Committee (approval no. 714). Informed consent was obtained from all patients.

Patients received a single daily oral administration of ADV 10 mg, in addition to ongoing LAM treatment (100 mg/day). The dosing interval of ADV was modified by the attending physician when serum creatinine level increased to >1.2 mg/dl. Liver cirrhosis was defined by the presence of stage 4 fibrosis on histopathological examination and/or clinical evidence of portal hypertension.

### Blood tests and serum viral markers

Routine biochemical tests were performed using standard procedures before and during therapy at least once every 3 months. Levels of HBsAg, HBeAg, and anti-HBe were determined using radioimmunoassay kits (Abbot Diagnostics, Chicago, IL, USA) or Chemiluminescent enzyme immunoassay (CLEIA; Lumipulse System, Fujirebio, Inc. Tokyo, Japan). Serum HBV DNA was quantified using the polymerase chain reaction (PCR)-based Amplicor HBV Monitor assay (Roche Diagnostics, Indianapolis, IN; lower limit of detection, 2.6 log copies/mL).

### Determination of nucleotide sequences of HBV DNA

DNA was extracted from 100 μL of serum. PCR reactions for detection of the reverse transcriptase (rt) region (nt 130–1161) of HBV DNA were performed in two parts. The first and second PCR reactions for detection of the first part of the rt region were performed using primers BGF1 (sense; 5′-CTGTGGAAGGCTGGCATTCT-3′) and BGR2 (antisense; 5′-GGCAGGATAGCCGCATTGTG-3′), and PreSBamH1 (sense; 5′-CTTGGGATCCAGAGCTAC AGCATGG-3′) and BR112 (antisense; 5′-TTCCGTCG ACATATCCCATGAAGTTAAGGGA-3′), respectively, under conditions of initial denaturation for 4 min, 35 cycles of amplification with 94 °C for 1 min, 55 °C for 2 min, 72 °C for 3 min, and a final extension at 72 °C for 7 min. The first and second PCR reactions for detection of the second part of the same region were performed using primer pairs B11F (sense; 5′-GGCCAAGTCTGTACAA CATC-3′) and B12R (antisense; 5′-TGCAGAGGTG AAGCGAAGTG-3′), and B11F and B14R (antisense; 5′-GATCCAGTTGGCAGCACACC-3′), respectively, under the same conditions. The amplified PCR products were used for direct sequencing or cloning methods as previously described [19, 20]. When mutations as a mixed viral population with the wild type sequence for direct sequencing were present, PCR was performed using a cloning method. Sequences of 9–26 independent clones from the sample were determined and analyzed. Measurement of sequences in the rt region was performed at the start of ADV treatment, and on viral breakthrough (VBT) during ADV plus LAM combination therapy. VBT was defined as any increase in serum HBV-DNA by >1 log copies/mL from the nadir or redetection of serum HBV-DNA at levels tenfold the lower limit of detection of the HBV-DNA assay after having an undetectable result. Moreover, sequences for serum HBV DNA level of ≥4 log copies/mL after 1 or 2 years of ADV plus LAM combination therapy were also measured.

### Measurement of LAM-, ADV-, ETV- and TDF-resistant variants using ultra-deep sequencing

Ultra-deep sequencing was performed using the Ion Personal Genome Machine (PGM) Sequencer (Life Technologies), as described previously [21]. An Ion Torrent adapter-ligated library was prepared using an Ion Xpress Plus Fragment Library Kit (Life Technologies). Briefly, 100 ng of fragmented genomic DNA was ligated to the Ion Torrent adapters P1 and A. The adapter-ligated products were nick-translated and PCR-amplified for a total of eight cycles. Subsequently, the library was purified using AM-Pure beads (Beckman Coulter, Brea, CA) and the concentration was determined using the StepOne Plus Real Time PCR (Life Technologies) and Ion Library Quantitation Kit in accordance with the manufacturer's instructions. Emulsion PCR was performed using Ion OneTouch (Life Technologies) in conjunction with an Ion OneTouch 200 Template Kit v2 (Life Technologies). Enrichment for templated ion spheres particles (ISPs) was performed using the Ion OneTouch Enrichment System (Life Technologies) in accordance with the manufacturer's instructions. Templated ISPs were loaded onto an Ion 314 chip and subsequently sequenced using 130 sequencing cycles in accordance with the Ion PGM 200 Sequencing Kit user guide. Total output read length per run is over 10 M base (0.5 M-tag, 200 base read). The results were analyzed with the CLC Genomics Workbench software (CLCbio, Aarhus, Denmark). A control experiment was included to validate the error rates in ultra-deep sequencing of the viral genome. In this study, amplification products of the second-round PCR were ligated with plasmid and transformed in Escherichia coli in a cloning kit (TA Cloning; Invitrogen, Carlsbad, CA). A plasmid-derived rt sequence was determined as the template by the control experiment. Coverage per position for aa180, aa181, aa184, aa194, aa202, aa204, aa233, aa236 and aa250 in the rt region was 63320, 63890, 67737, 49273, 57410, 57211, 40155, 34801 and 42914, respectively. Thus, using the control experiment based on the plasmid encoding rt sequence, amino acid mutations were defined as amino acid substitutions at a ratio of more than 0.25 % of total coverage. This frequency ruled out putative errors caused by the deep sequence platform used in this study.

### HBV genotype

The major genotypes of HBV were determined using the enzyme-linked immunosorbent assay (ELISA, Institute of Immunology, Tokyo, Japan) or the PCR-invader assay (BML, Inc, Tokyo, Japan) according to the method described by Usuda et al. [22] or Tadokoro et al. [23].

### Statistical analysis

Differences between groups were examined for statistical significance using the $\chi^2$ or Fisher's exact test where appropriate. Independent risk factors predicting the achievement of HBeAg seroclearance were studied using stepwise Cox regression analysis. The following 14 potential predictors of HBeAg seroclearance were assessed in this study: age, sex, pretreatment with IFN, severity of liver disease (CH or liver cirrhosis), duration from LAM to ADV, substitution of rtM204, HBV genotype, and levels of aspartate aminotransferase (AST), ALT, bilirubin, albumin, γ-glutamyl transpeptidase, platelets, and HBV DNA. Each was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. All factors found to be at least marginally associated with HBeAg seroclearance ($P < 0.10$) were tested in the multivariate Cox proportional hazards model, and hazard ratios and 95 % confidence intervals (CIs) were calculated to assess the relative risk confidence. The above calculations were performed using the Windows IBM SPSS version 19.0.0 software (IBM Corp., Armonk, NY, USA). A Kaplan–Meier estimate was also performed using the SPSS software.

## Results

### Study population

Clinical and virological profiles of the 406 patients at the start of ADV plus LAM combination therapy are shown in Table 1. At the start of combination therapy, 81 patients (20 %) had cirrhosis and 208 (51 %) were positive for HBeAg. Fourteen (3 %), 25 (6 %), 364 (90 %), 2 (0.5 %), and 1 (0.2 %) patients were infected with HBV genotypes A, B, C, D, and F, respectively. During the clinical course, 48 of 406 patients (12 %) showed an elevation in serum creatinine >1.2 mg/dL, and their ADV dose was accordingly reduced to 10 mg every second day.

### Response to ADV plus LAM combination therapy

The ratio of patients with undetectable serum HBV DNA levels (<2.6 log copies/mL) was 63 % (231/367), 72 % (254/352), 75 % (249/331), 79 % (235/297), 82 % (210/256), 80 % (137/171), and 85 % (94/110) at years 1 through 7, respectively (Fig. 1a). Among HBeAg-positive patients at baseline, undetectable rates of serum HBV DNA levels gradually increased from 1 to 7 years (42, 57, 65, 70, 76, 75, 83 % at years 1 through 7, respectively; $n = 208$). In contrast, ratios in HBeAg-negative patients at baseline were >80 % at all points (86, 89, 88, 90, 91, 87, 89 % at years 1 through 7, respectively; $n = 193$). The undetectable rates of serum HBV DNA in HBeAg-negative patients

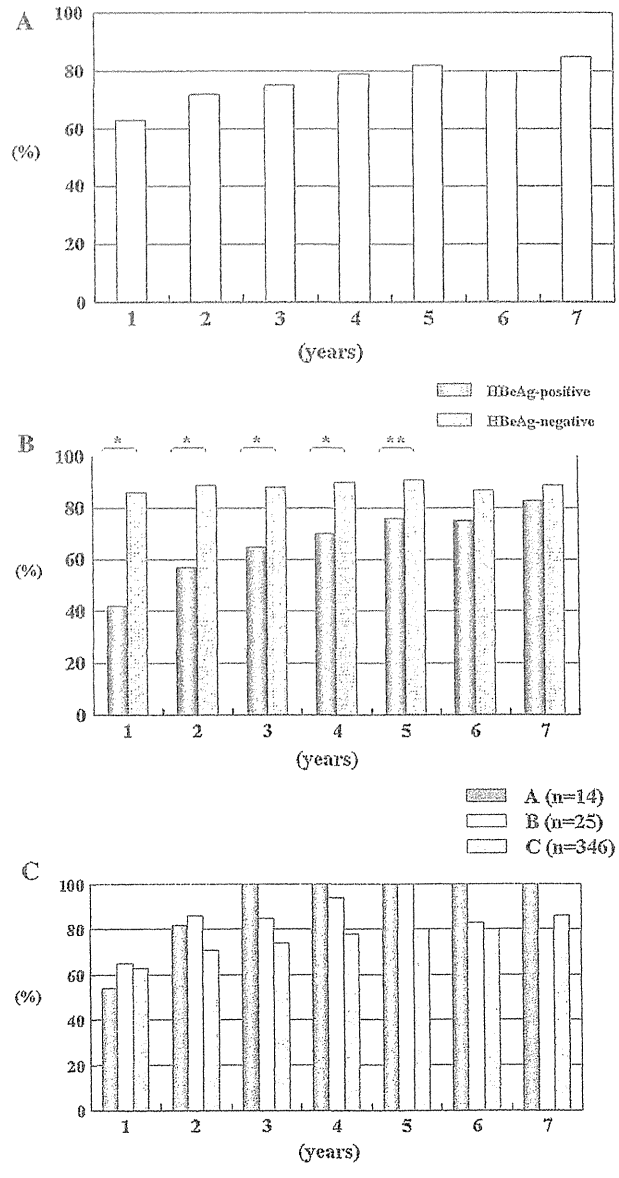


**Fig. 1** Undetectable serum HBV DNA levels (<2.6 log copies/mL) in years 1 through 7, respectively. **a** All patients. **b** HBeAg status. A *single asterisk* indicates a statistical significance of $P < 0.0001$ and a *double asterisk* indicates $P = 0.0044$, as determined at the $\chi^2$ test. **c** Genotypes A, B and C

were significantly higher than those in HBeAg-positive patients at years 1 through 5 ($P < 0.0001$ at years 1 through 4, and $P = 0.0044$ at year 5) (Fig. 1b).

By genotype, serum HBV DNA levels were undetectable after 3 years in 100 % of those with genotype A (54, 82, 100, 100, 100, 100, 100 % at years 1 through 7, respectively; $n = 14$), and in >80 % after 2 years in those with genotype B (65, 86, 85, 94, 100, 83, 80 %, at years 1 through 7, respectively; $n = 25$). In contrast, ratios in patients with genotype C gradually increased from 1 to 7 years (63, 71, 74, 78, 80, 80, 86 %, at years 1 through 7, respectively; $n = 364$) (Fig. 1c).

Moreover, the ratio of patients with ALT normalization (≤30 IU/L) was 66 % (250/380), 73 % (262/358), 78 % (255/327), 77 % (226/292), 77 % (194/251), 76 % (125/165), and 77 % (81/105) at years 1 through 7, respectively.

## HBeAg clearance

Eighty-four of 208 HBeAg-positive patients (40 %) achieved seroclearance of HBeAg. Cumulative HBeAg seroclearance rates from the commencement date of ADV plus LAM combination therapy were 13 % at 1 year, 24 % at 3 years, 35 % at 5 years, and 52 % at 7 years (Kaplan–Meier method; Supplementary Figure). No patients experienced the reappearance of HBeAg after seroclearance. Six factors found to be associated with the achievement of HBeAg seroclearance in univariate analysis were: AST upper limit of normal (ULN: 30 IU/L)×2<($P = 0.017$), bilirubin 1.1 < mg/dL ($P = 0.020$), ALT ULN×3 <($P = 0.040$), history of IFN therapy ($P = 0.068$), platelets 150<×$10^3$ μL ($P = 0.074$), and non C genotype ($P = 0.081$). In multivariate analysis, independent factors predicting the achievement of HBeAg seroclearance were history of IFN therapy ($P = 0.009$), AST ($P = 0.016$), bilirubin ($P = 0.030$), and genotype ($P = 0.042$) (Table 2).

## HBsAg clearance

Eight of 406 patients (1.9 %) achieved seroclearance of HBsAg (Supplementary Table). All patients were older than 40 years, and all but one was male. Three, two, and three patients were infected with HBV genotypes A, B, C, respectively; two patients were HBeAg-positive at baseline of combination therapy; and five patients had a history of IFN therapy. The duration of HBsAg seroclearance was 2.1–6.8 years.

## Genotypic analysis of ADV- and ETV-resistant mutants at baseline of combination therapy and clinical course

Genotypic resistance to LAM, ADV, ETV or TDF was analyzed in baseline samples before the start of ADV plus LAM combination therapy. Substitutions were assessed by direct sequencing or cloning, namely those at rtL180 or rtM204 associated with LAM resistance; rtA181, rtI233, or rtN236 associated with ADV resistance; rtT184, rtS202, or rtM250 associated with ETV resistance; and rtA194 associated TDF resistance. At baseline, substitutions associated with resistance to ADV or ETV were identified in 11 patients (2.7 %) (Table 3). RtA181S/T mutations without substitution at rtM204 were identified in four patients, whereas rtA181T mutation with substitution at rtM204 on the same clones was identified in three patients. RtA181T mutation and rtM204V/I mutation, which existed together on other clones, was identified in two patients. Substitutions related with ETV resistance were identified in the remaining two patients. All but one (Pt. 11) patient was HBeAg-positive and most were younger (<40 years old) and had a high viral load at baseline of LAM therapy. In the remaining 395 patients, rtM204 mutations without substitutions associated with resistance to ADV, ETV or TDF were identified in 358 patients, whereas 37 patients had no substitutions associated with resistance to LAM, ADV, ETV or TDF.

**Table 2** Factors associated with HBeAg seroclearance due to ADV plus LAM combination therapy on univariate and multivariate analyses

| Parameter | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | Hazard ratio (95 % CI) | *P* | Hazard ratio (95 % CI) | *P* |
| AST (≤UNL×2/UNL×2<) | 1.717 (1.102–2.676) | 0.017 | 1.750 (1.112–2.754) | 0.016 |
| Bilirubin (≤1.1/1.1<) | 1.783 (1.095–2.903) | 0.020 | 1.743 (1.056–2.876) | 0.030 |
| ALT (≤UNL×3/UNL×3<) | 1.577 (1.008–2.468) | 0.040 | | |
| History of IFN therapy (−/+) | | 0.068 | 1.824 (1.164–2.857) | 0.009 |
| Platelets (≤150 × $10^3$/150 × $10^3$<) | | 0.074 | | |
| Genotype (C/non C) | | 0.081 | 2.096 (1.025–4.274) | 0.042 |

*HBeAg* hepatitis B e antigen, *ADV* adefovir dipivoxil, *LAM* lamivudine, *CI* confidence interval, *AST* aspartate aminotransferase, *UNL* upper limit of normal: 30 IU/L, *ALT* alanine aminotransferase, *IFN* interferon

**Table 3** Characteristics of patients with resistance to ADV, ETV or TDF at baseline of ADV plus LAM combination therapy

| No. | Baseline of LAM therapy | | | | | Baseline of ADV plus LAM combination therapy | |
|---|---|---|---|---|---|---|---|
| | Age | Sex | Genotype | HBeAg | HBV DNA level | Mutation type (rt region) | Duration from start of LAM to emergence of mutation (years) |
| 1 | 29 | M | C | + | 7.6< | A181S | 3.3 |
| 2 | 32 | M | C | + | 7.6< | A181T | 1.3 |
| 3 | 23 | M | C | + | 7.6 | A181T | 2 |
| 4 | 34 | M | C | + | nd | A181T | 5 |
| 5 | 35 | M | C | + | 7.6< | A181T (17/19), L180M + M204V (2/19) | 1 |
| 6 | 37 | M | C | + | 6.5 | A181T (7/24), M204I (15/24), L180M + M204V (2/24) | 1.3 |
| 7 | 51 | M | C | + | 7.4 | A181T + M204I | 1.3 |
| 8 | 38 | F | C | + | nd | A181T + M204I (7/13), M204I (6/13) | 4 |
| 9 | 33 | M | C | + | nd | A181T + M204I (10/21), A181T + M204V(1/21), M204I (10/21) | 1.3 |
| 10 | 25 | F | D | + | nd | L180M + S202G + M204V | 5 |
| 11 | 31 | F | C | − | 7.6< | L180M + M204V + M250L | 6 |

No. of clones with combined mutations in rt region/total clones are shown in parentheses

*ADV* adefovir dipivoxil, *ETV* entecavir, *TDF* tenofovir disoproxil fumarate, *LAM* lamivudine, *HBV* hepatitis B virus, *HBeAg* hepatitis B e antigen, *nd* not done, *rt* reverse transcriptase, *M* male, *F* female

Following ADV plus LAM combination therapy, HBV DNA levels of four patients (Pt. 5, 6, 8, 10) were undetectable (<2.6 log copies/mL) (Fig. 2a), while those of the remaining seven were ≥2.6 log copies/mL. One patient (Pt. 7) achieved HBeAg clearance at 2 weeks, while HBeAg reappeared in a second patient (Pt. 11) at 40 weeks. Ratios of patients with undetectable levels of HBV DNA were 9 % (1/11) at 1 year, 22 % (2/9) at 2 years and 50 % (4/8) at 3 years. Three patients (Pt. 1, 2, 9) received TDF plus LAM or TDF plus ETV therapy after ADV plus LAM combination therapy due to insufficient virological response. Mutations of rtA181T + rtM204I, rtA181T + rtM204V and rtM204I in Pt. 9 changed to rtA181T + rtN236T and rtL180V + rtM204V after 3 years of combination therapy, and HBV DNA level was again thereafter elevated.

## Genotypic analysis of ADV- and ETV-resistant mutants during combination therapy and clinical course

Genotypic resistance to ADV, ETV or TDF was analyzed during ADV plus LAM combination therapy in 395 patients without ADV- or ETV-resistant mutants at baseline. During combination therapy, substitutions associated with resistance to ADV or ETV were identified in 12 patients (3 %) (Table 4). All patients were genotype C and had a high viral load (>5.0 log copies/ml) at baseline of combination therapy. Substitutions of rtM204 were identified in all but one patient (Pt. 19) at baseline. RtA181V/S/

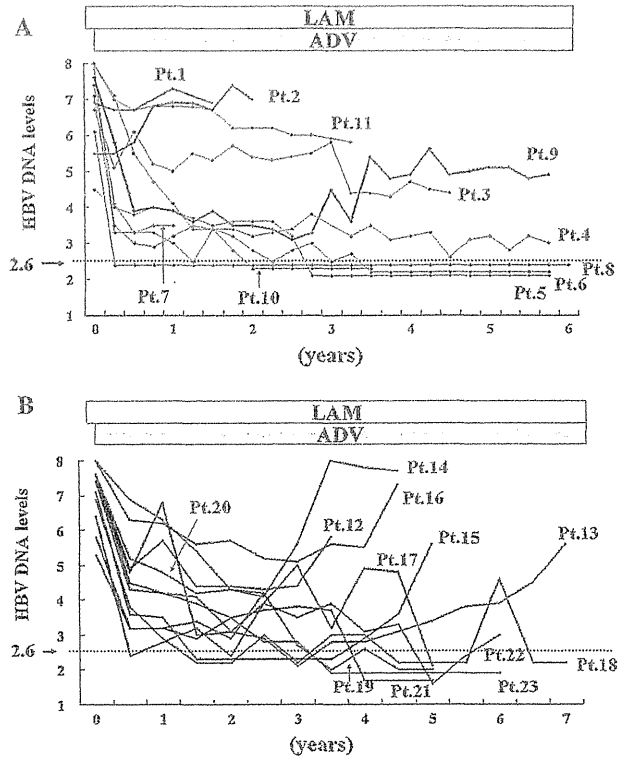


**Fig. 2** Clinical course (HBV DNA load) of patients. **a** Patients with resistance associated with ADV or ETV at baseline of ADV plus LAM combination therapy. **b** Patients with resistance associated with ADV or ETV during ADV plus LAM combination therapy

Table 4 Characteristics of patients with emergence of resistance to ADV, ETV or TDF during ADV plus LAM combination therapy

| No. | Baseline of ADV plus LAM combination therapy | | | | | | During ADV plus LAM combination therapy | |
|---|---|---|---|---|---|---|---|---|
| | Age | Sex | Genotype | HBeAg | HBV DNA level | rtM204 mutant type | Mutation type(rt) | Duration from start of ADV + LAM to emergence of mutations (years) |
| 12 | 32 | M | C | + | 7.6 | M204I/V | A181T + N236T (12/16), L180M + N236T (1/16), A181T (1/16), L180M + A181T + M204V (1/16), L180M + M204V + N236T (1/16) | 1 |
| 13 | 29 | M | C | + | 7.6 | M204I/V | A181T + M204I + M250L (13/18), L180M + M204V + M250L (2/18), L180M + T184I + M204I + M250L(1/18), L180M + M204I + M250L(1/18), A181T + M204I (1/18) | 5 |
| 14 | 58 | M | C | + | 7.6 < | M204I | L180M + T184I + M204I + M250L(16/26), L180M + T184I + M204I(6/26), A181T(4/26), | 3.5 |
| 15 | 49 | M | C | + | 5.1 | M204I/V | A181V + M250L | 5 |
| 16 | 46 | M | C | + | 7.6 | M204V | A181T + N236T | 3 |
| 17 | 30 | F | C | + | 7.4 | M204I | A181T | 0.2 |
| 18 | 40 | M | C | + | 6.9 | M204I | A181S | 4 |
| 19 | 40 | M | C | + | 5.3 | M204 | A181S | 2.3 |
| 20 | 49 | M | C | + | 7.6 | M204V | A181V | 0.1 |
| 21 | 63 | M | C | − | 5.8 | M204I | A181T(10/11), A181T + M204I(1/11) | 2 |
| 22 | 56 | M | C | − | 6.4 | M204V | A181S | 0.6 |
| 23 | 36 | M | C | + | 7.4 | M204I | M180M + A181T(5/9), L180M + A181T + M204I + M250I (3/9), L180M + M204I + M250I(1/9) | 1 |

No. of clones with combined mutations in the rt region/total clones are shown in parentheses

*ADV* adefovir dipivoxil, *ETV* entecavir, *TDF* tenofovir disoproxil fumarate, *LAM* lamivudine, *HBV* hepatitis B virus, *HBeAg* hepatitis B e antigen, *rt* reverse transcriptase, *M* male, *F* female

T mutation with or without substitution at rtM204 was identified in all patients, whereas rtT184I or rtM250I/L mutation with or without substitution at rtM204 was identified in 4 patients. Moreover, rtA181T + N236T double mutation related with ADV resistance was identified in two patients (Pt. 12 and 16). Interestingly, substitutions of rtM204 were not detected in five patients (Pt 15, 17, 18, 19, 22) when these ADV- or ETV-related mutations emerged.

Following ADV plus LAM combination therapy, the ratio of patients with undetectable levels of HBV DNA was 0 % (0/12) at 1 year, 25 % (3/12) at 2 years, 27 % (3/11) at 3 years, and 20 % (2/10) at 4 years (Fig. 2b). The HBV DNA levels of five patients (Pt. 12–16) were re-elevated after a decrease, and these patients were then switched to a different treatment (TDF plus LAM or TDF plus ETV in four patients and ETV plus ADV in one). Two of these five patients (Pt. 12 and 16) had rtA181T + rtN236T double mutation-related ADV resistance, while three (Pt. 12–14) had a wide variety of mutations. In contrast, HBV DNA levels of patients who had HBeAg clearance (Pt. 17–19, 23) during ADV plus LAM combination therapy were sustained at ≤5 Log copies/mL after 1 year, and only three patients (Pt. 19, 21, 22) showed sustained levels of ≤2.6 Log copies/mL after 4 years.

## Evolution of LAM-, ADV-, ETV- and TDF-resistant variants using ultra-deep sequencing

In 10 of 12 patients with emergent substitutions associated with resistance to ADV or ETV during combination therapy, LAM-, ADV-, ETV- and TDF-resistant variants were analyzed by ultra-deep sequencing at baseline (Table 5). Patients 13 and 20 could not be analyzed due to insufficient stored serum. RtA181T/V mutations were detected in all 7 patients by ultra-deep sequencing at baseline, although 6 of these 7 patients had very low frequency (<1 %) variants. Interestingly, rtA181S mutation in 3 patients could not be

**Table 5** Detection of resistance to ADV, ETV or TDF by ultra-deep sequencing at baseline in patients with emergence of resistance during ADV plus LAM combination therapy

| No. | Baseline of ADV plus LAM combination therapy (ultra-deep sequencing) | | | | | | | | | During therapy |
|---|---|---|---|---|---|---|---|---|---|---|
| | rtL180 | rtA181 | rtT184 | rtA194 | rtS202 | rtM204 | rtI233 | rtN236 | rtM250 | Mutation type(rt) |
| 12 | L (50.7 %) | A (96.4 %) | T (99.9 %) | A (99.9 %) | S (99.9 %) | I (59.1 %) | I (99.8 %) | N (99.9 %) | M (99.8 %) | A181T, |
| | M (49 %) | **T (3.5 %)** | | | | V (34.5 %) | | | | N236T |
| 14 | L (81.2 %) | A (99.4 %) | T (99.9 %) | A (99.7 %) | S (99.8 %) | I (99.6 %) | I (99.7 %) | N (99.8 %) | M (99.5 %) | A181T, T184I, |
| | M (15.6 %) | **T (0.56 %)** | | | | | | | I (0.38 %) | M250L |
| 15 | L (75.3 %) | A (97.5 %) | T (99.7 %) | A (99.7 %) | S (99.7 %) | I (70.6 %) | I (99.7 %) | N (99.8 %) | M (99.6 %) | A181V, |
| | M (24.4 %) | S (1.5 %) | | | | V (27.2 %) | | | | M250L |
| | | **V (0.75 %)** | | | | | | | | |
| 16 | M (99.3 %) | A (99.7 %) | T (99.9 %) | A (99.7 %) | S (99.8 %) | V (99.5 %) | I (99.7 %) | N (99.8 %) | M (99.4 %) I (0.51 %) | A181T |
| | L (0.26 %) | **T (0.27 %)** | | T (0.27 %) | | | | | | |
| 17 | L (99.8 %) | A (99.7 %) | T (99.9 %) | A (99.9 %) | S (99.9 %) | I (80.3 %) | I (99.7 %) | N (99.8 %) | M (99.7 %) | A181T |
| | | **T (0.25 %)** | | | | M (19.5 %) | | | | |
| 18 | L (87.9 %) | A (98.7 %) | T (99.9 %) | A (99.4 %) | S (99.5 %) | I (98.2 %) | I (99.7 %) | N (99.8 %) | M (98.9 %) | A181S |
| | M (11.9 %) | T (1.3 %) | | T (0.55 %) | | V (1.7 %) | | | I (0.97 %) | |
| 19 | L (99.8 %) | A (98.8 %) | T (99.9 %) | A (99.8 %) | S (99.8 %) | M (99.5 %) | I (99.6 %) | N (99.7 %) | M (99.6 %) | A181S |
| | | T (0.89 %) | | | | | | | | |
| 21 | L (98.8 %) | A (98.2 %) | T (99.9 %) | A (99.8 %) | S (99.8 %) | I (72.3 %) | I (99.6 %) | N (99.7 %) | M (99.6 %) | A181T |
| | M (0.96 %) | V (0.99 %) | | | | M (27.0 %) | | | | |
| | | S (0.48 %) | | | | V (0.49 %) | | | | |
| | | **T (0.35 %)** | | | | | | | | |
| 22 | M (99.4 %) | A (99.8 %) | T (99.8 %) | A (99.8 %) | S (99.8 %) | V (99.8 %) | I (99.6 %) | N (99.8 %) | M (99.6 %) | A181S |
| 23 | L (87.5 %) | A (99.1 %) | T (99.9 %) | A (99.9 %) | S (99.8 %) | I (99.4 %) | I (99.8 %) | N (99.8 %) | M (99.6 %) | A181T, M250I |
| | M (12.3 %) | **T (0.81 %)** | | | | M (0.48 %) | | | **I (0.31 %)** | |

Bold values indicate emergent substitutions during combination therapy

*ADV* adefovir dipivoxil, *ETV* entecavir, *TDF* tenofovir disoproxil fumarate, *LAM* lamivudine, *rt* reverse transcriptase

detected at baseline. In contrast, rtT184I, rtN236T or M250I/L mutations were detected in 1 of 4 patients with emergent mutations during combination therapy.

## Discussion

Although ADV plus LAM combination therapy is a standard rescue treatment for patients with LAM-refractory HBV, the virological benefits of long-term therapy have not yet been fully assessed. Here, we evaluated the long-term efficacy of ADV plus LAM combination therapy in 406 LAM-refractory patients over a median follow-up period of 5.4 years. We also investigated baseline factors associated with HBeAg clearance and HBsAg clearance. We found long-term combination therapy produced a gradual virological improvement. In particular, virological response was higher in patients who were HBeAg-negative at baseline, and genotype A and B. Toyama et al. [24] recently evaluated the long-term (median 41 months, 158 patients) efficacy of add-on ADV treatment for patients with LAM-resistant HBV and reported a rate of virological response of 90.8 % at 4 years. Inoue et al. [25] reported that HBV-DNA levels were undetectable (<2.6 log copies/mL) on long-term ADV plus LAM combination therapy (median 47 months; 28 patients, including 7 genotype B) in 56, 80, 86, and 92 % of patients at 12, 24, 36, and 48 months, respectively, whereas Aizawa et al. [26] reported undetectable levels on the same long-term regimen (median 46 months, 72 patients) in 61, 74, 81, 84, and 85 % at 12, 24, 36, 48, and 60 months, respectively, a pattern of response that was similar to our present findings. These differences in virologic response among these Japanese studies might have been due to treatment duration, genotype, or number of patients. Nevertheless, all these long-term studies in Japanese showed a gradual increase in virological response rate for 7 years, and that combination therapy with ADV plus LAM was effective for LAM-refractory patients without multidrug-resistant HBV.

The rate of HBeAg clearance at the end of follow-up in our study of 40 % was compatible with previous reports [13, 24]. The strongest predictor of HBeAg clearance on multivariate analysis was IFN history, as in a previous report [24]. Moreover, we recently reported that HBsAg clearance during NA therapy in patients with HBeAg was influenced by previous IFN therapy and HBV genotype [27]. These results suggest that previous IFN therapy might have an immunomodulatory effect on NA therapy. In addition, baseline levels of AST and bilirubin were also significantly associated with HBeAg clearance in this study. Our results agree with those of many clinical studies that have shown baseline transaminase levels to be the strongest predictor of HBeAg seroconversion in response to both IFN [11] and NA therapy [6, 28]. On the other hand, the rate of HBsAg clearance at the end of follow-up in the present study was only 1.9 %. As mentioned above, we reported that HBsAg clearance during NA therapy was influenced by previous IFN therapy and HBV genotype as well as HBsAg level at baseline or by a decrease in HBsAg level within 6 months [27]. That study [27] included patients originally treated with LAM monotherapy or ETV therapy who switched to LAM monotherapy along with ADV plus LAM combination therapy. In this regard, further study to evaluate factors affecting HBsAg clearance in ADV plus LAM combination therapy is necessary.

We previously reported the emergence of ADV-resistant mutations (rtA181T, rtA181S and rtA181T + rtN236T) in 3 of 132 patients at baseline and in 2 during subsequent combination therapy for a period of 2 years [17]. Moriconi et al. [29] reported that rtA181S and rtT184S mutations, either alone or with rtM204 mutation, at baseline in combination therapy in patients with viral breakthrough during LAM monotherapy correlated negatively with virologic response. Moreover, Heo et al. [30] reported that the presence of the rtA181V/T mutation at baseline was associated with a decreased rate of virologic response at 12 months of combination therapy. In the present study, we analyzed more patients with multidrug resistance during combination therapy over a longer clinical course. Substitutions associated with resistance to ADV or ETV were identified at baseline in 11 of 406 patients (2.7 %), most of whom were HBeAg-positive, of younger age, and had a high viral load. Moreover, a virological response during combination therapy was obtained in only four patients. On this basis, substitution of rtA181 without rtM204 mutation might correlate with a poor virological response in combination therapy. In contrast, virological response rate in patients with mutations associated with ETV (Pt. 10 and 11) was 50 %. Inoue et al. [25] detected ETV-resistant mutations of rtT184S and rtS202C during ADV plus LAM combination therapy, and noted that these patients also showed an ADV resistance profile on in vitro analysis. Moreover, a previous report showed that A181S, A181S + M204I, and L180M + T184S + M204V/I mutations were associated with a poor response to ADV plus LAM combination therapy [29]. In light of these results, A181S mutation and A181T without rtM204I/V mutation at baseline might be associated with multidrug resistance.

On the other hand, substitutions associated with resistance to ADV or ETV were identified in 12 of 395 patients (3 %) during combination therapy. Two patients (Pt. 12 and 16) in this group and a patient (Pt. 9) with rtA181T + M204V/I mutations at baseline developed rtA181T + rtN236T double mutation-related ADV resistance. Considering our clinical study, rtA181T + rtN236T

double mutation correlated with a poor virological response. Moreover, a wide variety of mutations (Pt. 12–14) might be correlated with a poor virological response. Inoue et al. reported that 1 of 28 patients developed virologic breakthrough after combination therapy and sequence analysis identified a wide variety of mutations, including L180M + A200V + M204V + N236T, L180M + A200V + M204V, L180M + M204V, L180M + T184S + M204V and L180M + S202C + M204V [25]. The replication capacity of each clone differed [25], and accordingly a wide variety of mutations might be associated with the development of multidrug resistance. Although rtA181S mutation emerged in three patients (Pt. 18, 19, 22), their HBV DNA level was sustained below 5 log copies/mL. This might be explained by the fact that two of these patients (Pt. 18, 19) had HBeAg clearance during combination therapy while the third (Pt. 22) was HBeAg-negative at baseline. In contrast, Lampertico et al. [31] reported that 9 of 145 (6 %) LAM-resistant patients developed rtA181T/V mutation before and during combination therapy for 4 years, but that HBV DNA levels progressively declined to become undetectable in 7 (78 %). In that report, however, rtA181T and rtA181V mutations were detected as a mixed population together with the wild-type sequence rtA181 in all serum samples. In our study, in contrast, rtA181S/T/V mutations were the major population and may accordingly have influenced the poor virologic response. In any case, response to combination therapy may be influenced by amino acid substitutions other than the well-known mutations associated with LAM, ADV, or ETV resistance, and further in vivo and in vitro studies are required.

Moreover, rtA181T/V mutations were detected by ultra-deep sequencing at baseline in 7 of 10 patients with emergent substitutions associated with resistance to ADV or ETV during combination therapy. It was possible that these mutant viruses increased during combination therapy. However, rtA181S, rtT184I or rtN236T or M250L were not detected at baseline. These data indicate that resistant variants of a minor population increased in some cases, whereas de novo resistant variants emerged during combination therapy in others. However, the number of patients analyzed by ultra-deep sequencing in this study was small; and we did not obtain data from patients without emergent substitutions associated with resistance during combination therapy. Further studies should be performed to interpret the significance of the presence of low frequency variants detected by ultra-deep sequencing.

In conclusion, this study shows that long-term ADV plus LAM combination therapy is effective for LAM-refractory patients. A history of IFN therapy, AST, bilirubin, and genotype were important factors in predicting HBeAg seroclearance. However, some patients did not achieve complete viral suppression of HBV DNA level (<2.6 Log copies/mL). We speculate that incomplete suppression might favor further selection of drug-resistant mutants, albeit that the frequency of multidrug resistance in the present study (5.7 %, 23/406) was low. Moreover, the presence of rtA181S mutation at baseline and emergence of rtA181T + rtN236T double mutation or a wide variety of mutations during combination therapy might be associated with a poor virological response. Several recent reports have indicated the effectiveness of TDF for ADV- or ETV-refractory patients [32–34]. Where indicated, HBV DNA and virological analysis should be carefully monitored.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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# Long-term entecavir therapy results in falls in serum hepatitis B surface antigen levels and seroclearance in nucleos(t)ide-naïve chronic hepatitis B patients

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SUMMARY. Entecavir (ETV) is reported to result in suppression of hepatitis B virus DNA (HBV DNA) replication with minimal drug resistance. However, information on the long-term effect of such therapy on serum hepatitis B surface antigen (HBsAg) level and elimination of HBsAg is not available. ETV therapy was started in 553 nucleos(t)ide-naïve patients with chronic hepatitis B infection (HBeAg positive: 45%) in our hospital. Serum HBsAg levels were measured serially by the Architect assay. The median baseline HBsAg was 2180 IU/mL (0.12–243 000 IU/mL), and median follow-up period was 3.0 years, with 529, 475, 355, 247 and 163 patients followed-up for 1, 2, 3, 4 and 5 years, respectively. At year 5, the mean log HBsAg decline from baseline was −0.48 log IU/mL, and the cumulative HBsAg clearance rate was 3.5%. Multivariate analysis identified HBV DNA level at baseline (<3.0 log copies IU/mL, odd ratio = 10.2; 95% confidence interval = 1.87–55.5, $P = 0.007$) and HBsAg level (<500 IU/mL, odd ratio = 29.4; 95% confidence interval = 2.80–333, $P = 0.005$) as independent predictors of HBsAg seroclearance. These results indicate that although serum HBsAg level declines gradually during ETV therapy, HBsAg seroclearance remains a rare event.

*Keywords:* chronic hepatitis, entecavir, hepatitis B surface antigen, hepatitis B virus.

## INTRODUCTION

Approximately 400 million people worldwide have chronic hepatitis B (CHB) infection, the majority of whom live in the Asia-Pacific region [1,2]. CHB patients with elevated viral load are at risk of cirrhosis, liver failure and hepatocellular carcinoma. Within the past 10 years, nucleos(t)ide analogs (NAs) have been approved in Japan for the treatment of CHB, and recent investigations have shown that entecavir (ETV) effectively suppresses hepatitis B virus DNA (HBV DNA) replication with minimal drug resistance [3–5].

Quantification of serum hepatitis B surface antigen (HBsAg) has been recently advocated as a marker of disease activity in CHB, and the correlation between HBV DNA and HBsAg level disappears after ETV therapy [6,7]. Very low rates of HBsAg clearance by antiviral therapies such as NAs have been reported in the past [4,8–13]. Other groups have also shown that serum HBsAg level can accurately predict the outcome of pegylated interferon therapy in CHB [14,15]. In this regard, pegylated interferon therapy is more successful than ETV at reducing serum HBsAg [16]. Nonetheless, the duration of follow-up period in the majority of the above studies is relatively short. On the other hand, the kinetics of serum HBsAg measurement during long-term NAs therapy remains unknown. Recent studies showed that serum HBsAg levels fall gradually during lamivudine (LAM) therapy [13]. However, little is known about serum HBsAg kinetics during long-term ETV therapy in CHB patients. In the present study, we assessed serum HBsAg kinetics, including the rate of HBsAg clearance, during long-term ETV treatment of NA-naïve CHB patients.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate transaminase; CHB, chronic hepatitis B; CIs, confidence intervals; ETV, entecavir; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBV-DNA, hepatitis B virus DNA; ORs, odds ratios; PCR, polymerase chain reaction; ULN, upper limit of normal.

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## PATIENTS AND METHODS

### *Patients*

We performed a retrospective analysis of 553 patients with CHB and cirrhosis who received ETV treatment at the Department of Hepatology, Toranomon Hospital, Tokyo, between March 2004 and March 2012, and adhered to the treatment for more than 6 months. All patients were negative for hepatitis C serological markers, but all had detectable HBsAg for at least 6 months prior to the commencement of ETV therapy. None had received other NAs previously. Each patient was treated with ETV at 0.5 mg/day for at least 6 months.

The diagnosis of hepatitis and cirrhosis was established by needle biopsy, peritoneoscopy and/or clinically before treatment. The clinical criteria for the diagnosis of chronic hepatitis included elevated alanine aminotransferase (ALT) over 6 months and absence of clinical evidence of portal hypertension, such as oesophageal varices, ascites, hepatic encephalopathy, together with features suggestive of cirrhosis on ultrasonography. Chronic hepatitis and cirrhosis were diagnosis in 408 and 145 patients, respectively.

Informed consent was obtained from each patient enrolled in the study, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the ethics committee of Toranomon Hospital.

The primary outcome for this study was HBsAg clearance. The endpoint of the follow-up was HBsAg clearance or last visit before March 2013. At least every 1–3 months, liver function and virological markers of HBV infection were assessed in every patient. Serum HBsAg titre was measured in frozen serum samples (stored at −80 °C) collected at baseline and once annually over a period of 1–5 years.

### *Markers of HBV infection*

Serum HBsAg titres were measured using the Architect HBsAg QT assay kit (Abbott Laboratories, Tokyo, Japan). The lower and upper limits of detection of this kit are 0.05 and 250 IU/mL, respectively. To expand the upper range from 250 to 125 000 IU/mL, serum samples that went off the scale were diluted stepwise to 1:20 and 1:500 with Architect diluents as described in the product document. Hepatitis B e antigen (HBeAg) was determined by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). HBV DNA was quantified using the Amplicor monitor assay (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.6–7.6 log copies/mL. The major genotypes of HBV were determined using an ELISA kit (Institute of Immunology) or PCR-invader assay (BML, Inc, Tokyo, Japan) according to the methods described previously [17,18].

### *Statistical analysis*

Categorical data were compared between groups using the chi-square test or Fisher's exact test. Continuous variables with nonparametric distribution were analysed by the Mann–Whitney *U*-test, while those with a parametric distribution were analysed by the Student's *t*-test. All *P*-values were two-tailed, and $P < 0.05$ was considered statistically significant. Cox regression analyses were used to assess those variables that correlated significantly with HBsAg clearance. All baseline factors that were found to be significantly associated with HBsAg clearance by univariate analysis were entered into a multivariate analysis. Independent baseline factors associated with clearance of HBsAg were calculated using a stepwise Cox regression analysis. Data analysis was performed using the Statistical Package for Social Science version 11.0.1J (SPSS, Chicago, IL, USA).

## RESULTS

### *Study population*

Table 1 lists the characteristics of participating patients at baseline. Of the 553 patients, 68% were males, and the median of age was 48 years. At baseline, the HBV DNA

**Table 1** Characteristics of patients at the start of entecavir therapy

| | |
|---|---|
| *n* | 553 |
| Sex, male/female | 377/176 |
| Age, years | 48 (17–82) |
| Family history of HBV | 357 (66.8%) |
| Cirrhosis | 145 (26.2%) |
| Previous IFN therapy | 128 (23.1%) |
| Median duration of treatment, years (range) | 3.0 (0.5–7.5) |
| Laboratory data | |
| Aspartate aminotransferase (AST), IU/L | 50 (14–1595) |
| Alanine aminotransferase (ALT), IU/L | 65 (7–2121) |
| Total bilirubin, mg/dL | 0.7 (0.2–14.5) |
| γGT, IU/L | 40 (9–679) |
| Albumin, gd/L | 3.9 (1.9–4.7) |
| Alpha fetoprotein, ng/mL | 5 (1–1319) |
| HBeAg positive | 249 (45.0%) |
| Viral load, $\log_{10}$ copies/mL | 6.5 (<2.6–>7.6) |
| HBsAg, IU/mL | 2180 (0.12–243 000) |
| *HBeAg positive | 5400 (1.01–243 000) |
| *HBeAg negative | 1375 (0.12–29 000) |
| HBV genotype, A/B/C/D/H/unknown | 18/75/441/1/1/17 |

Data are number of patients or median (range).

level was 6.5 log copies/mL, and 45% of the patients were HBeAg positive. Furthermore, 18, 75 and 441 patients were infected with CHB virus genotype A, B and C, respectively.

### *HBsAg titres*

The baseline median HBsAg level was 2180 IU/mL. Baseline HBsAg correlated moderately with HBV DNA levels in HBeAg-positive patients ($r = 0.261$, $P < 0.001$), but not in HBeAg-negative patients ($r = -0.019$, $P = 0.747$).

Figure 1a,b shows the fall in HBsAg at the end of the 5-year study period. The mean fall in HBsAg level from baseline was −0.21 log IU/mL at year 1, −0.27 at year 2, −0.34 at year 3, −0.42 at year 4 and −0.48 at year 5. The baseline HBsAg levels and the changes in HBsAg levels according to HBeAg status, HBV genotype and baseline HBs levels are shown in Figs 2, 3 & Fig. S1. The median baseline HBsAg level of HBeAg-positive patients (5400 IU/mL) was significantly higher than that of HBeAg-negative patients (1375 IU/mL, $P < 0.001$) (Fig. 2a). The mean changes in HBsAg levels in HBeAg-positive and HBeAg-negative patients were −0.52 and −0.44 log IU/mL at year 5, respectively. Furthermore, there were significant differences in the decline of HBsAg levels at years 1, 2 and 3 between HBeAg-positive and HBeAg-negative patients ($P < 0.001$, 0.01 and 0.05, respectively, Fig. 2c). The median baseline HBsAg levels tended to be higher in patients with genotype C (2520 IU/mL) than those with genotype B (877 IU/mL, $P < 0.001$, Fig. S1a). The mean changes in HBsAg levels were −0.80 and −0.43 log IU/mL for patients with genotypes B and C at year 5, respectively. However, there was no significant difference in the decline between the two groups (Fig. S1c).

Patients were further stratified according to baseline HBsAg levels into <100, 100–1000 and >1000 IU/mL. The mean changes in HBsAg levels from baseline at year 5 were −0.68, −0.35 and −0.50 log IU/mL among HBsAg <100, 100–1000 and >1000 IU/mL groups, respectively. There were significant differences in the decline of HBsAg levels at years 1, 2, 3 and 4 between baseline HBsAg 100–1000 and >1000 IU/mL ($P < 0.001$, <0.001, 0.002 and 0.01, respectively). There were also significant differences in the fall in HBsAg level at years 1 and 2 from the baseline between the HBsAg <100 and 100–1000 IU/mL groups ($P = 0.03$ and 0.005, respectively). However, there was no significant difference in the fall in HBsAg from baseline between the HBsAg <100 and >1000 IU/mL groups (Fig. 3).

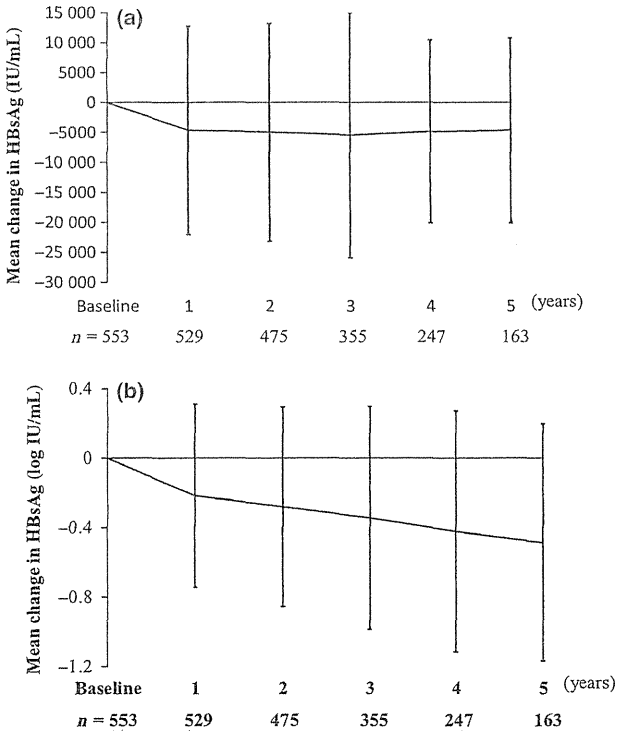


**Fig. 1** (a) Mean decline in HBsAg relative to the baseline for all patients treated with ETV (real value). (b) Mean decline in HBsAg relative to the baseline for all patients treated with ETV (logarithmic axis). HBsAg, hepatitis B virus surface antigen; ETV, entecavir.

### *HBsAg seroclearance during ETV therapy*

Table 2 shows the clinical and virological characteristics of patients who showed HBsAg seroclearance. Seven patients (two infected with genotype B, five with genotype C) achieved HBsAg seroclearance during ETV therapy. Only one patient was HBeAg positive at baseline, with HBeAg seroconversion occurring after 84 days. Five patients (71.4%) developed antibody to HBsAg. The cumulative HBsAg clearance rates were 0.2% at year 1, 1.0% at year 3 and 3.5% at year 5 (Fig. S2). Multivariate analysis identified HBV DNA level (<3.0 log copies/mL, $P = 0.007$) and HBsAg level (<500 IU/mL, $P = 0.005$) at the start of treatment as significant factors associated with HBsAg seroclearance (Table 3).

Moreover, among the 89 patients with baseline HBsAg levels <500 IU/mL, 6 (6.7%) achieved HBsAg seroclearance. The mean changes in HBsAg levels for HBsAg seroclearance and no HBsAg seroclearance were −0.73 and −0.082 log IU/mL at year 1 and −1.55 and −0.46 log IU/mL at year 5, respectively. Among the 40 patients with baseline HBV DNA levels <3.0 $\log_{10}$ copies/mL, 4 (10.0%) achieved HBsAg seroclearance. The median baseline HBsAg level was significantly higher in patients who showed no HBsAg seroclearance(616.5 IU/mL) compared with those who showed HBsAg seroclearance (0.63 IU/mL, $P = 0.005$). The mean changes in HBsAg levels in patients with HBsAg seroclearance and those without such seroclearance were −1.30 and −0.35 log IU/mL at year 1 and −1.43 and −0.47 log IU/mL at year 5, respectively.

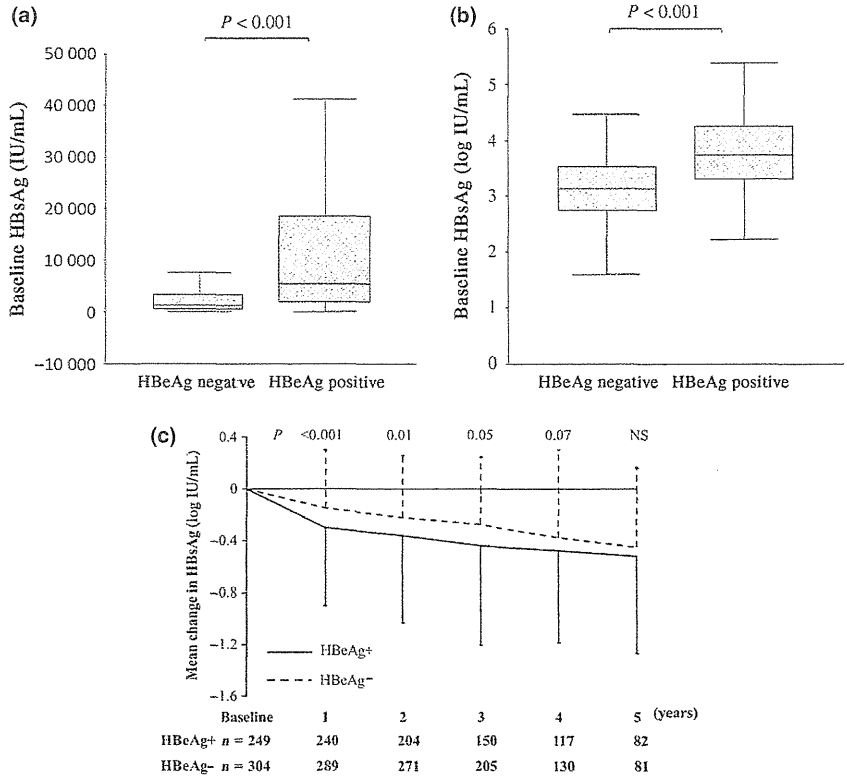


**Fig. 2** (a) Box-and-whisker plots of baseline HBsAg level in HBeAg-positive and HBeAg-negative patients (real value). In these plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. (b) Box-and-whisker plots of baseline HBsAg level in HBeAg-positive and HBeAg-negative patients (logarithmic axis). In these plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. (c) Mean HBsAg decline relative to the baseline for HBeAg-positive patients and HBeAg-negative patients (real value). *P*-values by Mann–Whitney *U*-test. HBsAg, hepatitis B virus surface antigen; HBeAg, hepatitis B e antigen.

## DISCUSSION

We have already reported that ETV is effective in suppressing HBV DNA replication with minimal drug resistance [5]. Recently, serum HBsAg kinetics has been evaluated as a marker for monitoring treatment of CHB, and the relation between HBV DNA and HBsAg level disappears after NA treatment, following the profound suppression of HBV DNA [6,7,13]. To our knowledge, there is little or no information on the long-term changes in serum HBsAg levels in nucleoside-naïve patients treated with ETV.

In this study, the annual fall in HBsAg was 0.097 log IU/mL during ETV therapy, which is similar to the HBsAg decline rate reported during the natural history and patients treated with LAM [13,19]. On the other hand, it was reported that serum HBsAg decreased at a rate of 0.71 log IU/mL/year during pegylated interferon therapy in HBeAg-negative patients [15]. These differences in the response to therapy are due to the inhibitory effects of NAs on viral replication through the suppression of HBV polymerase, persistent production of HBsAg through a pathway distinct from that of HBV DNA [20]. We also reported, in the present study, the changes in HBsAg levels based on HBeAg status and HBV genotype. The results showed significant differences in the rate of decline of HBsAg level. However, the rate of fall in HBsAg level was always gradual, and the above factors did not seem to influence HBsAg seroclearance. Previous studies indicated that genotypes A and D have an impact on the decline and clearance of HBsAg during NA therapy [8,9,12]. With regard to HBV genotype, our study only investigated genotypes B and C due to the small number of patients attending our hospital who were infected with other genotypes.

Hepatitis B virus surface antigen seroclearance remains the ultimate endpoint of CHB treatment. In the present study, multivariate analysis identified baseline HBV DNA level (<3.0 log copies/mL) and baseline HBsAg level (<500 IU/mL) as significant and independent determinants

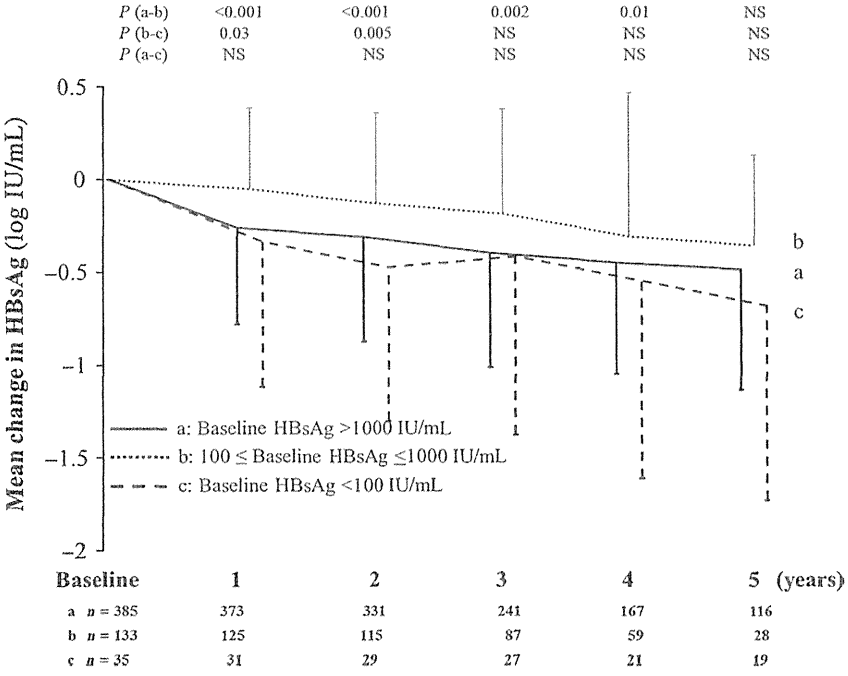


Fig. 3 Mean fall in HBsAg relative to the baseline, stratified by baseline HBsAg levels, <100 IU/mL, 100–1000 IU/mL, and >1000 IU/mL (logarithmic axis). *P*-values by Mann–Whitney *U*-test. HBsAg, hepatitis B virus surface antigen.

**Table 2** Characteristics of the seven patients who showed HBsAg seroclearance

| | At start of ETV therapy | | | | | | From baseline to year 1 | | |
|---|---|---|---|---|---|---|---|---|---|
| No. | Age (year)/ Sex | HBsAg (IU/mL) | HBV DNA (log copies/ mL) | HBeAg status | HBV genotype | Liver histology | Change in HBsAg (log IU/mL) | Time to HBsAg seroclearance (years) | HBsAg seroconversion |
| 1 | 56/M | 0.12 | 4.5 | − | C | LC | +1.19 | 4.8 | + |
| 2 | 45/M | 0.35 | <2.6 | − | B | CH | −0.69 | 1.7 | + |
| 3 | 66/M | 0.39 | <2.6 | − | C | CH | −0.89 | 2.3 | − |
| 4 | 72/F | 0.86 | <2.6 | − | C | CH | −1.23 | 3.1 | − |
| 5 | 61/F | 30.6 | <2.6 | − | C | CH | −2.40 | 2.0 | + |
| 6 | 54/M | 74.2 | 3.9 | − | C | CH | −0.36 | 3.6 | + |
| 7 | 65/M | 15 200 | >7.6 | + | B | CH | −5.48 | 0.7 | + |

LC, Liver cirrhosis; CH, chronic hepatitis; HBsAg, hepatitis B virus surface antigen; HBV DNA, hepatitis B virus DNA; HBeAg, hepatitis B e antigen; ETV, entecavir.

of HBsAg seroclearance. Previous studies identified baseline HBsAg level as a predictor of ETV-related HBsAg decline [7], and annual decline rate of HBsAg of 0.5 log IU/mL as a predictor of NA-related HBsAg seroclearance [12,13,21]. Among patients with baseline HBsAg levels <100 and 100–1000 IU/mL, the HBsAg decline rate was greater in patients with lower baseline HBsAg level, and a decline in HBsAg levels of >0.5 log IU/mL was observed in five of seven patients (71%) who achieved HBsAg seroclearance. Furthermore, a decline of >0.5 IU/mL in HBsAg level and HBsAg seroclearance was noted in four of 12 patients (33.3%) with both low HBV DNA level (<3.0 log copies/mL) and low HBsAg level (<500 IU/mL) at baseline. The results suggest that long-term ETV therapy is effective with regard to HBsAg seroclearance in these patients.

Entecavir was discontinued in two of five patients after HBsAg seroconversion after 1 and 3 months of treatment, respectively, and none showed HBsAg seroreversion at the end of 30 months of post-treatment follow-up.

**Table 3** Results of univariate and multivariate analyses for host and viral factors associated with HBsAg clearance

| | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| Parameter | OR (95% CI) | *P* | OR (95% CI) | *P* |
| Sex (male) | 1.14 (0.22–5.87) | 0.876 | | |
| Age (>50 years) | 7.75 (0.93–64.4) | 0.058 | | |
| Family history of HBV infection | 2.28 (0.26–19.6) | 0.450 | | |
| Previous IFN therapy | 1.20 (0.23–6.22) | 0.824 | | |
| Presence of cirrhosis | 0.39 (0.04–3.25) | 0.385 | | |
| ALT (> ×3 upper limits of normal) | 0.94 (0.18–4.87) | 0.947 | | |
| Total bilirubin (>1.0 mg/dL) | 0.65 (0.07–5.47) | 0.698 | | |
| HBsAg (<500 IU/mL) | 33.3 (4.03–25.0) | 0.001 | 29.4 (2.80–333) | 0.005 |
| HBeAg (negative) | 5.88 (0.70–50.0) | 0.103 | | |
| HBV DNA (<3.0 $\log_{10}$copies/mL) | 25.6 (5.49–125) | <0.001 | 10.2 (1.87–55.5) | 0.007 |
| HBV genotype C | 0.45 (0.08–2.35) | 0.347 | | |

HBsAg, hepatitis B virus surface antigen; HBV DNA, hepatitis B virus DNA; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase

In the present study, the cumulative HBsAg clearance rate was 3.5% at year 5, which is lower than the HBsAg clearance rate reported during the long-term natural history of infection [22,23]. We reported previously that seroclearance of HBsAg in treated and untreated patients is influenced by HBeAg status and baseline HBsAg [23]. Randomized control clinical trials are necessary to identify differences in HBsAg clearance rates between ETV-treated and ETV-untreated patients.

In the present study, none of the 128 patients who were treated previously with IFN achieved HBsAg seroclearance. We have already reported that previous IFN therapy is associated with HBsAg seroclearance in HBeAg-positive patients treated with lamivudine [12]. The reason for the different outcome may be related to differences in ALT levels or HBV DNA level at baseline between the ETV group and lamivudine group. Alternatively, the different response may be related to differences in viral mutation.

In summary, serum HBsAg levels decreased gradually during ETV therapy in NA-naïve CHB patients (by approximately 0.1 log IU/mL/year). The cumulative HBsAg clearance rate was 3.5% at year 5, and baseline low serum HBsAg and HBV DNA level were identified as two significant and independent determinants of HBsAg seroclearance. These finding suggest that HBs seroclearance is probably a rare event during ETV therapy.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST

Hiromitsu Kumada has received speaker's honoraria from Bristol-Myers Squibb. All other authors declare no conflict of interest.

## FINANCIAL SUPPORT

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1:** (A) Box-and-whisker plots of baseline HBsAg level in patients with genotypes B and C (real value). In these plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. (B) Box-and-whisker plots of baseline HBsAg level in patients with genotypes B and C (logarithmic axis). In these plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. (C) Mean decline in HBsAg relative to the baseline for patients with genotypes B and C (logarithmic axis). *P* values by Mann-Whitney U-test.

**Figure S2:** Cumulative HBsAg clearance rates analyzed with the Kaplan-Meier test.

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CASE REPORT

# Virologic breakthrough in a patient with chronic hepatitis B by combination treatment with tenofovir disoproxil fumarate and entecavir

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**Abstract:** Tenofovir disoproxil fumarate (TDF) is widely used to treat hepatitis B virus (HBV) patients in the USA and Europe. No confirmed report of resistance selection during treatment with TDF in treatment-naïve and nucleoside/nucleotide analog-treated chronic hepatitis B patients has yet been reported. Here, we report for the first time a patient with chronic hepatitis B and cirrhosis who emerged with virologic breakthrough during combination therapy with TDF and entecavir (ETV), against ETV-resistant virus. A 51-year-old Japanese woman with hepatitis B e-antigen (HBeAg), whose genotype was C, received ETV monotherapy continuously followed by TDF and ETV combination therapy, because her HBV DNA levels had been >3.5 log copies/mL. At the start of combination therapy, amino acid substitutions of the reverse transcriptase (rt) gene, rtL180M, rtT184I/M, and rtM204V, were detected. After this, serum HBV DNA decreased to less than 2.1 log copies/mL and remained at this level until 31 months of combination therapy, when it again began to increase. Amino acid substitutions of rtL180M, rtS202G, and rtM204V emerged and were associated with an increase in serum HBV DNA at virologic breakthrough. Long-term therapy with TDF against the ETV-resistant virus has the potential to induce virologic breakthrough and resistance, and careful follow-up should be carried out.

**Keywords:** hepatitis B virus, resistant

## Introduction

Hepatitis B virus (HBV) infection is a common disease that can induce a chronic carrier state and is associated with the risk of progressive disease and hepatocellular carcinoma.[1] Interferon (IFN) and several nucleoside/nucleotide analogs (NAs), such as lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), and tenofovir disoproxil fumarate (TDF), are currently approved for the treatment of chronic hepatitis B (CHB) in most countries.[2–5] Because NA analogs inhibit reverse transcription of the HBV polymerase but do not directly interfere with the formation of covalently closed circular DNA (cccDNA), they require long-term administration, which is usually accompanied by the emergence and selection of drug-resistant mutations in the viral polymerase.[6]

TDF is widely used to treat HBV patients in the USA and Europe. This agent is equally effective against multiple HBV genotypes (A–H) as well as against LAM-resistant isolates.[7] No confirmed report of resistance selection during treatment with TDF in treatment-naïve CHB patients has yet been reported.[8–10] In a recent study, long-term TDF monotherapy provided durable antiviral efficacy for 240 or up to 288 weeks (6 years) of treatment, and comprehensive genotypic and phenotypic analyses detected no evidence of TDF resistance.[11,12] Additionally, longer treatment duration

did not increase the incidence of virologic breakthrough.[12] Moreover, TDF monotherapy has demonstrated the long-term (median 23 months) efficacy of this agent in NA-experienced patients with treatment failure, and virologic breakthrough was not observed in any patient during the entire observation period.[13]

Here, we report for the first time a patient with CHB and cirrhosis who emerged with virologic breakthrough during TDF and ETV combination therapy against ETV-resistant virus.

## Case report

A 51-year-old Japanese woman with CHB underwent a checkup in February 1999 and was found to be seropositive for hepatitis B surface antigen (HBsAg), with mild alanine aminotransferase (ALT) elevation. Hepatitis B e-antigen (HBeAg) was positive, and serum HBV DNA was >7.6 log copies/mL (Amplicor HBV Monitor assay; F Hoffman-La Roche Ltd, Basel, Switzerland). The HBV genotype was C, and human immunodeficiency virus (HIV) status was negative. She was diagnosed with cirrhosis by peritoneoscopy and liver biopsy (moderate hepatitis [A2] and severe fibrosis [F4]) in February 2000. She received LAM (100 mg/day) monotherapy from September 2006. The nadir of HBV DNA was 2.5 log copies/mL in January 2007. HBV DNA levels gradually increased, and LAM-resistant virus emerged (reverse transcriptase [rt] M204I). Treatment was switched from LAM to ETV (0.5 mg/day) in October 2007 (HBV DNA 3.9 log copies/mL) following the emergence of ETV-resistant virus (rtL180M, rtS202G, and rtM204V) and higher elevation in HBV DNA. However, she discontinued therapy of her own volition from February 2009 to May 2010. She returned to our hospital in May 2010 because of general fatigue and ascites, at which time serum HBV DNA was >7.6 log copies/mL, ALT was 687 IU/L, and bilirubin was 3.8 mg/dL. Treatment with ETV (0.5 mg/day) was restarted immediately, and ALT and serum HBV DNA levels gradually decreased. However, because HBV DNA levels remained at >3.5 log copies/mL until September 2010, she was started on TDF (300 mg/day) and ETV combination therapy (HBV DNA 3.9 log copies/mL). Serum HBV DNA then decreased to less than 2.1 log copies/mL (COBAS® TaqMan® HBV Test, v2.0; F Hoffman-La Roche Ltd) at November 2011 (month 14 of TDF and ETV treatment) and remained at this level until April 2013 (month 31 of TDF and ETV treatment), when it again began to increase (HBV DNA 3.9 log copies/mL). Moreover, ALT was elevated in September 2013 (Figure 1). Compliance with TDF and ETV was good throughout the course of treatment.

During treatment, nucleotide sequences of the polymerase gene were determined by polymerase chain reaction (PCR) direct sequencing, as previously described.[14] The viral polymerase reverse transcriptase (rt) gene at the baseline of LAM treatment (September 2006) showed the wild type sequence

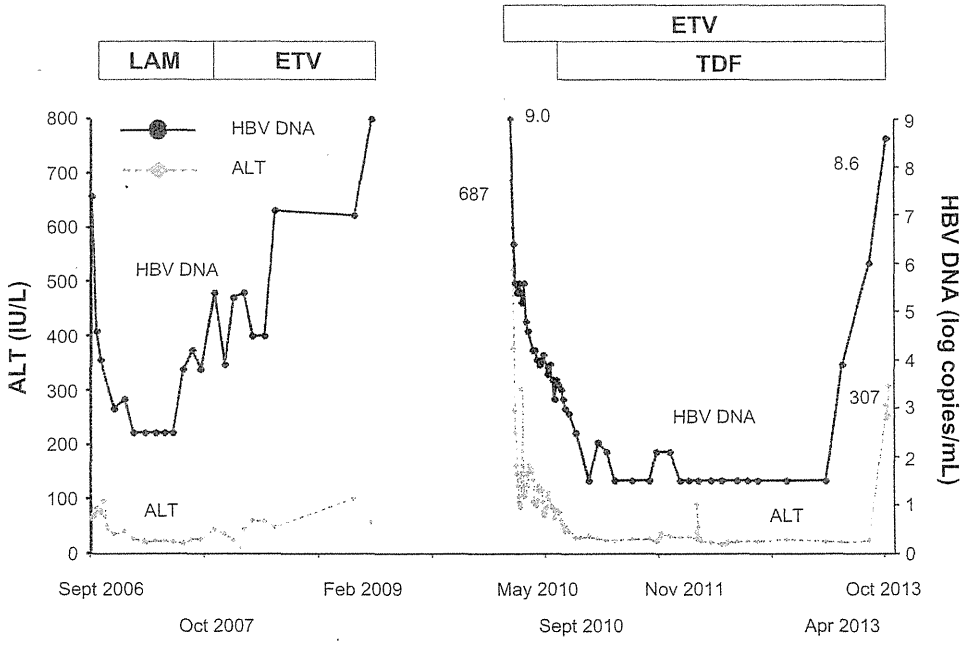


**Figure 1** Clinical course of lamivudine or entecavir and tenofovir disoproxil fumarate therapy.
**Abbreviations:** ALT, alanine aminotransferase; ETV, entecavir; HBV, hepatitis B virus; LAM, lamivudine; TDF, tenofovir disoproxil fumarate.

(with no LAM, ADV, ETV, or TDF resistance substitutions). In October 2007 (after switching from LAM to ETV), an amino acid substitution of the rt gene, rtM204I (LAM resistance substitution), was detected. Moreover, amino acid substitutions of rtL180M, rtS202G, and rtM204V (ETV resistance substitutions) emerged during ETV treatment in February 2009. The rt gene analysis at the baseline of ETV retreatment (May 2010) returned the wild type sequence (with no LAM or ETV resistance substitutions). In June 2010 (week 3 of ETV retreatment), amino acid substitutions of rtL180M, rtT184I, and rtM204V (ETV resistance substitutions) were simultaneously detected. Moreover, amino acid substitutions of rtL180M, rtT184M, and rtM204V coexisted with the above mutants at the end of June 2010 (week 6 of ETV retreatment). In October 2010 (week 4 of TDF and ETV treatment), these amino acid substitutions were replaced by wild type virus (no ETV resistance substitutions). Since April 2013 (month 31 of TDF and ETV treatment), amino acid substitutions of rtL180M, rtS202G, and rtM204V have emerged and have been found to be associated with an increase in serum HBV DNA (Figure 2). In comparison with those at the start of TDF therapy, the amino acid substitutions changed from rtL180M, rtT184M, and rtM204V to rtL180M, rtS202G, and rtM204V, and no other amino acid substitutions apart from these in the rt region were observed. Further, there were no substitutions that could be associated with reduced TDF susceptibility (rtA181V/T, rtN236T, or rtA194T) in April 2013.

## Discussion

Genotypic resistance to TDF has been detected in several patients with HIV-HBV coinfection. The substitution rtA194T (plus rtL180M + rtM204V) has been associated with TDF resistance,[15] albeit that a second report failed to confirm this.[16] It has been shown that rtA181V + rtN236T double mutants are resistant to TDF in vitro, but clinical data suggest that patients with rtA181 or rtN236T remain susceptible to TDF.[17] The substitution rtP177G and rtF249A reduced susceptibility to TDF in an in vitro study, but no clinical findings have yet been reported.[18] Moreover, rescue therapy with ETV and TDF in CHB patients harboring viral resistance patterns (for LAM, ADV, or ETV) or showing only partial antiviral responses to preceding therapies was efficient in patients both with and without advanced liver disease.[19] To date, there have been no confirmed reports of resistance selection during treatment with TDF for CHB.[9–12] Moreover, virologic breakthrough occurs infrequently and has been associated with nonadherence to medication in the majority of cases.[12]

To our knowledge, this is the first report of a patient with virologic breakthrough during TDF therapy. In our case, compliance with TDF and ETV was good throughout the

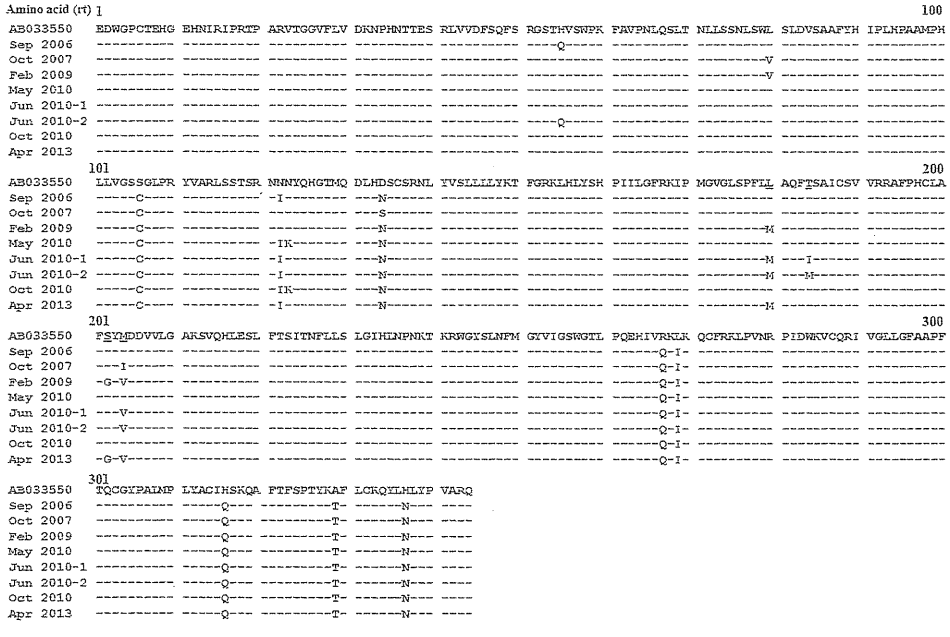


**Figure 2** Evolution of the viral polymerase reverse transcriptase protein sequence (amino acids 1–344) during lamivudine, entecavir, and tenofovir disoproxil fumarate therapy.

**Notes:** The AB033550 strain was reported by Okamoto et al.[23] In June 2010, two kinds of strain were identified, June 2010–1 and –2.

**Abbreviation:** rt, reverse transcriptase.

course of treatment. Virologic breakthrough in compliant patients is generally related to viral resistance.[6] Amino acid substitutions of rtL180M, rtS202G, and rtM204V have emerged in cases in which serum HBV DNA increased during TDF and ETV therapy. Moreover, these amino acid substitutions changed from rtL180M, rtT184M, and rtM204V to rtL180M, rtS202G, and rtM204V. This clinical course suggests that these amino acid substitutions are resistant to TDF and ETV therapy, although in vitro confirmation is necessary. Kim et al reported that among 18 patients who failed multiple NA treatments, including LAM, ADV, and ETV, 17 patients achieved virologic response and one patient showed a viral reduction of 3.9 log IU/mL, nearly reaching virologic response within 24 months.[20] These findings indicate that genotypic resistance to ETV does not affect the probability of an initial virologic response to TDF therapy.[20] Petersen et al reported that four patients harboring ETV-resistant virus achieved a virologic response within 9 months.[19] Recently, Seto et al reported 142 Asian CHB patients with at least 6 months exposure to other NAs (including ETV) who received TDF with or without LAM. With a median 2.25 years of follow-up, 45 patients had detectable viremia in at least one time point.[21] For these 45 patients, which included ten with virologic breakthrough, both line probe assay and direct sequencing revealed no new amino acid substitutions, including substitutions that could be associated with reduced TDF susceptibility (rtA181V/T, rtN236T, or rtA194T). Moreover, Karatayli et al reported that HBV DNA, in seven of eight patients with ETV resistance mutations (T184F/A/L/I, S202G, and M250V), became undetectable with TDF and LAM after 6 months of treatment.[22] In vitro drug susceptibility showed that TDF displayed one- to twofold resistance to ETV-resistant viral strains (N123D + H124Y + L180M + S202G + M204V + Y257H, I163V + L164M + L180M + S202G + M204V + C256S, and H124Y + L180M + S202G + M204V + Y257H). However, in other cases, the treatment period was relatively shorter. In our case, virologic breakthrough occurred at month 31 of TDF and ETV therapy, and the ETV-resistant strain (L180M + S202G + M204V) of our case was not identical with that in the in vitro drug susceptibility study above. Clarification of virologic breakthrough and resistance of TDF against patients with NA-resistant virus, especially ETV, will likely require further studies with a longer time frame.

In conclusion, this study shows that long-term treatment of ETV-resistant virus with TDF has the potential to induce virologic breakthrough and resistance, and careful follow-up should be done.

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## Disclosure

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