Emergence of a novel lamivudine-resistant hepatitis B virus variant with a substitution outside the YMDD motif

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Abbreviations:

HBeAg, hepatitis B e Antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; ORF, open reading frame; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; RT, reverse transcriptase
Abstract

Lamivudine is a major drug approved for treatment of chronic hepatitis B virus infection. Emergence of drug-resistant mutants with amino acid substitutions in the YMDD motif is a well documented problem during long-term lamivudine therapy. Here we report a novel lamivudine-resistant strain of HBV with intact YMDD motif, which included an amino acid substitution rtA181T in the reverse transcriptase (RT) domain of HBV polymerase. The substitution also induced a unique amino acid substitution (HBs W172L) to overlapping hepatitis B surface (HBs) protein. The YMDD mutant strains were not detected even by using the sensitive PNA-mediated polymerase chain reaction (PCR) clamping method. The detected nucleotide substitution was accompanied by the emergence of an additional nucleotide substitution that induced amino acid change (S331C) in the spacer domain. The rtA181T mutant strain displayed a 3-fold decrease in susceptibility to lamivudine in in vitro experiments in comparison with wild type. In vivo analysis using the human hepatocyte chimeric mouse confirmed resistance of this mutant strain against lamivudine. We developed a method to detect this novel rtA181T mutation and a previously reported rtA181T mutation with HBs stop codon using PCR restriction fragment length polymorphism (RFLP) and identified one patient with the latter pattern among 40 patients with lamivudine resistance. In conclusion, although the incidence is not high, we have to be careful regarding the emergence of lamivudine-resistant mutant strains with intact YMDD motif. (228 words)
Introduction

Hepatitis B virus (HBV) is a small, enveloped DNA virus that causes chronic hepatitis and often leads to liver cirrhosis and hepatocellular carcinoma (4, 12, 33). To date, interferon and three nucleoside and nucleotide analogs (lamivudine, adefovir dipivoxil, and entecavir) have been approved by the United States Food and Drug Administration for the treatment of chronic HBV infection. Lamivudine, an oral cytosine nucleoside analogue, potently inhibits hepatitis B virus (HBV) replication by interfering with RNA dependent DNA polymerase (10, 16, 22). Lamivudine therapy suppresses HBV replication in most patients and improves transaminase levels and liver histology (16, 22, 25, 30). However, prolonged therapy results in the emergence of drug-resistant mutants in 24% and 70% of patients after 1 and 4 years of therapy, respectively, followed by increases in viral load and re-elevation of transaminase levels (18).

Most lamivudine-resistant strains show amino acid substitutions in the YMDD (tyrosine-methionine-aspartate-aspartate) motif in the C domain of HBV polymerase. In addition to the emergence of the YMDD mutation, rtL180M and rtV173L mutations in the B domain of HBV polymerase are frequently observed (1, 9). In vitro analyses have confirmed that the rtL180M mutation augments the level of lamivudine-resistance and enhances viral replication, while the rtV173L mutation enhances only viral replication (9, 23). On the other hand, only a few uncommon mutations associated with lamivudine-resistance have been reported so far (3, 7, 24, 34). In the C domain of HBV polymerase, rtM204S and rtD205N were detected in patients with lamivudine resistance (3, 7). In the B domain, rtL180C and rtA181T were associated with
lamivudine resistance (7, 24, 34). Yeh et al. (34) reported the emergence of rtA181T mutants in 4 of 23 patients who received long-term lamivudine therapy. The mutant appeared concomitantly or after emergence of YMDD motif mutant and persisted thereafter. The nucleotide substitution in the FLLA motif resulted in early termination of overlapping HBs gene by creating a stop codon (TGG to TGA). Yeh et al. (34) demonstrated that the mutation reduced the susceptibility to lamivudine \textit{in vitro}. They also detected such mutant in a patient with leukemia and speculated that truncated HBs gene might be related to the development of leukemia (7).

Analyzing nucleotide and amino acid sequences of HBV in patients who developed a breakthrough, we identified a novel mutant that showed nucleotide substitutions in the B domain of the reverse transcriptase. The G residues of nucleotides 669 and 670 were substituted to T and A, respectively, and associated with amino acid substitution of rtA181T. The substitutions also induced amino acid substitution W172L into the overlapping HBs protein. Since the nucleotide substitution was associated with nucleotide and amino acid substitution in the putative spacer region of the polymerase, we checked the importance of these substitutions for resistance of lamivudine \textit{in vitro}.

We also analyzed the resistance of this new strain \textit{in vivo} using human hepatocyte chimeric mouse (27, 31). Furthermore, we analyzed the susceptibility of the mutant strain to adefovir and entecavir. When used alone or in combination with lamivudine, these drugs are known to be effective against wild-type as well as lamivudine-resistant HBV (2, 5, 14, 17, 32). Infrequent emergence compared with lamivudine has been reported for both of these two drugs (2, 5). We also developed a detection system to identify this novel and previously reported (7, 34) nucleotide substitutions to study the
incidence of such mutations.
Material and Methods

Antiviral compounds

Lamivudine \([-\beta-L-2',3'-\text{dideoxy-3’-thiacytidine}]\) was provided by GlaxoSmithKline (Stevenage, Herts). Adefovir \([9-\text{[2-(phosphonomethoxy)ethyl]-adenine}]\) was provided by Gilead Sciences (Foster City, CA) and entecavir \([2\text{-amino-1,9-dihydro-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one, monohydrate}]\) was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Wallingford, CT).

Analysis of virological markers

HBsAg, HBeAg and antibody against HBeAg (anti-HBe) were determined by enzyme immunoassay kits (Abbot Diagnostics, Chicago, IL). HBV-DNA was measured by real-time PCR using the Light Cycler (Roche, Mannheim, Germany) by the polymerase chain reaction (PCR). The primers used for amplification were

\[5'-\text{TGTGGGATGCCATGGACATTGAC-3'}\] and \[5'-\text{GGTGAACATGTTCGAGAC-3'}\].

The amplification condition included initial denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 5 seconds and extension at 72°C for 6 seconds. The lower detection limit of this assay was 300 copies.

Cloning of HBV DNA and plasmid construction
HBV DNA was extracted from 100 μl of serum samples by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 μl H2O. Full-length HBV DNA was amplified using the above HBV DNA samples by the method of Gunter et al (13). Nucleotide sequence positions were numbered from the unique EcoRI site. The 1.4 genome lengths HBV DNA amplified from the serum of a patient who showed lamivudine resistance was cloned into a plasmid vector pTRE (TAKARA BIO, Tokyo) (patient’s strain). In brief, the PCR product amplified using serum from the patient was cleaved with BamHI and ApaI (HBV positions 1400-2600) and cloned into pcDNA3 (Invitrogen, San Diego, CA), which was named pcDNA3-1. Similarly, the PCR product was cleaved with ApaI and BamHI (HBV positions 2600-3215, 1-1400) and cloned into pBluescript SK+ (Stratagene, La Jolla, CA), which was named pB-1. KpnI-BamHI fragment from pB-1 and KpnI-ApaI fragment from pcDNA3-1 were cloned into pcDNA3-1. Finally, the plasmids were cleaved with HindIII and NotI within the multi-cloning site, and cloned into a plasmid vector pTRE. As a laboratory strain, we employed a plasmid containing 1.4 genome length wild type genotype C HBV (wild type strain; GenBank accession number AB206816) (31). To introduce the nucleotide substitutions into the S331C/rtA181T (pt) and wt (lab), site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis kit (Stratagene). The eight plasmids with/without amino acid substitutions in the spacer and reverse transcriptase domain were created and listed in Table 1.

Cell culture, transfection and determination of IC50
HepG2 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C in 5% CO₂. Cells were seeded to semi-confluence in 6-well tissue culture plates. Transient transfection of the plasmids into HepG2 cell lines was performed using TransIT-LT1 (Mirus, Madison, WI) according to the instructions provided by the supplier. To determine 50% inhibitory concentrations (IC₅₀s) for each anti-viral drug, various concentrations of lamivudine, adefovir and entecavir were added after 24 hours to the culture plate containing the cells, and harvested after five days. The medium containing the drugs was changed at days 1, 3 and 4. Plasmid encoding β-galactosidase (β-gal) was co-transfected to adjust the transfection efficiency. The β-gal enzyme assay was performed using the β-gal Enzyme Assay System (Promega, Madison, WI). All experiments were performed in triplicate. GraphPad prism (GraphPad Prism Software, Inc., USA) was used to determine the best-fit values for individual dose-response equations.

Analysis of replicative intermediate of HBV by Southern blot hybridization and quantitation

The cells were harvested at 3 or 5 days after transfection and lysed with 250 μl of lysis buffer (10 mM Tris-HCl [pH 7.4], 140 mM NaCl, and 0.5% (v/v) NP-40) followed by centrifugation for 2 min at 15,000 x g. The core-associated HBV genome was immunoprecipitated by mouse anti-core monoclonal antibody 2A21 (Institute of Immunology, Tokyo) and subjected to Southern blot analysis after SDS / proteinase K digestion followed by phenol extraction and ethanol precipitation. The DNA was detected with full-length HBV-DNA probe labeled by the DIG DNA labeling and
detection kit (Roche Diagnostics, Basel, Switzerland) according to the instructions provided by the manufacturer. Quantitative analysis was performed by real-time PCR with cyber green using Light Cycler. The HBV-specific primers used for amplification were 5'-TTTGGGCATGGACATTGAC-3' and 5'-GGTGAACAATGTTCCGGAGAC-3'. The amplification condition included initial denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 5 seconds and extension at 72°C for 6 seconds. The lower detection limit of this assay was 300 copies.

**Evaluation of effects of antiviral drugs on mutant strains using human hepatocyte chimeric mice**

Human hepatocyte chimeric mice were generated and used in the drug evaluation studies as described previously (27, 31). Briefly, human hepatocytes were transplanted into urokinase-type plasminogen activator-transgenic SCID mice, which are immunodeficient and develop liver failure. The transplanted cells were characterized in terms of *in vivo* growth potential and functions. The human hepatocytes progressively repopulated the murine host liver and were susceptible to cultured cell line-produced HBV. All animal protocols were performed in accordance with the guidelines of the local committee for animal experimentation. The mice were inoculated with 50 μl of serum samples containing wild type and newly identified drug resistant strains. Serum samples obtained from mice were stored at −80°C before further analyses. After confirming the stable high-level HBV viremia, the mice were administered food
containing 30 mg/kg/body/day lamivudine. The nucleotide sequences of wild and mutant strains were confirmed by sequencing analysis.

Detection of rtA181T mutants by PCR with restriction fragment length polymorphism (RFLP)

HBV DNA extracted from serum samples were amplified by PCR using primers 5’-GCCCGTTTTGTCTCTCTTCCA-3’ and 5’-ACCACTGAACAAATGGCAGCTACGA-3’ . The reverse primer was designed to introduce EspI site (GCTCAGC) into only wild type sequences. The PCR was performed in a total volume of 25 μl, consisting of a reaction buffer (100 mmol/L Tris-HCl [pH 8.3], 50 mmol/L KCl and 15 mmol/L MgCl2), 0.2 mmol/L each of dNTP, 1 μl of the DNA solution, 10 pmol of each primer and 1U of Taq DNA polymerase (Gene taq, Wako Pure Chemicals, Tokyo) with 0.2 μg of anti-Taq high (Toyobo Co., Osaka, Japan). The amplification conditions included an initial denaturation at 94 °C for 2 min, 35 cycles of amplification (denaturation at 94 °C for 1 min, annealing of primer at 58°C for 1 min, extension at 72°C for 2 min), followed by final extension at 72°C for 7 min. Two μl of PCR products were digested with five units of restriction enzyme EspI and subjected to electrophoresis in a 3.5 % agarose gel.

Statistical analysis

Data are expressed as mean ± SD. Group comparisons were performed using the Student t-test. A P value of less than 0.05 was considered statistically significant.
Results

Isolation of a novel lamivudine-resistant strain with intact YMDD motif

The novel lamivudine-resistant strain of HBV was isolated from a 44-year-old Japanese man with chronic HBV infection (Fig. 1A). In this patient, lamivudine successfully reduced the HBV at the initial stage of treatment, but viral breakthrough was observed at 24 months after the beginning of therapy. The patient was very punctual and confirmed that he took lamivudine with perfect compliance. The HBV viral load reached up to 8.5 log copies/ml, but nucleotide sequence analysis showed no YMDD mutation. The YIDD or YVDD mutants were not detected even by using a PNA-mediated PCR clamping method sensitive for detection of YMDD mutants (6). The analysis also showed that this isolate belonged to genotype C of HBV. Comparison of nucleotide sequences by the direct sequence method obtained before and after the viral breakthrough showed three nucleotide substitutions that induced two amino acid substitutions in both spacer (pol S331C) and reverse transcriptase domains of the polymerase (pol A527T or rtA181T) (Figs. 1B and 2). The latter nucleotide substitutions induced an amino acid change in the overlapping HBs protein (W172L) (Fig. 2). Twelve HBV genomes were cloned from the serum of this patient after viral breakthrough and eleven of them showed the above amino acid substitutions. Only one clone showed wild type sequence. The new strain of HBV became undetectable when lamivudine therapy was discontinued and this strain out-competed the wild type strain upon re-administration of the drug (Fig.1B). These results prompted us to study the significance of each of these mutations.
Effect of substitutions on HBV replication

To assess the effect of nucleotide substitutions on HBV replication, four plasmids containing 1.4 genome length patient-specific HBV genome (Table 1) were generated and transfected into HepG2 cells. In comparison with the patient’s wild type strain, the replication capacities of the S331C, rtA181T and S331C/rtA181T mutants were not different (94%, 82% and 96%, respectively), suggesting that these mutants can replicate at almost the same rate as the wild type strain (Fig. 3).

Susceptibility of mutants to lamivudine in vitro

To analyze the role of the polS331C and rtA181T mutations in lamivudine resistance, four patient-specific strains and four laboratory strains were transfected into HepG2 cells (Fig. 4, Table 1). A single amino acid substitution in the spacer region did not contribute to resistance in both patient and laboratory strains. In contrast, amino acid substitution in the polymerase (rtA181T) induced 3.0- and 3.9-times greater resistance than patient and laboratory strains (p<0.001), respectively. The presence of both of these amino acid changes induced 3.0- and 4.3-times greater resistance to each of the above strain. Thus, the spacer mutation had little effect on the susceptibility to lamivudine (Table 1).

We also compared the rtA181T mutant identified in this study with the rtA181T mutant reported previously, which had premature termination in the HBs protein (7, 34), for replication ability and susceptibility to lamivudine. Although the production of HBs antigen to culture supernatants were different between the two strains (52.5 ± 8.2 and
4.4 ± 0.6 IU/ml, respectively), there was no noticeable difference in replication ability and lamivudine sensitivity between the two mutants (data not shown).

Assessment of drug resistance of novel mutations in vivo using human hepatocyte chimeric mice

To confirm the lamivudine resistance of the novel mutant strain, two human hepatocyte chimeric mice were each inoculated with a serum sample obtained from the patient who developed breakthrough without mutations in the YMDD motif (Fig. 1A). The serum was obtained during breakthrough while the patient was still taking the drug. Twelve weeks after the inoculation of the serum samples, both mice developed high level viremia (7.8 and 6.6 log copies/ml, respectively). Direct sequence analysis showed that the nucleotide sequence of the virus that replicated in the chimeric mice was in accordance with the mutant strain. Cloning and sequencing analysis showed that only one of 12 clones obtained from the inoculum was wild type; while the remaining eleven clones were rtA181T mutant with intact YMDD motif. We also analyzed the serum of the two infected mice before and after lamivudine therapy. All 11 and 15 clones before and all 11 and 12 clones during therapy had rtA181T mutation (data not shown). Two other mice were inoculated with wild type HBV obtained from a patient not treated with lamivudine as a control, and both mice also developed high level viremia (8.3 and 9.3 log copies/ml, respectively). Thirteen weeks later, the viremia reached plateau and the mice were fed food containing lamivudine. After six weeks of treatment, the mean viral load decreased by 2.8 log copies/ml in the wild type, whereas it decreased by only 0.39 log copies/ml in the mutant (p<0.001, Fig. 5).
Susceptibility of mutants to adefovir and entecavir in vitro

We also analyzed the effects of adefovir and entecavir against the S331C/rtA181T mutant using transient transfection assay with HepG2 cells. The IC_{50} values of the mutant strain and wild type for both of these two drugs were almost the same (Table 2).

Detection of rtA181T mutant in patients treated with lamivudine

In this study, we developed a PCR-RFLP method to detect the rtA181T mutants, by which we were able to detect mutant strains even when mixed with the wild type (Fig. 6). The system also detected the rtA181T (HBs stop) mutant reported by Chien et al. (7) and Yeh et al. (34). Using this method, we analyzed 40 patients who showed viral breakthrough (increase in viral load equal or more than one log) during lamivudine therapy. We found that only one of these 40 patients was positive (Fig. 6A). Nucleotide sequence analysis of serum samples obtained from this patient showed that the mutant strain had rtA181T mutation with truncated HBs antigen, as reported previously (7, 34). The YMDD motif of HBV detected in this patient was of the wild type. All 39 remaining patients with viral breakthrough were positive for YIDD and/or YVDD mutants. The PCR-RFLP analysis of these 39 samples showed that four of these samples contain small amount of rtA181T mutants (Fig. 6B). Nucleotide sequence analyses of these samples showed that they contained only a small amount of rtA181T mutants with truncated HBs antigen (Fig. 6C).

Finally, we examined the presence of YMDD or rtA181T mutants in 8 patients who showed poor response with lamivudine treatment (HBV viral load above 6.0 log...
copies/ml after six months of treatment). None of these patients tested positive for both of these mutations (data not shown).
Discussion

In this study, we identified a novel lamivudine-resistant strain of HBV with intact YMDD motif in a patient who received long-term lamivudine therapy. YMDD mutant was not detected even by using sensitivity enhanced detection method, which was reported previously by our group (6). The double nucleotide substitutions (GG to TA) induced amino acid substitutions in both polymerase (rtA181T) and HBs antigen (HBs W172L). One may assume that the compliance of the patient was poor. However, the patient was very punctual and swore that he took lamivudine with perfect compliance.

Our study demonstrated that the rtA181T mutation reduced the susceptibility to lamivudine by 3.0 – 3.9-fold in vitro (Table 1). Furthermore, we also confirmed lamivudine resistance of this mutant strain in vivo using the human hepatocyte chimeric mice. The amino acid substitution in the RT domain is similar to that reported previously (7, 34). However, in contrast to our results, the mutant strains in the latter reports emerged with or after the mutation in the YMDD motif mutants (YIDD or YVDD) and took over them (34). There are two additional different points between the substitutions we identified and those of Yeh et al (34). Firstly, the HBs antigen was prematurely terminated in the mutant strain reported by Yeh et al (34). In this regard, a similar amino acid substitution of the B domain of the polymerase FLLA motif was reported in the woodchuck hepatitis virus (WHV) treated with lamivudine (15, 28). The HBs antigen in these WHV mutant strains also had premature stop codon. These findings suggest that the mutant strains identified in HBV and WHV cannot replicate and spread by themselves because of the lack of HBs antigen. Such strains are thought
to replicate \textit{in vivo} supplied HBs antigen from wild type strains as helper. In contrast, the novel strain identified in this study had no premature termination of HBs gene. The \textit{in vitro} study suggested that the strain had similar replication ability compared with wild type. Furthermore, we also showed that the strain infected and reached high viral load in human hepatocyte chimeric mice. Although the inoculum contained only a small amount of wild type strain (one of 12 clones), all clones obtained from mouse serum were mutant strain (rtA181T). Considering these results and the fact that the index patient showed high viral titer after breakthrough (more than 7.6 log copies/ml), this mutant strain can spread and replicate by itself and had strong replicative ability.

Secondly, the substitutions identified in this study appeared with nucleotide and amino acid substitution in the spacer region of the polymerase (S331C). There are only a few studies that reported the function of the spacer domain (19, 21, 28), leaving the biological significance of this region unknown. The substitution in the spacer region reappeared with the A181T mutation in the RT domain in the index patient after restarting lamivudine therapy. Although our study showed no significant contribution of this mutation to drug resistance (Figs. 3 and 4, Table 1), the significance of the mutation in this region (fingers in the HBV polymerase homology model [8]) should further be investigated.

Recently, the amino acid substitutions rtA181T and rtA181V have been reported to emerge with resistance against adefovir (11, 32). Tillmann et al. (29) reported one patient who developed the rtA181T mutation during famcyclovir breakthrough. The A556T mutation of WHV, analogous to the rtA181T mutation of HBV, has been reported to be associated with lamivudine resistance (15, 28). These results indicate that
the amino acid substitutions at position 181 may associate with resistance against many nucleoside analogues including lamivudine, famcyclovir and adefovir. Although our in vitro study indicated that the rtA181T mutant had no resistance against adefovir and the animal study showed that the combination therapy with lamivudine and adefovir effectively reduced the virus load in woodchuck (15), such combination therapy did not produce sufficient suppression of HBV in the index patient (Fig. 1A). Amino acid substitution at position 181 has to be further analyzed with regard to resistance against anti-HBV drugs.

The rtA181T mutation detection system using PCR-RFLP developed in this study is a useful tool as we were able to distinguish wild type from all mutants with nucleotide substitutions at the region. The system also enabled us to monitor the fluctuation of wild/mutant ratio during antiviral therapy against HBV (Figs. 1 and 6). The incidence of rtA181T mutants with intact YMDD motif is rare in Japanese patients with chronic HBV infection treated with lamivudine. Interestingly, 4 of the 39 (10%) patients who developed lamivudine breakthrough and were positive for YMDD mutants were found to have small amount of rtA181T mutant strains. Different from the previous report (34), the mutants did not take over another strain and were not preceded by exacerbation. We have to follow up these patients carefully for further population change of mutants and for exacerbation of hepatitis.

A recent study reported that the prevalence of genotype A HBV infection is increasing in Japan and the incidence of disease chronicity is higher than other genotypes (26). It is thus expected that increasing number of sexually active population will receive nucleoside analogue therapy against HBV and multiple mutant strains can
potentially emerge and spread along with long-term treatment. There is an increasing possibility of emergence of novel mutants resistant to multiple anti-HBV drugs. The importance and significance of the rtA181 mutations, including the novel mutant strain identified in this study, should be investigated further to develop more useful treatment strategies.
Acknowledgment

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Figure Legends

**Figure 1.** (A) Clinical course of a patient who developed breakthrough without emergence of YMDD mutant during lamivudine therapy. Letters a - e with arrow indicated time points of serum sampling for direct sequencing and PCR RFLP study.

(B) Nucleotide sequence analysis of the reverse transcriptase/polymerase gene of hepatitis B virus by direct sequencing. Refer to Figure 1A for time points of serum sampling. (a) just before lamivudine treatment, (b) after breakthrough, (c) after cessation of lamivudine treatment, (d) just before re-administration of lamivudine, (e) during adefovir and lamivudine therapy. Note that the wild type re-appeared during the cessation of therapy (c and d), but it disappeared after re-administration of the drug (e).

**Figure 2.** Comparison of nucleotide sequences and amino acid sequences of overlapping two open reading frames, reverse transcriptase/polymerase and HBs gene of the hepatitis B virus before and after viral breakthrough. Sequences obtained from serum samples before (a) and after (b) were compared. Refer to Figure 1A for time points of serum sampling (a - e). Nucleotide sequence numbers are those of typical HBV (ex, accession AB206816 [ref. 31]) which starts from unique EcoRI site.

**Figure 3.** Replication ability of wild type HBV and three types of mutants (S331C, rtA181T, S331C/rtA181T). Plasmids containing 1.4 genome length of HBV were transiently transfected into HepG2 cells. (A) The replicative intermediates were analyzed by Southern blot hybridization. Core-associated replicative intermediates of HBV DNA were isolated.
from HepG2 cells at 3 days after transfection. The positions of relaxed circular (RC) and replication intermediates (RI) are indicated. (B) Quantitative analyses of core associated intermediates of HBV. Experiments were performed in triplicates. Values are relative to those of wild type and are expressed as mean ± SD. * NS, not significant compared to wild type.

**Figure 4.** *In vitro* analyses of susceptibility of wild type HBV and three mutants (S331C, rtA181T, S331C/rtA181T) to lamivudine after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4 genome lengths HBV and treated with the indicated amount of lamivudine. (A) Southern blot analysis of replicative intermediate. Representative figures of wild type and the S331C/rtA181T mutant are shown. The positions of relaxed circular (RC) and replication intermediates (RI) form of HBV DNA are indicated. (B) Dose-response curve of the four HBV strains against lamivudine. They were used to estimate the lamivudine IC$_{50}$ values for each HBV strains. Values are relative to no lamivudine treatment controls of each strain. Experiments were performed in triplicates. Values are expressed as mean ± SD.

**Figure 5.** *In vivo* analyses of the effect of lamivudine on wild type and S331C/rtA181T mutant hepatitis B virus (HBV). Four human hepatocyte chimeric mice were inoculated with serum samples containing wild type or mutant strain of HBV. One of the animals fed with lamivudine died six weeks after the beginning of therapy.
**Figure 6.** Detection of rtA181T mutant by PCR and RFLP assay. PCR amplified DNA fragments were treated with *Esp*I, which digests only wild type sequences, and separated in a 3.5% agarose gel. (A) Agarose gel electrophoresis of PCR-RFLP products. Wild type and rtA181T mutant plasmids were used as controls. Refer to the time points of serum sampling in Fig. 1A for a to e of patient 1 and refer to Figure 1B for comparison with nucleotide sequence analyses. F and g indicated the time points before and after viral breakthrough for Patient 2. (B) Agarose gel electrophoresis of PCR-RFLP products using HBV-DNA samples obtained from 39 patients who showed lamivudine breakthrough. 35 of 39 samples were wild type as shown in lanes 1 and 2. The remaining four samples (lanes 3-7) showed partial digestion, suggesting a mixture of wild and mutant strains. (C) Nucleotide sequence analysis of a sample suggested the presence of wild and mutant type mixture by PCR-RFLP (lane 5 of panel B).
Table 1. In vitro susceptibility of S331/rtA181 mutation to lamivudine

<table>
<thead>
<tr>
<th>Strain</th>
<th>S331/rtA181</th>
<th>IC₅₀ (μM)</th>
<th>Fold resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>— /—</td>
<td>0.19±0.01</td>
<td>1</td>
</tr>
<tr>
<td>Patient’s strain</td>
<td>S331C</td>
<td>C / —</td>
<td>0.23±0.01</td>
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<tr>
<td></td>
<td>rtA181T</td>
<td>— / T</td>
<td>0.58±0.08</td>
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<tr>
<td></td>
<td>S331C/rtA181T</td>
<td>C / T</td>
<td>0.57±0.06</td>
</tr>
<tr>
<td>Laboratory strain</td>
<td>S331C</td>
<td>C / —</td>
<td>0.3±0.05</td>
</tr>
<tr>
<td></td>
<td>rtA181T</td>
<td>— / T</td>
<td>0.88±0.2</td>
</tr>
<tr>
<td></td>
<td>S331C/rtA181T</td>
<td>C / T</td>
<td>0.98±0.12</td>
</tr>
</tbody>
</table>

Experiments were performed in triplicates. Values are expressed as mean ± SD. * NS, not significant, ** P<0.001 compared to wild type.
Table 2. *In vitro* susceptibility of S331/rtA181 mutation to lamivudine, adefovir and entecavir

<table>
<thead>
<tr>
<th>Patient' strain</th>
<th>S331 / rtA181</th>
<th>Lamivudine</th>
<th>Adefovir</th>
<th>Entecavir</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
<td>Fold resistance</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
</tr>
<tr>
<td>wt</td>
<td>— / —</td>
<td>0.19±0.01</td>
<td>1</td>
<td>0.37±0.1</td>
</tr>
<tr>
<td>S331C/rtA181T</td>
<td>C / T</td>
<td>0.57±0.06</td>
<td>3**</td>
<td>0.36±0.08</td>
</tr>
</tbody>
</table>

Experiments were performed in triplicates. Values are expressed as mean±SD. *NS, not significant, **P<0.001 compared to wild type.
Figure 1A, Yatsuji et al.

- **HBeAg/HBeAb**
  - a: +/−
  - b: −/−
  - c: −/−
  - d: +/−
  - e: +/−

- **ALT (IU/L)**
  - 0
  - 200
  - 400
  - 600

- **HBV-DNA (Log.copies/ml)**
  - 3.7
  - 6
  - 8

- **Treatments**
  - LAM 100 mg/day
  - ADV 10 mg/day
Figure 1B, Yatsuji et al.
Figure 2, Yatsuji et al.

<table>
<thead>
<tr>
<th>Nucleotide sequence</th>
<th>TP</th>
<th>spacer</th>
<th>RT</th>
<th>RNaseH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>preS1</strong></td>
<td>a)</td>
<td>-a-a-c-a-g-t-a-a-</td>
<td>c-t-g-g-c-t-c-a-g-</td>
<td>S-ORF</td>
</tr>
<tr>
<td>b)</td>
<td>t</td>
<td></td>
<td>t-a</td>
<td>P-ORF</td>
</tr>
<tr>
<td>nt 82</td>
<td></td>
<td>nt 669 / 670</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**P-ORF**

|  | a) | ...R N S K P C... | ...F L L A Q F... | ...Y M D D... |
|  | b) | ...C... | ...T... | ...M... |
|  | pol 331 | | pol 527 (rt 181) | pol 550 (rt 204) |

**S-ORF**

|  | a) | ...S G T V N P... | ...R F S W L S... | ...I W M M... |
|  | b) | ...T... | ...L... | ...W... |
|  | preS2 31 | | | |
|  |  | S 172 | | S 195 |
Figure 3B, Yatsuji et al.
Figure 4A, Yatsuji et al.

Lamivudine (μM)

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>0.1</th>
<th>0.3</th>
<th>1.0</th>
<th>3.0</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RI</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

wt

S331C/rtA181T
Figure 4B, Yatsuji et al.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt</td>
<td>b</td>
<td>c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rta181T</td>
<td>d</td>
<td>e</td>
<td>f</td>
</tr>
<tr>
<td></td>
<td></td>
<td>f</td>
<td>g</td>
<td></td>
</tr>
</tbody>
</table>

236 bp- 206 bp-