until the development of BTH during the course of lamivudine treatment.

METHODS

Patients

THIS WAS A retrospective, nonrandomized study that enrolled 929 HBV DNA-positive-patients receiving 100 mg of lamivudine daily and followed up for a period of 1 year or longer between 1995 and 2006. Since long-term treatment with lamivudine was associated with a high frequency of YMDD motif mutant and BTH (BTH can be defined as abnormal variations in serum transaminase level due to YMDD motif mutant), we analyzed patients who had a possibility to switch to other nucleoside analogues. Patients (n = 395) with ALT ≤ 40 IU/L during follow-up (for 48 patients who developed BTH, data was used until 1 month before the patient developed BTH). Patients were not treated with either adefovir or entecavir during follow-up (for patients who used adefovir or entecavir because of BTH development, data was used until the point before the patient started adefovir or entecavir treatment). Patients were negative for anti-hepatitis C virus (HCV) (third-generation enzyme immunoassay; Chiron, Emerville, CA) and negative for HCV RNA with PCR (Amplicor; Roche Diagnostic Systems, Pleasanton, CA), did not have hepatocellular carcinoma, nore other forms of liver injury such as hemochromatosis, Wilson's disease,

primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease.

Informed consent was obtained from each patient included in the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Patients were stratified into 2 groups based on the duration of lamivudine treatment – one receiving lamivudine for < 3 years ($n = 125$) and the other for ≥ 3 years ($n = 234$). In addition, we also analyzed patients based on their ALT level (IU/L) grouped into ≤20, 21–30, and 31–40, and HBV DNA (log copies/mL) divided into < 2.6, 2.6–5.0, and ≥ 5.1.

During treatment, patients were followed up each month for liver function and serum markers of HBV infection. The serum sample of the patients were collected and preserved at –80 °C. All the collected samples up to this time period were analyzed for HBV DNA in June 2001. From July 2001, the serum samples were collected and analyzed once a month at the clinical treatment facility.

YMDD motif mutants were determined at the baseline and monitored at 6 months and during the study as well as at the development of breakthrough hepatitis. YMDD motif mutants were analyzed in the serum preserved at –80 °C altogether.

Markers of HBV infection

The HBeAg was estimated by enzyme-linked immunosorbent assay (ELISA) (F-HBe; Sysmex, Kobe). HBV DNA was determined by PCR followed by hybridization (Amplicor HBV Monitor: Roche Molecular Systems, Branchburg, NJ), and the results were expressed in log copy per milliliter over a range of 2.6–7.6. The 6 major genotypes of HBV (A–F) were determined serologically by ELISA (HBV GENOTYPE EIA; Institute of Immunology) and the PCR-invader method with genotype-specific probes.[15] YMDD motif mutants were determined by PCR followed by restriction fragment length polymorphism (RFLP)[8] or enzyme-linked minisequence assay with commercial assay kits (PCR-ELMA; Genome Science).

Statistical analyses

Frequencies were compared between groups by the χ^2-test, Fisher's exact test, and HBV DNA values by Mann–Whitney *U*-test. Emergence of YMDD motif mutants and BTH were compared in the Kaplan–Meier life table by using the production limit method. A *P*-value < 0.05 was considered significant. Analyses of all data were performed with SAS 9.1.3.

RESULTS

DURING THE PERIOD of 12 years from 1995 to 2006, 929 HBV DNA-positive patients received 100 mg of lamivudine daily. From the total of 929 patients who received lamivudine for 1 year or more, 359 patients who maintained an ALT level of ≤ 40 IU/L were stratified based on the duration of lamivudine treatment and divided into 2 groups – one receiving lamivudine for < 3 years ($n = 125$) and the other for ≥ 3 years ($n = 234$). Demographic features and clinical background of the two study groups were uniformly matched with no significant differences in age, sex, serum transaminase levels, HBV DNA, hepatitis B e-antigen (HBeAg), and HBV genotype (Table 2). The median ALT values were 112 IU/L and 145 IU/L in both the groups, respectively, and the median HBV DNA level was identical at 6.1 log copies/mL in both the groups.

Incidence of YMDD motif mutant and BTH after lamivudine treatment for < 3 years

The incidence of YMDD motif mutant within 3 years of treatment with lamivudine by ALT (IU/L) level was 27% in 53 patients maintaining an ALT level of ≤ 20 (group A), 58% in 46 patients maintaining an ALT level of ≤ 30 (group B); and 63% in 26 patients maintaining an ALT level of ≤ 40 (group C), with statistical differences among the 3 groups ($P = 0.002$). The incidence of BTH was 2% in group A, 7% in group B, and 48% in group C ($P < 0.001$). The lowest incidence of YMDD motif mutant and BTH was noted in patients with ALT level of ≤ 20 (IU/L) (Fig. 1a,b). Follow-up for patients who developed BTH was discontinued upon the detection of YMDD motif mutant.

The incidence of YMDD motif mutant within 3 years of treatment with lamivudine based on the HBV DNA (log copies/mL) level was 28% in patients maintaining an HBV DNA level of < 2.6; 83% in patients maintaining an HBV DNA level of 2.6–5.0; and 100% in patients maintaining an HBV DNA level of ≥ 5.1, with significant differences among the 3 groups ($P < 0.001$). The incidence of BTH was 4%, 30%, and 40%, respectively, in patients with HBV DNA level of < 2.6, 2.6–5.0, and ≥ 5.1 log copies/mL ($P = 0.004$) (Fig. 2a,b). The lowest incidence of YMDD motif mutant and BTH was seen in patients maintaining an HBV DNA level of < 2.6 log

Table 2 Background of 359 patients using lamivudine treatment for ≥ 1 year at the start of lamivudine therapy

Factors	Duration of lamivudine therapy		
	< 3 years *n* = 125	≥ 3 years *n* = 234	Differences (*P*-value)
Age (years)	23–75 (43)†	18–76 (43)†	NS‡
Male	93 (73%)	182 (77.1%)	NS‡
HBV infection in mother	47 (37%)	82 (35%)	NS‡
Chronic hepatitis	109 (85%)	212 (90%)	NS‡
AST (IU/L)	15–866 (80)†	19–2593 (83)†	NS‡
ALT (IU/L)	11–2092 (112)†	14–2142 (145)†	NS‡
Total bilirubin (mg/dL)	0.2–3.8 (0.7)†	0.2–10.6 (0.7)†	NS‡
γ-GTP (IU/L)	16–440 (54)†	13–468 (65)†	NS‡
HBV DNA (log copy/mL)	<2.6–>7.6 (6.1)†	<2.6–>7.6 (6.1)†	NS‡
HBeAg	66(52%)	107 (45%)	NS‡
HBV genotype (A, B, C, ND)	4:15:98:8	5:21:207:1	NS‡

†Median value where indicated. ‡Not significant. ALT, alanine transaminase; AST, aspartate aminotransferase; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; γ-GTP, gamma glutamyl transferase.

copies/mL. The BTH incidence was particularly high in patients with an HBV DNA level of ≥ 5.1, which was 40% within 1 year.

The incidence of YMDD motif mutant within 3 years of treatment with lamivudine in patients based on both the ALT (IU/L) and HBV DNA (log copies/mL) level during the course of lamivudine treatment was evaluated (Table 3).

In patients maintaining HBV DNA < 2.6 and ALT ≤ 20, the incidence of YMDD motif mutant and BTH was 7% and 2%, respectively. Whereas in patients with HBV DNA level of < 2.6 and ALT 21–30, the incidence of YMDD motif mutant was higher at 16% and BTH was 0%, and in patients with ALT 31–40, YMDD motif mutant and BTH was further higher at 42% and 17%, respectively.

In patients with HBV DNA level at 2.6–5.0 and ALT ≤ 20, the incidence of YMDD motif mutant was 33% in patients with 0% incidence of BTH. Nevertheless, in patients maintaining HBV DNA at 2.6–5.0 but with ALT 21–30, the incidence of YMDD motif mutant was 73% and BTH was 18%; whereas in patients with ALT 31–40, the incidence of YMDD motif mutant was 50% and BTH was 42%.

In patients maintaining HBV DNA ≥ 5.1 and ALT 31–40, both YMDD motif mutant and BTH was 100%.

Incidence of YMDD motif mutant and BTH after lamivudine treatment for ≥ 3 years

In patients treated with lamivudine for 3 years or more, the incidence of YMDD motif mutant by ALT (IU/L) level was 58% in 113 patients in group A, 60% in 84

Table 3 Incidences of tyrosine-methionine-aspartate-aspartate (YMDD) mutant and breakthrough hepatitis (BTH) by hepatits B virus (HBV) DNA and alanine transaminase (ALT) level in patients during lamivudine treatment for < 3 years (125 patients)

HBV DNA† (Amplicor: log copies/mL)	ALT level (IU/L)†					
	≤ 20		21–30		31–40	
	YMDD	BTH	YMDD	BTH	YMDD	BTH
< 2.6	3/41 (7%)	1/41 (2%)	5/32 (16%)	0/32 (0%)	5/12 (42%)	2/12 (17%)
2.6–5.0	4/12 (33%)	0/12 (0%)	8/11 (73%)	2/11 (18%)	6/12 (50%)	5/12 (42%)
≥ 5.1	0	0	3/3 (100%)	0/3 (0%)	2/2 (100%)	2/2 (100%)

†The HBV DNA and ALT levels are shown based on the treatment duration of lamivudine.

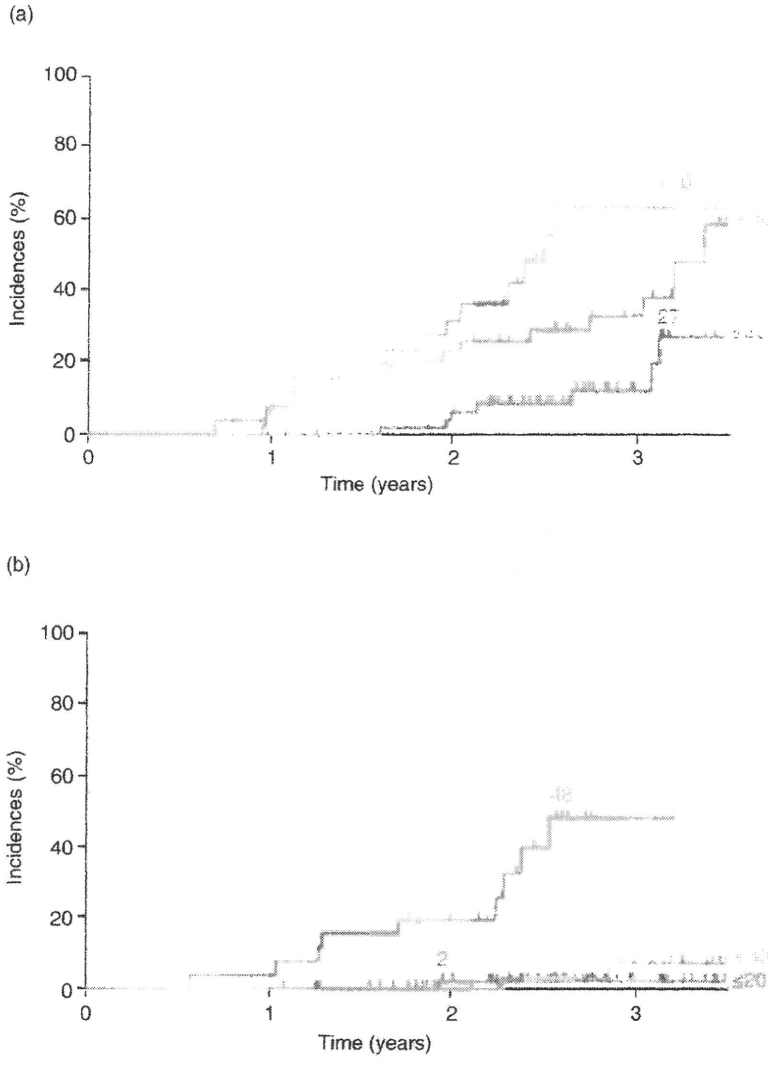

Figure 1 The incidence of tyrosine-methionine-aspartate-aspartate (YMDD) motif mutant and breakthrough hepatitis was noted in patients with alanine aminotransferase level of ≤ 20 (IU/L) (a) Incidence of YMDD mutants over time ($P = 0.0017$). (b) Incidence of break throughhepatitis over time ($P < 0.0001$).

patients in group B, and 80% in 37 patients in group C ($P = 0.002$), and that of BTH in the corresponding groups was 7%, 14%, and 57% ($P < 0.001$) (Fig. 3a,b).

In patients treated with lamivudine for ≥ 3 years, the increased incidence of YMDD motif mutant by HBV DNA (log copies/mL) level was 65% in patients maintaining an HBV DNA level of < 2.6, 78% in patients maintaining an HBV DNA level of 2.6–5.0, and 92% in patients maintaining an HBV DNA level of ≥ 5.1, and that of BTH in the corresponding groups was 10%, 18%, and 77% ($P < 0.001$) (Fig. 4a,b).

The incidence of YMDD motif mutant in ≥ 3 years treatment with lamivudine in patients by both ALT (IU/L) and HBV DNA (log copies/mL) levels during the course of lamivudine treatment was also analyzed (Table 4).

In patients maintaining HBV DNA < 2.6 and ALT ≤ 20, the incidence of YMDD motif mutant and BTH was 38% and 7%, respectively. At the same HBV DNA level of < 2.6 and ALT 21–30, the incidence of YMDD motif mutant was 48% and BTH was 8%; whereas at ALT 31–40, YMDD motif mutant was 36% and BTH was 9%.

In patients maintaining HBV DNA 2.6–5.0 and ALT ≤ 20, the incidence of YMDD motif mutant and BTH was 60% and 4%, respectively. At the same HBV DNA

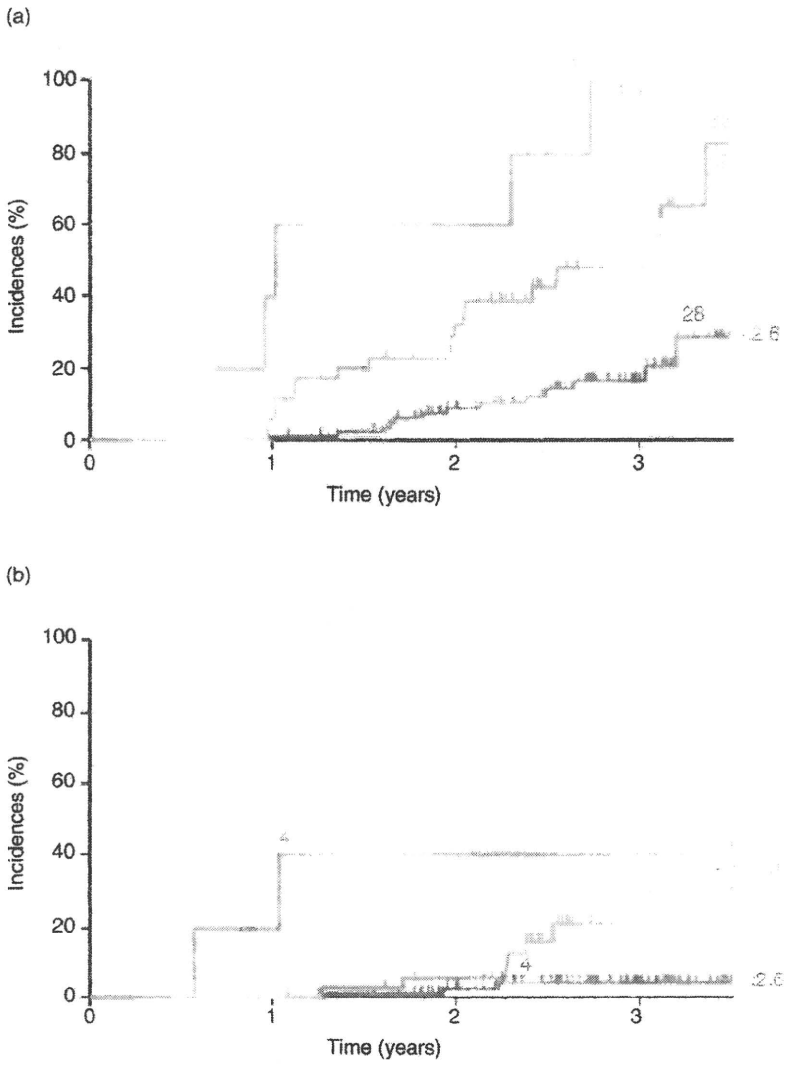

Figure 2 incidence of BTH was 4%, 30%, and 40%, respectively, in patients with HBV DNA level of < 2.6, 2.6–5.0, and ≥ 5.1 log copies/mL ($P = 0.004$). (a) Incidence of YMDD mutants over time ($P = 0.0001$). (b) Incidence of breakthrough hepatitis over time ($P < 0.0037$).

level, 2.6–5.0 and ALT 21–30, the incidence of YMDD motif mutant was 86% and BTH was 18%; whereas at ALT 31–40, YMDD motif mutant was 92% and BTH was 42%.

In patients maintaining HBV DNA ≥ 5.1 and ALT 31–40, YMDD motif mutant was 93% and BTH was 86%.

DISCUSSION

LONG-TERM THERAPY for CHBV can lead to the development of HBV drug-resistant mutants. Early detection of the YMDD motif mutants in lamivudine-treated patients and timely switch to other nucleoside analogues with low viral resistance is crucial to prevent viral and biochemical flares and ineffective therapeutic response. Although development of YMDD mutants results in decreased viral susceptibility to lamivudine, viral replication rate is lower in mutant strains than in wild type.[6]

Among the 359 patients who received lamivudine for > 1 year and maintained an ALT level of ≤ 40 IU/L, the rate of YMDD motif mutant was 11% (1 year), 29% (2 year), 42% (3 year), 49% (4 year) and 61% (5 year). BTH occurrences were 3% (1 year), 8% (2year), 13% (3 year), 15% (4 year) and 19% (5 year). The rate of

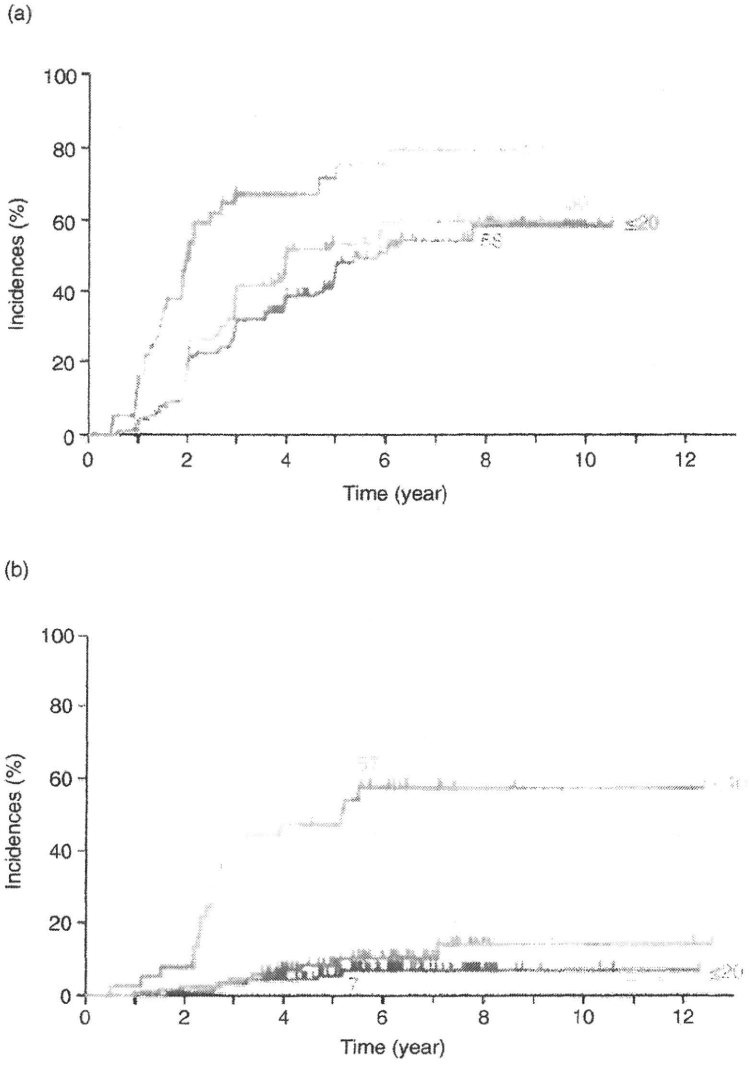

Figure 3 In patients treated with lamivudine for 3 years or more, the incidence of tyrosine-methionine-aspartate-aspartate (YMDD) motif mutant by alanine aminotransferase (IU/L) level was 58% in 113 patients in group A, 60% in 84 patients in group B, and 80% in 37 patients in group C ($P = 0.002$), and that of BTH in the corresponding groups was 7%, 14%, and 57% ($P < 0.001$). (a) Incidence of YMDD mutants over time ($P = 0.0015$). (b) Incidence of breakthrough hepatitis over time ($P < 0.0001$).

YMDD motif mutant and BTH were low after 3 or more years of treatment with lamivudine. Therefore, the year of switching treatment from lamivudine to other nucleic acid analogue will be at 3 years. Accordingly, in this study, we examined patients treated with lamivudine for < 3 and ≥ 3 years.

Among the patients treated with lamivudine for < 3 years, the lowest incidence of YMDD motif mutant and BTH was seen in patients with ALT < 20 IU/L maintaining HBV DNA level of 2.6–5.0. The other category for lowest incidence was in patients with ALT 21–30 IU/L and HBV DNA level of < 2.6 log copies/mL. In this study, within 3 years of treatment with lamivudine, the group of patients with the recommended HBV DNA (< 2.6 log copies/mL) and ALT maintained at 21–30 IU/L may be considered eligible to be switched to entecavir therapy as per Japanese guidelines. We, however, believe it is important to consider the prognosis for patients who are switched from lamivudine to entecavir. Similarly, in patients maintaining HBV DNA level in the range of 2.6–5.0 log copies/mL and ALT < 20 IU/L, switching to dual therapy with adefovir in combination with lamivudine depends on the related viral breakthrough. In a study by Li Zhou *et al.*,[16] some patients with YMDD motif mutants had significantly lower HBV DNA and ALT levels compared with baseline

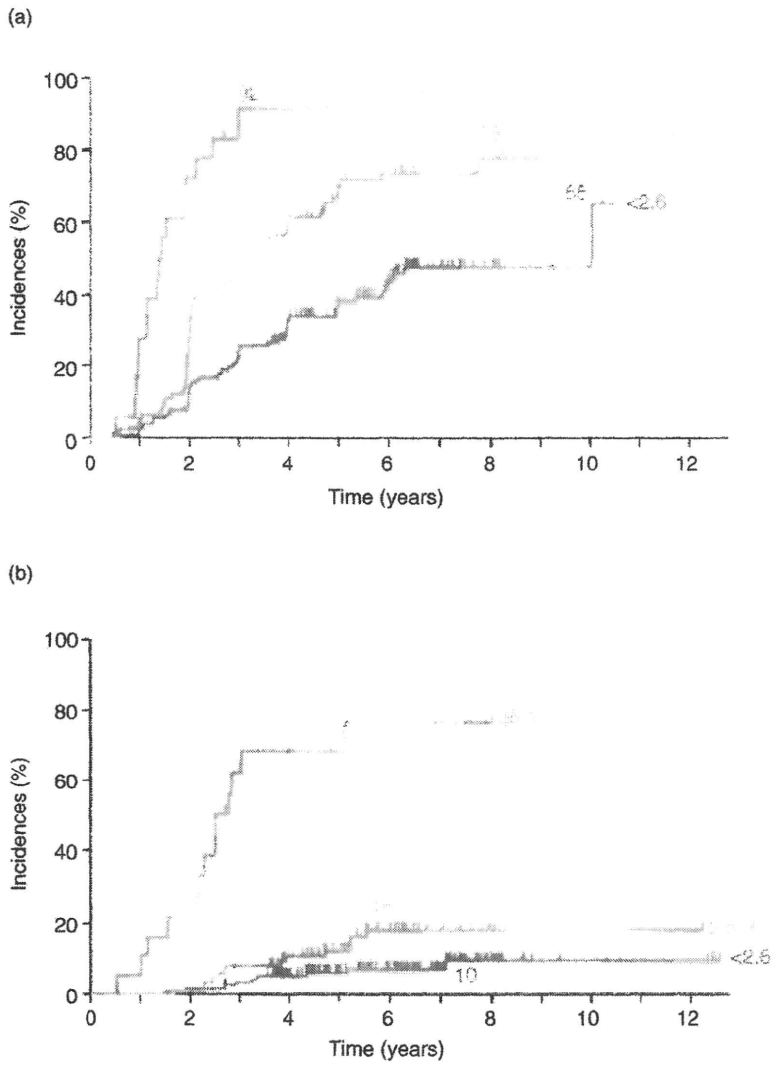

Figure 4 In patients treated with lamivudine for ≥ 3 years, the increased incidence of tyrosine-methionine-aspartate-aspartate (YMDD) motif mutant by hepatitis B virus (HBV) DNA (log copies/mL) level was 65% in patients maintaining an HBV DNA level of < 2.6, 78% in patients maintaining an HBV DNA level of 2.6–5.0, and 92% in patients maintaining an HBV DNA level of ≥ 5.1, and that of BTH in the corresponding groups was 10%, 18%, and 77% ($P < 0.001$). (a) Incidence of YMDD mutants over time ($P = 0.0001$). (b) Incidence of breakthrough hepatitis over time ($P < 0.0001$).

values, which might be due to decreased replication efficiency of the HBV mutants.

HBeAg, severe liver disease, high HBV DNA, and low ALT levels at the baseline were factors accelerating the development of BTH. This was in confirmation of previous results.[17–19] Development of BTH, however, was not influenced by HBV genotypes. This is probably due to the response in HBeAg-positive patients, which was comparable among those with different genotypes though it differed among HBeAg-negative patients.[20]

In a study of Japanese adult patients treated with lamivudine for > 12 months, the YMDD motif mutation was detected in 26% patients, with 23, 16, and 21 patients correspondingly positive for YIDD, YVDD, and YIDD + YVDD mutants. The occurrence of mutations steadily increased and two, five, and 52 patients with genotypes A, B, and C, respectively developed resistance.[21] Lamivudine retreatment could induce rapid re-emergence of YMDD motif mutants with associated viral and hepatic flares[22] and should be avoided. Next, we were interested to know if any difference in sensitivity existed in detecting YMDD mutants by the two different methods used in this study, PCR-RFLP and PCR-ELMA. We studied the rate of detection of YMDD motif mutant by both methods in 20 patients who received lamivudine for more than two years. The detection rate

Table 4 Incidences of tyrosine-methionine-aspartate-aspartate (YMDD) mutant and breakthrough hepatitis (BTH) by hepatitis B virus (HBV) DNA and alanine transaminase (ALT) level in patients during lamivudine treatment for ≥ 3 years (234 patients)

HBV DNA† (Amplicor: log copies/mL)	ALT level (IU/L)†					
	≤ 20		21–30		31–40	
	YMDD	BTH	YMDD	BTH	YMDD	BTH
< 2.6	23/60 (38%)	4/60 (7%)	29/61 (48%)	5/61 (8%)	4/11 (36%)	1/11 (9%)
2.6–5.0	30/50 (60%)	2/50 (4%)	19/22 (86%)	4/22 (18%)	11/12 (92%)	5/12 (42%)
≥ 5.1	3/3 (100%)	1/3 (33%)	0/1 (0%)	0/1 (0%)	13/14 (93%)	12/14 (86%)

†The HBV DNA and ALT levels are shown based on the treatment duration of lamivudine.

between PCR-RFLP and PCR-ELMA was similar; eight patients (40%) and nine patients (45%), respectively.[23]

CONCLUSION

CORRELATION OF ALT and HBV DNA levels with YMDD motif mutant and viral breakthrough can be used as an indirect method of estimating susceptibility to develop lamivudine resistance. The low incidence of YMDD motif mutant and BTH associated with an HBV DNA level of < 2.6 log copies/mL and ALT level of ≤ 30 IU/L and an HBV DNA level of 2.6–5.0 log copies/mL and ALT level of ≤ 20 IU/L during only less than 3 year-treatments can be utilized as a clinically relevant tool to monitor patients' criteria in switching to other nucleoside analogue drugs. Using these simple methods, which can be easily pursued in clinical practice, it may be feasible in the future to switch from lamivudine to other nucleoside analogue drugs with low rates of inducing resistant mutants in CHBV patients. This is important considering the risk of continuous lamivudine treatment causing YMDD motif mutant and BTH.

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Original Article

Development of HCC in patients receiving adefovir dipivoxil for lamivudine-resistant hepatitis B virus mutants

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Aim: To identify factors for the development of hepatocellular carcinoma (HCC) in the patients who receive adefovir add-on lamivudine for treatment of lamivudine-resistant hepatitis B virus (HBV) mutants.

Methods: A total of 247 patients who developed lamivudine-resistant HBV mutants, with an increase of HBV DNA ≥ 1 log copies/mL, received adefovir dipivoxil 10 mg add-on lamivudine 100 mg daily during a median of 115 weeks (range: 25–282 weeks). They were followed for the development of HCC by imaging modalities every 3–6 months.

Results: HCC developed in 18 of the 247 (7.3%) patients. Eight factors were in significant association with the development of HCC by the univariate analysis. They included age, cirrhosis, platelet counts, levels of bilirubin, aspartate aminotransferase (AST), alanine aminotransferase and α-fetoprotein, as well as YMDD mutants at the start of adefovir dipivoxil. By the multivariate analysis, AST levels, YIDD mutants, cirrhosis and age were independent factors for the development of HCC. By the Kaplan-Meier analysis, AST levels ≥ 70 IU/L, YIDD mutants, cirrhosis and age ≥ 50 years increased the risk of HCC ($P = 0.018$, $P = 0.035$, $P = 0.002$ and $P = 0.014$, respectively). HCC developed more frequently in the patients with than without cirrhosis at the start of adefovir (10/59 [16.9%] vs. 8/188 [4.3%], $P = 0.002$).

Conclusion: HCC can develop in cirrhotic patients receiving adefovir add-on lamivudine. Hence, the patients with baseline AST ≥ 70 IU/L and YIDD mutants would need to be monitored closely for HCC.

Key words: adefovir dipivoxil, chronic hepatitis B, hepatitis B virus, hepatocellular carcinoma, lamivudine, rescue therapy

INTRODUCTION

WORLDWIDE, AN ESTIMATED 400 million people are infected with hepatitis B virus (HBV) persistently, and one million die of decompensated cirrhosis and/or hepatocellular carcinoma (HCC) annually.[1,2] Interferon (IFN) was introduced for treatment of chronic hepatitis B, and it has been replaced for pegylated-IFN.[3] Due to substantial side-effects and requirement for injection, however, IFN-based therapies are not favored.

In 1998, lamivudine was approved as the first nucleot(s)ide analogue for treatment of chronic hepatitis B,[4] and then adefovir in 2002.[5] Due to its lower costs and safety records, lamivdine has gained a wide popularity for treatment of chronic hepatitis B. However, drug-resistant mutants arise in parallel with the duration of lamivudine, in 12.5% after 1 year, in 43.8% after 3 years, and 62.5–70.2% after 5 years.[6,7] For preventing breakthrough hepatitis induced by lamivudine-resistant HBV mutants, additional adefovir dipivoxil 10 mg daily has been recommended;[8,9] it is more effective than switching to adefovir monotherapy and has fewer chances of developing drug-resistant mutants.[10,11]

Since 1995, 930 patients with chronic hepatitis have been treated with lamivudine in the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo.[12] HBV mutants with mutations in the thyrosine-methionine-aspartic acid-aspartic acid (YMDD) motif elicited in the 247 (26.5%) patients, and they started to receive additional adefovir since December, 2002.[13,14] However, HCC developed in 18 (7.3%) of them during the combination therapy for 25–282 weeks; HCC has

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Received 25 April 2009; revision 6 June 2009; accepted 9 June 2009.

not been reported in any of the patients who have received adefovir add-on lamivudine for 5 years.[15–17] Hence, factors for the development of HCC in the patients receiving adefovir add-on lamivudine were sought for in a retrospective study.

METHODS

Patients

OVER A PERIOD of 13 years, from September 1995 to September 2007, 930 patients with chronic hepatitis B received long-term lamivudine treatment at the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo. Drug-resistant YMDD mutants developed in 247 (26.5%) of them, accompanied by an increase in HBV DNA ≥ 1 log copies/mL, and they received adefovir 10 mg in addition to lamivudine 100 mg daily during the median of 115 weeks (range: 25–282 weeks). They have been followed for liver function and virological markers of HBV infection monthly, as well as blood counts and tumor makers including alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II). Cirrhosis was diagnosed by laparoscopy or liver biopsy, and in the patients who had not received them, by clinical data, imaging modalities and portal hypertension. HCC was diagnosed by hypervascularity on angiography and/or histological examination, characteristic features of computed tomography, magnetic resonance imaging and ultrasonography. An informed consent was obtained from each patient in this study, and the protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a *priori* approval by the institution's human research committee.

Markers of HBV infection

Hepatitis B e antigen (HBeAg) was determined by enzyme-linked immunosorbent assay (ELISA) with commercial kits (HBeAg EIA, Institute of Immunology, Tokyo). HBV DNA was quantitated by the Amplicor monitor assay (Roche Diagnostics, Tokyo) with a dynamic range over 2.6–7.6 log copies/mL. Genotypes of HBV were determined serologically by the combination of epitopes expressed on the pre-S2 region product, which is specific for each of the seven major genotypes (A–G),[18,19] with use of commercial kits (HBV Genotype EIA, Institute of Immunology).

Detection of YMDD mutants

YMDD mutants were determined by polymerase chain reaction (PCR)-based enzyme-linked mini-sequence assay (PCR-ELIMA) with commercial kits (Genome Science Laboratories, Tokyo).

Statistical analyses

Categorial variables were compared between groups by the χ^2 test, and non-categorical variables by the Mann–Whitney *U*-test. A *P*-value < 0.05 was considered significant. Factors associated with HCC by univariate analysis were evaluated by the multivariate analysis by the stepwise Cox proportional hazard model. Development of HCC with time was analyzed by the Kaplan–Meier method, and differences were evaluated by the log-rank test. Data were analyzed by the SPSS software, version 11.0 (Chicago, IL).

RESULTS

Baseline characteristics of the patients who did and who did not develop hepatocellular carcinoma during adefovir add-on lamivudine treatment

TABLE 1 COMPARES characteristics at the start of adefovir between the 18 patients who developed HCC and the 229 who did not. Eight factors were associated with the development of HCC by the univariate analysis. They included age, cirrhosis, platelet counts, bilirubin, AST, alanine aminotransferease (ALT) and α-fetoprotein (AFP) levels, as well as YMDD mutants. HCC developed more frequently in the patients with than without cirrhosis at the start of adefovir (10/59 [16.9%] vs. 8/188 [4.3%], $P = 0.002$). There were 61 (26.6%) patients who had cirrhosis at the start of adefovir. Of them, one of the 18 (2.2%) with HCC and 18 of the 229 (2.2%) without HCC presented with decompensation; no patients developed decompensation after the start of adefovir.

Rates of HBV DNA disappearance from serum (< 2.6 log copies/mL) were: 55% (113/207) at 1 year, 71% (119/168) at 2 years, 77% (78/101) at 3 years and 85% (35/41) at 4 years. Rates of AST nomarlization (< 38 IU/L) were: 87% (179/207) at 1 year, 90% (151/168) at 2 years, 92% (93/101) at 3 years and 95% (39/41) at 4 years; and those of ALT normalization (< 50 IU/L) were: 88% (183/207) at 1 year, 91% (153/168) at 2 years, 93% (94/101) at 3 years and 98% (40/41) at 4 years. There were no differences in the rate of HBV DNA disappearance from serum between the patients with and without HCC: 57% (8/14) vs. 54% (105/193) at 1 year ($P = 1.0$); 86% (12/14) vs. 70% (107/154) at 2 years ($P = 0.229$); and 89% (8/9) vs.

Table 1 Characteristics of patients who did and did not develop hepatocellular carcinoma (HCC) at the start of adefovir†

	HCC developed ($n = 18$)	HCC did not develop ($n = 229$)	Differences *P*-value
Duration of lamivudine before the start of adefovir	128 (31–346)	144 (13–617)	0.321
Age (years)	52 (35–75)	45 (26–75)	0.008
Men	15 (83%)	183 (80%)	1.000
Cirrhosis	10 (56%)	51 (22%)	0.004
Platelets ($\times 10^3/mm^3$)	12.0 (4.6–19.7)	16.3 (3.1–31.9)	0.001
Albumin (g/dL)	3.6 (2.3–4.7)	3.9 (2.8–4.7)	0.073
Bilirubin (mg/dL)	0.8 (0.5–15.5)	0.7 (0.2–6.0)	0.046
Creatinine (mg/dL)	0.8 (0.5–1.0)	0.8 (0.4–1.6)	0.950
AST (IU/L)	119 (55–248)	66 (14–1413)	0.003
ALT (IU/L)	151 (61–576)	104 (13–1563)	0.035
AFP (ng/dL)	8 (2–130)	4 (1–282)	0.026
HBV genotypes			0.228
C	18 (100%)	189 (87%)	
Others	0	27 (13%)	
HBeAg	8 (44%)	132 (58%)	0.323
HBV DNA (log copies/mL)	7.1 (4.4–>7.6)	7.1 (<2.6–>7.6)	0.623
YMDD mutants			0.041
YIDD	13 (72%)	109 (45%)	
YVDD	5 (28%)	62 (25%)	
YI/VDD	0	56 (23%)	

†Values are the median with the range in parentheses or *n* with percent in parentheses.
AFP, alpha-fetoprotein; ALT, alaine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus.

92% (85/92) at 3 years ($P = 0.555$). Rates of normalized AST levels in the patients with and without HCC were: 50% (7/14) vs. 90% (173/193) at 1 year ($P < 0.001$); 79% (11/14) vs. 91% (140/154) at 2 year ($P = 0.166$); and 67% (6/9) vs. 95% (87/92) at 3 year ($P = 0.037$). Rates of ALT normalization in the patients with and without HCC were: 71% (10/14) vs. 90% (174/193) at 1 year ($P = 0.037$); 79% (11/14) vs. 90% (139/154) at 2 year ($P = 0.189$); and 56% (5/9) vs. 92% (85/92) at 3 year ($P = 0.015$). Thus, normalization of AST and ALT was less frequent in the patients with than without HCC.

Characteristics of the 18 patients who developed HCC are compared between the baseline and at the development of HCC (Table 2). At the start of adefovir, 10 (56%) of them had developed cirrhosis and 16 (89%) had AST levels ≥ 70 IU/L. HBV DNA was not detectable in 10 (56%) of them at the development of HCC. Of the eight patients with detectable HBV DNA levels (≥ 2.6 log copies/mL), five (63%) developed HCC within 1 year after the start of adefovir. AST was elevated (> 38 IU/L) in eight patients, including four (50%) without detectable HBV DNA levels.

Factors independently associated with the development of hepatocellular carcinoma

Eight factors associated with the development of HCC by the univariate analysis, including age, cirrhosis, platelet counts, bilirubin, AST, ALT and AFP levels, as well as YMDD mutants (Table 1), were evaluated by the multivariate analysis. AST ≥ 70 IU/L, YIDD mutants, age ≥ 50 years and cirrhosis at the baseline were independent risk factors for the development of HCC (Table 3). There were no differences in the distribution of YIDD, YVDD and the mixture thereof among the patients with distinct AST, ALT or HBV DNA levels or between those with and without cirrhosis at the start of adefovir. HBV mutants with mutations resistant to adefovir (rtA181T/S, rtN236T) occurred in two of the 247 (0.8%) patients; none of them developed HCC.

The median time between the elevation of HBV DNA > 5.0 log copies/mL and the administration of adefovir was 124 (range: 0–815) days for the 13 patients who developed HCC and 147 (0–3268) days for the 166 patients who did not ($P = 0.605$). The median time between the elevation of ALT > 43 IU/L and the start of