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

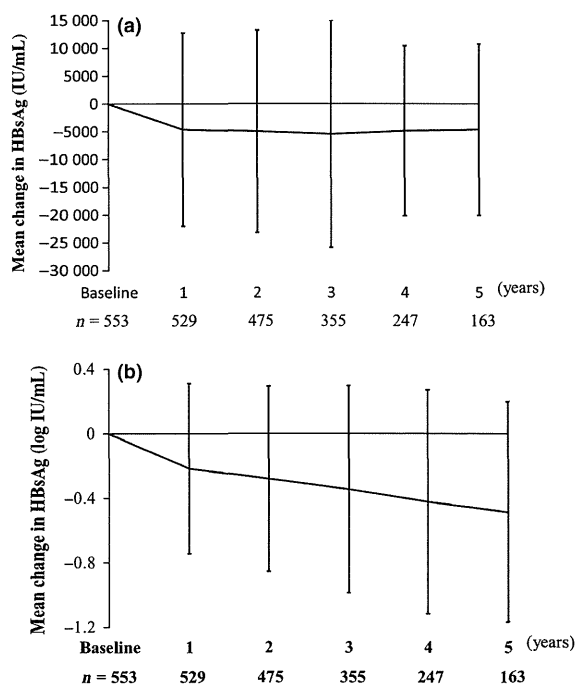

Fig. 1 **(a)** Mean decline in HBsAg relative to the baseline for all patients treated with ETV (real value). **(b)** Mean decline in HBsAg relative to the baseline for all patients treated with ETV (logarithmic axis). HBsAg, hepatitis B virus surface antigen; ETV, entecavir.

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).

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.

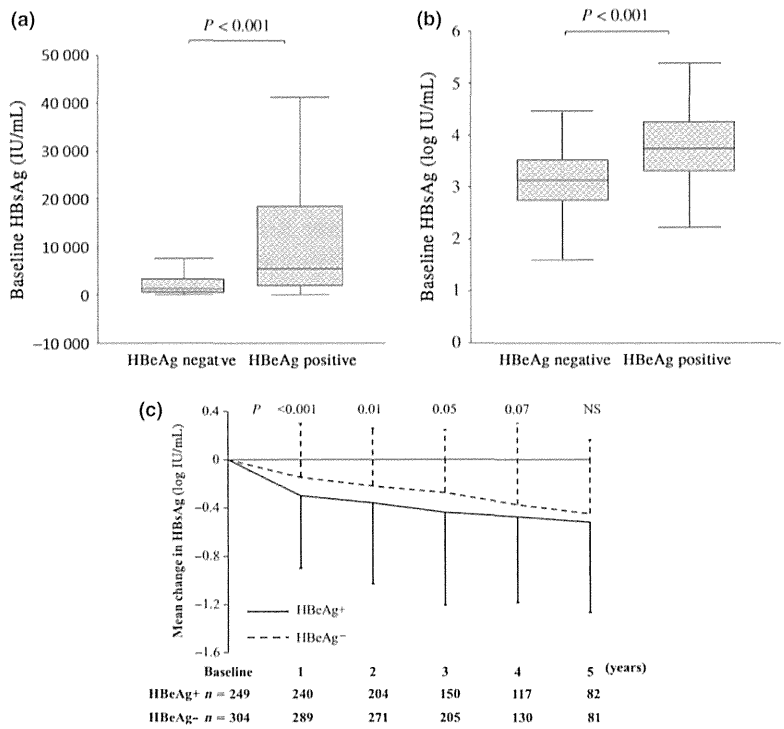

Fig. 2 (a) Box-and-whisker plots of baseline HBsAg level in HBeAg-positive and HBeAg-negative patients (real value). In these plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars 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

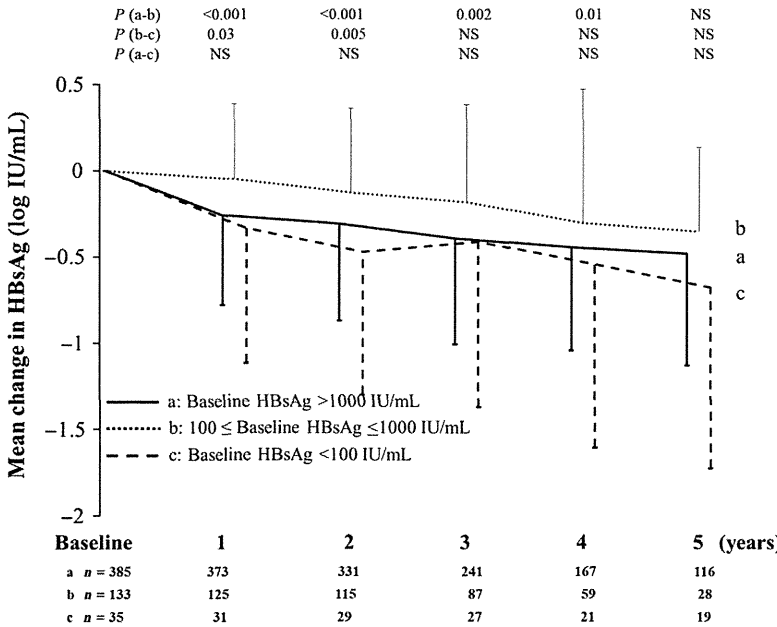

Fig. 3 Mean fall in HBsAg relative to the baseline, stratified by baseline HBsAg levels, <100 IU/mL, 100–1000 IU/mL, and >1000 IU/mL (logarithmic axis). *P*-values by Mann–Whitney *U*-test. HBsAg, hepatitis B virus surface antigen.

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

This study was supported in part by a Grant-in-aid from the Ministry of Health, Labor and Welfare of Japan.

CONFLICT OF INTEREST

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

FINANCIAL SUPPORT

This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan.

REFERENCES

1 Lavanchy D, Hepatitis B. Virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepat* 2004; 11: 97–107.
2 Liaw YF, Leung N, Kao JH *et al.* Asian-Pacific consensus statement on the management of chronic hepatitis B. *Hepatol Int* 2008; 2: 263–283.
3 Yokosuka O, Takaguchi K, Fujioka S *et al.* Long-term use of entecavir in nucleoside-naive Japanese patients with chronic hepatitis B infection. *J Hepatol* 2010; 52: 791–799.
4 Chang TT, Lai CL, Kew Yoon S *et al.* Entecavir treatment for up to 5 years in patients with hepatitis B e antigen-positive chronic hepatitis B. *Hepatology* 2010; 51: 422–430.
5 Ono A, Suzuki F, Kawamura Y *et al.* Long-term continuous entecavir therapy in nucleos(t)ide-naïve chronic hepatitis B patients. *J Hepatol* 2012; 57: 508–514.
6 Fung J, Lai CL, Young J *et al.* Quantitative hepatitis B surface antigen levels in patients with chronic hepatitis B after 2 years of entecavir treatment. *Am J Gastroenterol* 2011; 106: 1766–1773.
7 Lee JM, Ahn SH, Kim HS *et al.* Quantitative hepatitis B surface antigen and hepatitis B e antigen titers

in prediction of treatment response to entecavir. *Hepatology* 2011; 53: 1486–1493.
8 Gish RG, Chang TT, Lai CL *et al.* Loss of HBsAg antigen during treatment with entecavir or lamivudine in nucleoside-naive HBeAg-positive patients with chronic hepatitis B. *J Viral Hepat* 2010; 17: 16–22.
9 Heathcote EJ, Marcellin P, Buti M *et al.* Three-year efficacy and safety of tenofovir disoproxil fumarate treatment for chronic hepatitis B. *Gastroenterology* 2011; 140: 132–143.
10 Kobayashi M, Suzuki F, Akuta N *et al.* Loss of hepatitis B surface antigen from the serum of patients with chronic hepatitis treated with lamivudine. *J Med Virol* 2007; 79: 1472–1477.
11 Liaw YF, Gane E, Leung N *et al.* 2-Year GLOBE trial results: telbivudine is superior to lamivudine in patients with chronic hepatitis B. *Gastroenterology* 2009; 136: 486–495.
12 Hosaka T, Suzuki F, Kobayashi M *et al.* Clearance of hepatitis B surface antigen during long-term nucleot(s)ide analog treatment in chronic hepatitis B: results from a nine-year longitudinal study. *J Gastroenterol* 2013; 48: 930–941.
13 Seto WK, Liu K, Wong DK *et al.* Reduction of hepatitis B surface antigen levels and HBsAg seroclearance in chronic hepatitis B patients receiving 10 years of nucleoside analogue therapy. *Hepatology* 2013; 58: 923–931.
14 Moucari R, Mackiewicz V, Lada O *et al.* Early serum HBsAg drop: a strong predictor of sustained virological response to pegylated interferon alfa-2a in HBeAg-negative patients. *Hepatology* 2009; 49: 1151–1157.
15 Brunetto MR, Moriconi F, Bonino F *et al.* Hepatitis B virus surface antigen levels: a guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. *Hepatology* 2009; 49: 1141–1150.
16 Reijnders JG, Rijckborst V, Sonneveld MJ *et al.* Kinetics of hepatitis B surface antigen differ between treatment with peginterferon and entecavir. *J Hepatol* 2011; 54: 449–454.
17 Usuda S, Okamoto H, Imawari H *et al.* Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in preS2-region product. *J Virol Method* 1999; 80: 97–112.
18 Tadokoro K, Kobayashi M, Yamaguchi T *et al.* Classification of hepatitis B virus genotypes by the PCR-Invader method with genotype-specific probes. *J Virol Method* 2006; 138: 30–39.
19 Seto WK, Wong DK, Fung J *et al.* A large case-control study on the predictability of hepatitis B surface antigen levels three years before hepatitis B surface antigen seroclearance. *Hepatology* 2012; 56: 812–819.
20 Thompson AJV, Nguyen T, Iser D *et al.* Serum hepatitis B surface antigen and hepatitis B e antigen titers: disease phase influences correlation with viral load and intrahepatic hepatitis B virus markers. *Hepatology* 2010; 51: 1933–1944.
21 Jaroszewicz J, Ho H, Markova A *et al.* Hepatitis B surface antigen (HBsAg) decrease and serum interferon-inducible protein-10 levels as predictive markers for HBsAg loss during treatment with nucleoside/nucleotide analogues. *Antivir Ther* 2011; 16: 915–924.
22 Chu CM, Liaw YF. HBsAg seroclearance in asymptomatic carriers of high endemic areas: appreciably high rates during a long-term follow-up. *Hepatology* 2007; 45: 1187–1192.
23 Kobayashi M, Hosaka T, Suzuki F et al. Seroclearance rate of hepatitis B surface antigen in 2,112 patients with chronic hepatitis in Japan during long-term follow-up. *J Gastroenterol* 2013; doi: 10.1007/s00535-013-0821-2.

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.

Drug Design, Development and Therapy

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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

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http://dx.doi.org/10.2147/DDDT.S65349

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

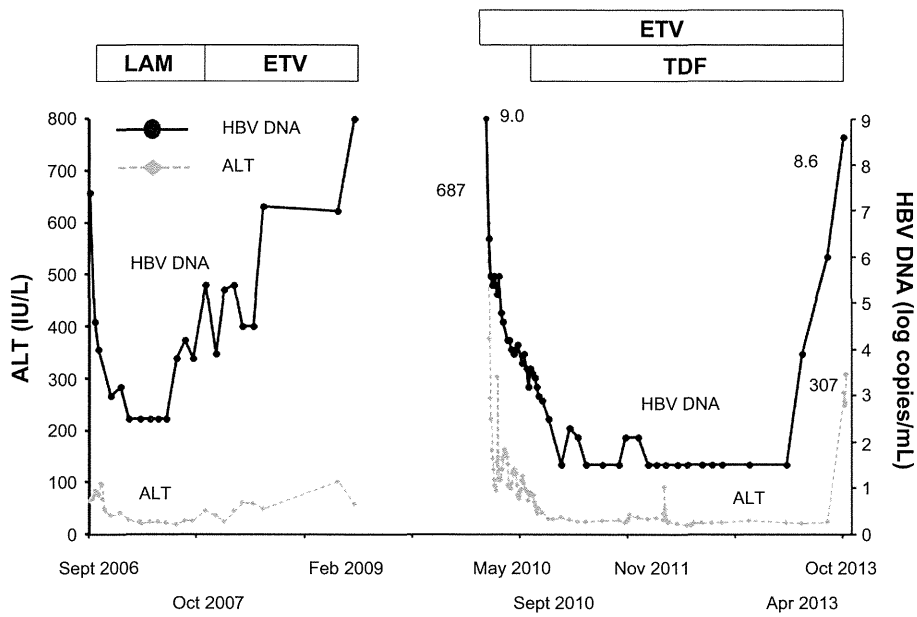

Figure 1 Clinical course of lamivudine or entecavir and tenofovir disoproxil fumarate therapy.
Abbreviations: ALT, alanine aminotransferase; ETV, entecavir; HBV, hepatitis B virus; LAM, lamivudine; TDF, tenofovir disoproxil fumarate.

(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

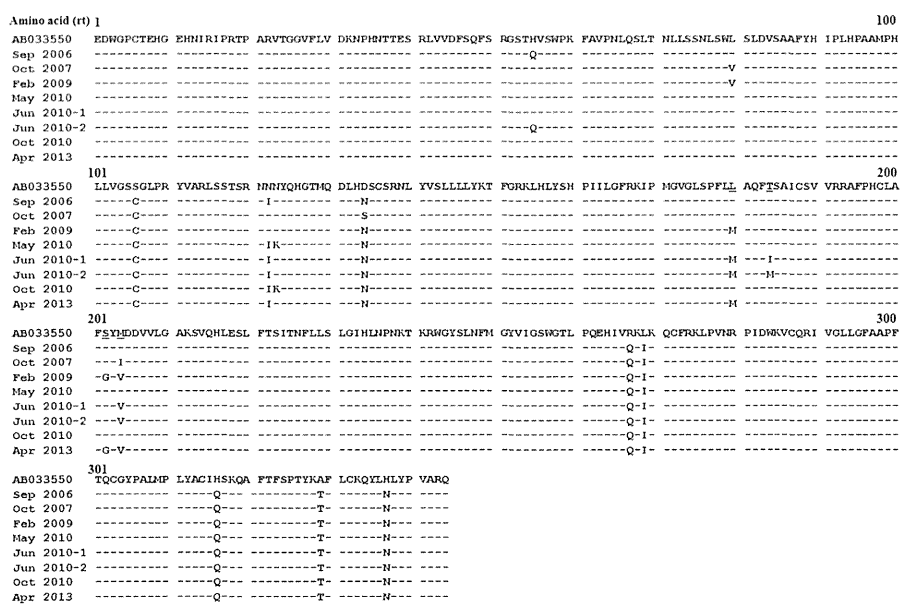

Figure 2 Evolution of the viral polymerase reverse transcriptase protein sequence (amino acids 1–344) during lamivudine, entecavir, and tenofovir disoproxil fumarate therapy.

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.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research (C) (grant number 24590999) from the Japan Society for the Promotion of Science and by a Grant-in-Aid from the Ministry of Health, Labor and Welfare of Japan.

Disclosure

Dr Kumada reports having received investigator, lecture, and consulting fees from Bristol-Myers KK, Tokyo, Japan and GlaxoSmithKline KK, Tokyo, Japan. The other authors report no conflicts of interest in this work.

References

1. Beasley RP, Hwang LY, Lin CC, Chien CS. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22,707 men in Taiwan. *Lancet*. 1981;2(8256):1129–1133.
2. Suzuki F, Suzuki Y, Tsubota A, et al. Mutations of polymerase, precore and core promoter gene in hepatitis B virus during 5-year lamivudine therapy. *J Hepatol*. 2002;37(6):824–830.
3. Marcellin P, Chang TT, Lim SG, et al; Adefovir Dipivoxil 437 Study Group. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *N Engl J Med*. 2003;348(9):808–816.
4. Ono A, Suzuki F, Kawamura Y, et al. Long-term continuous entecavir therapy in nucleos(t)ide-naïve chronic hepatitis B patients. *J Hepatol*. 2012;57(3):508–514.
5. Suzuki F, Arase Y, Suzuki Y, et al. Long-term efficacy of interferon therapy in patients with chronic hepatitis B virus infection in Japan. *J Gastroenterol*. 2012;47(7):814–822.
6. Zoulim F, Locarnini S. Hepatitis B virus resistance to nucleos(t)ide analogues. *Gastroenterology*. 2009;137(5):1593–1608.
7. Yang H, Qi X, Sabogal A, Miller M, Xiong S, Delaney WE. Cross-resistance testing of next-generation nucleoside and nucleotide analogues against lamivudine-resistant HBV. *Antivir Ther*. 2005;10(5):625–633.
8. Heathcote EJ, Marcellin P, Buti M, et al. Three-year efficacy and safety of tenofovir disoproxil fumarate treatment for chronic hepatitis B. *Gastroenterology*. 2011;140(1):132–143.
9. Snow-Lampart A, Chappell B, Curtis M, et al. No resistance to tenofovir disoproxil fumarate detected after up to 144 weeks of therapy in patients monoinfected with chronic hepatitis B virus. *Hepatology*. 2011;53(3):763–773.
10. Marcellin P, Gane E, Buti M, et al. Regression of cirrhosis during treatment with tenofovir disoproxil fumarate for chronic hepatitis B: a 5-year open-label follow-up study. *Lancet*. 2013;381(9865):468–475.
11. Gordon SC, Krastev Z, Horban A, et al. Efficacy of tenofovir disoproxil fumarate at 240 weeks in patients with chronic hepatitis B with high baseline viral load. *Hepatology*. 2013;58(2):505–513.
12. Kitrinos KM, Corsa A, Liu Y, et al. No detectable resistance to tenofovir disoproxil fumarate after 6 years of therapy in patients with chronic hepatitis B. *Hepatology*. 2014;59(2):434–442.
13. van Bömmel F, de Man RA, Wedemeyer H, et al. Long-term efficacy of tenofovir monotherapy for hepatitis B virus-monoinfected patients after failure of nucleoside/nucleotide analogues. *Hepatology*. 2010;51(1):73–80.
14. Suzuki F, Akuta N, Suzuki Y, et al. Clinical and virological features of non-breakthrough and severe exacerbation due to lamivudine-resistant hepatitis B virus mutants. *J Med Virol*. 2006;78(3):341–352.
15. Sheldon J, Camino N, Rodés B, et al. Selection of hepatitis B virus polymerase mutations in HIV-coinfected patients treated with tenofovir. *Antivir Ther*. 2005;10(6):727–734.

16. Delaney WE, Ray AS, Yang H, et al. Intracellular metabolism and in vitro activity of tenofovir against hepatitis B virus. *Antimicrob Agents Chemother*. 2006;50(7):2471–2477.
17. Qi X, Xiong S, Yang H, Miller M, Delaney WE. In vitro susceptibility of adefovir-associated hepatitis B virus polymerase mutations to other antiviral agents. *Antivir Ther*. 2007;12(3):355–362.
18. Qin B, Budeus B, Cao L, et al. The amino acid substitutions rtP177G and rtF249A in the reverse transcriptase domain of hepatitis B virus polymerase reduce the susceptibility to tenofovir. *Antiviral Res*. 2013;97(2):93–100.
19. Petersen J, Ratziu V, Buti M, et al. Entecavir plus tenofovir combination as rescue therapy in pre-treated chronic hepatitis B patients: an international multicenter cohort study. *J Hepatol*. 2012;56(3):520–526.
20. Kim YJ, Sinn DH, Gwak GY, et al. Tenofovir rescue therapy for chronic hepatitis B patients after multiple treatment failures. *World J Gastroenterol*. 2012;18(47):6996–7002.
21. Seto WK, Liu K, Wong DK, et al. Patterns of hepatitis B surface antigen decline and HBV DNA suppression in Asian treatment-experienced chronic hepatitis B patients after three years of tenofovir treatment. *J Hepatol*. 2013;59(4):709–716.
22. Karatayli E, Idilman R, Karatayli SC, et al. Clonal analysis of the quasispecies of antiviral-resistant HBV genomes in patients with entecavir resistance during rescue treatment and successful treatment of entecavir resistance with tenofovir. *Antivir Ther*. 2013;18(1):77–85.
23. Okamoto H, Tsuda F, Sakugawa H, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol*. 1988;69(10):2575–2583.

J Gastroenterol (2014) 49:470–480
DOI 10.1007/s00535-013-0779-0

The Japanese Society of Gastroenterology

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Renal dysfunction and hypophosphatemia during long-term lamivudine plus adefovir dipivoxil therapy in patients with chronic hepatitis B

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Received: 26 November 2012 / Accepted: 12 February 2013 / Published online: 26 March 2013

Abstract
Background Renal dysfunction and Fanconi's syndrome associated with hypophosphatemia caused by long-term administration of low-dose adefovir dipivoxil (ADV) has been reported in recent years. The aim of this retrospective study was to determine the incidence and factors associated with renal dysfunction and hypophosphatemia in patients with hepatitis B infection on long-term treatment with ADV and lamivudine (LAM).
Methods The study subjects were 292 patients treated with 10 mg/day ADV and 100 mg/day LAM for more than 6 months. We evaluated estimated glomerular filtration rate (eGFR), serum creatinine and serum phosphate level at the start of ADV and every 6 months.
Result During a median treatment duration of 64 months, 28 (9.6 %) patients developed renal impairment (defined as eGFR < 50 ml/min/1.73 m^2), and 73 (27.1 %) developed hypophosphatemia, including 14 with persistent hypophosphatemia. The cumulative incidences of renal impairment at 1, 3, and 5 years were 1.4, 7.5, 10.5 %, respectively, and those of hypophosphatemia were 6.8, 20.6, 26.7 %, respectively. Multivariate analysis identified old age, liver cirrhosis and hypertension as determinants of renal impairment, and male sex, HCC, low baseline serum phosphate as determinants of hypophosphatemia. Three of the 14 patients with persistent hypophosphatemia developed Fanconi's syndrome; their serum creatinine level remained normal, but eGFR was lower than at baseline.
Conclusion Long-term treatment of hepatitis B with low-dose (10 mg/day) ADV and LAM can potentially cause renal impairment and hypophosphatemia. We advocate regular monitoring of serum phosphate and evaluation of eGFR, in addition to serum creatinine, in such patients.

Keywords Adefovir dipivoxil · Hepatitis B virus · Renal dysfunction · Hypophosphatemia · Fanconi's syndrome · Osteomalacia

Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMI	Body mass index
CHB	Chronic hepatitis B
CHBI	Chronic hepatitis B infection
CI	Confidence interval
eGFR	Estimated glomerular filtration rate
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
IFN	Interferon
IP	Inorganic phosphate
LC	Liver cirrhosis

Introduction

Hepatitis B virus (HBV) infects more than 350 million people worldwide. Hepatitis B is a leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC)

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[1]. The goal of therapy is to reduce HBV replication to limit progressive liver disease and improve the natural history of chronic HBV infection (CHBI) [2]. Oral nucleotide analogs are used for antiviral therapy of patients with CHBI. Lamivudine (LAM) is the first nucleotide analogue indicated for CHBI [3]. However, long-term LAM therapy is associated with emergence of drug-resistant HBV mutation, and relapse of hepatitis [4–7]. Subsequent studies indicated that adefovir dipivoxil (ADV) alone or in combination with LAM provides effective antiviral therapy in patients with LAM-resistant HBV [8, 9]. However, ADV monotherapy of LAM-resistant HBV resulted in the appearance of virological breakthrough due to acquisition of ADV-resistant mutation [10]. Therefore, the Japanese guidelines recommend the use of the combination of ADV and LAM for patients with LAM-resistant HBV [11].

Renal impairment is one of the most serious side effects of ADV. Nephrotoxicity associated with ADV is dose-dependent. In CHBI phase III trials, significant renal toxicity was not observed during a median follow-up period of 64 weeks in patient treated with ADV at 10 mg/day [12]. However, renal dysfunction associated with long-term use of low-dose ADV has been documented in a few reports published in recent years [13–15]. Moreover, a few case reports also described hypophosphatemia associated with Fanconi's syndrome in association with the use of ADV at 10 mg/day [14, 16, 17].

On the other hand, there are only a few studies on the incidence of renal dysfunction and hypophosphatemia during long-term combination therapy of ADV and LAM. In the present study, we investigated the incidence of renal impairment and hypophosphatemia associated with long-term use of ADV–LAM combination in patients with CHBI and defined the characteristics of those patients who developed the above side effects.

Patients and methods

Patients

The study group comprised 292 Japanese patients who were treated with the combination therapy of ADV and LAM between November 2002 and December 2011 at Toranomon Hospital, Tokyo, Japan. Patients were included in this study if they met the following criteria: (1) patients with LAM-refractory CHBI who commenced ADV add-on LAM at Toranomon Hospital; (2) the starting dose of ADV was 10 mg/day; (3) normal renal function at the commencement of ADV (serum creatinine < 1.2 mg/dl and estimated glomerular filtration rate (eGFR) of ≥ 50 ml/min/1.73 m^2); (4) patients who received the combination therapy for more than 6 months. Furthermore, we excluded patients who had history of treatment with other nucleotide analogs and co-infection with hepatitis C virus or human immunodeficiency virus (HIV).

Study protocol

Patients visited our hospital every 1–3 months after the initiation of ADV treatment, and blood samples were obtained at every visit. We evaluated virological and biochemical markers at the start of ADV and every 6 months thereafter. The eGFR was calculated by the Japanese GFR equation [$194 \times Cr^{1.094} \times age^{0.287}$ ($\times 0.739$ for females)]. Renal impairment represented a decrease in eGFR to < 50 ml/min/1.73 m^2, while hypophosphatemia was defined by serum phosphate level of < 2.5 mg/dl. 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 presence of stage 4 fibrosis on histopathological examination and/or clinical evidence of portal hypertension.

The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and approved by the ethics committee of Toranomon Hospital.

Statistical analysis

Descriptive statistics were reported as proportion (%) for categorical variables, and median values (range) for continuous variables. The Mann–Whitney *U* test was used to compare two continuous variables, and Fisher's exact test or Chi square test was used to compare two categorical variables. The cumulative incidences of renal impairment and hypophosphatemia were calculated using the Kaplan–Meier method and group data were evaluated using the log-rank test. The Cox proportional hazard regression model was used to estimate univariate and multivariate risk factors for renal dysfunction and hypophosphatemia. Wilcoxon rank sum test was used to compare changes in the median values of eGFR and serum phosphate. Statistical significance was defined with two-tailed *P* value of < 0.05. Statistical analyses were performed using The Statistical Package for Social Sciences (version11; SPSS, Chicago, IL).

Results

Baseline characteristics

Table 1 lists the baseline clinical and laboratory characteristics at the start of ADV. The total duration of the combination therapy of ADV and LAM was 64.3 months (range: 6–118). The median age of the patient was 47 years

Table 1 Baseline characteristics

n	292
Age (years)	47 (25–75)
Male sex	228 (78.1 %)
Body weight (kg)	63 (39.9–92.5)
Body mass index (kg/m^2)	22.2 (15.8–36.9)
Treatment duration (months)	64.3 (6.0–118)
Current cirrhosis	67 (22.9 %)
Current and/or history of HCC	48 (16.4 %)
History of diabetes mellitus	17 (5.8 %)
History of hypertension	42 (14.4 %)
Genotype (A/B/C/others or unknown)	13/15/240/24 (4.5/5.1/82.2/8.2 %)
HBeAg (positive/negative/unknown)	114/176/2 (39.0/60.3/0.7 %)
Serum HBV-DNA (logIU/ml)	6.9 (< 2.1 to ≤9.0)
Total bilirubin (mg/dl)	0.7 (0.2–6.0)
Alanine aminotransferase (IU/ml)	86 (9–3156)
Albumin (g/dl)	3.9 (2.4–4.7)
Platelet($\times 10^4$/mm^3)	16.1 (3.1–45.2)
Creatinine (mg/dl)	0.8 (0.4–1.1)
eGFR (ml/min/1.73 m^2)	85.2 (51.2–179.9)
Inorganic phosphate (mg/dl)	3.2 (1.6–4.6)

Values are expressed as median (range), or number of patient (%)

eGFR estimated glomerular filtration rate, *HCC* hepatocellular carcinoma

(25–75), and patients were mostly men (78.1 %). Sixty-seven (22.9 %) patients had cirrhosis before starting ADV, and 48 patients (16.4 %) had a history of HCC or had HCC at study entry. Forty-two (14.4 %) patients had diabetes mellitus, and 17 (5.4 %) had arterial hypertension. The median body weight was 63 kg (39.9–92.5), and median BMI was 22.2 kg/m^2 (15.8–36.9). Baseline eGFR was 85.2 ml/min/1.73 m^2 (51.2–179.9), and phosphate was 3.2 mg/dl (1.6–4.6).

ADV-induced nephrotoxicity

Frequency of renal impairment

Twenty-eight (9.6 %) patients developed renal impairment during the combination therapy. The eGFR decreased 20–30 % from baseline in 67 (22.9 %) patients, 30–50 % in 54 (18.5 %) patients, and >50 % in 5 (1.7 %) patients. Figure 1 displays the cumulative incidence of renal impairment. Figure 1a shows the time to eGFR of < 50 ml/min/1.73 m^2 (i.e., renal impairment). The 1-, 3-, and 5-year cumulative incidence of renal impairment was 1.4, 7.5, and 10.5 %, respectively. Figure 1b shows the time to reduction in eGFR of ≥30 % from baseline. The 1-, 3-, 5-year

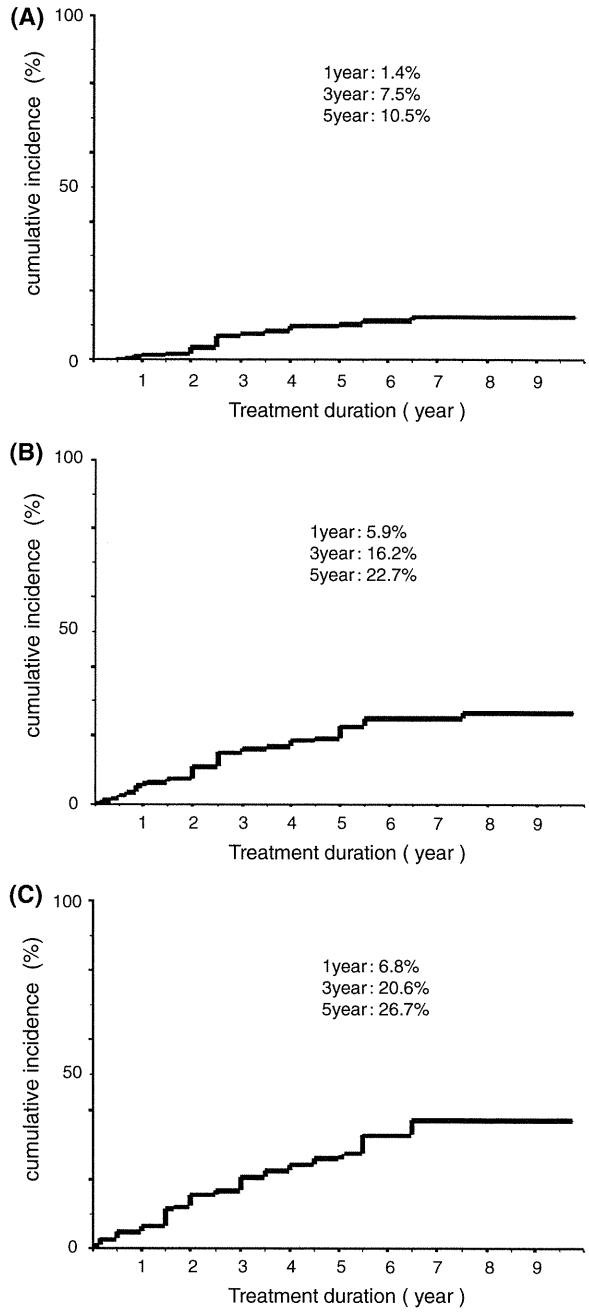

Fig. 1 Cumulative incidence of renal impairment and hypophosphatemia. **a** Cumulative incidence of reduction of eGFR to less than 50 ml/min/1.73 m^2 at 1-, 3-, and 5-years of treatment with ADV and LAM. **b** Cumulative incidence of reduction of eGFR by ≥30 % relative to baseline at 1-, 3-, and 5-years of treatment with ADV and LAM. **c** Cumulative incidence of hypophosphatemia among 269 patients with baseline IP of ≥2.5 mg/dl

cumulative incidence of reduction in eGFR ≥30 % was 5.9, 16.2, 22.7 %, respectively. We also evaluated renal function using serum creatinine. Serum creatinine increased to more

than 1.2 mg/dl in 34 (11.6 %) patients during the study period. The 1-, 3-, and 5-year cumulative incidence of serum creatinine of ≥1.2 mg/dl was 1.4, 6.51, and 11.4 %, respectively. The proportion of patients who developed renal impairment started to increase about 2 years after the commencement of ADV.

Time-course of renal impairment

Figure 2a shows serial changes in the median value of eGFR after the addition of ADV to LAM. We excluded from this analysis those patients in whom the dose of ADV was reduced at the point of modification. The eGFR of 264 patients without renal impairment remained stable throughout the study. On the other hand, the eGFR of 28 patients with renal impairment decreased rapidly within about 2 years after the addition of ADV.

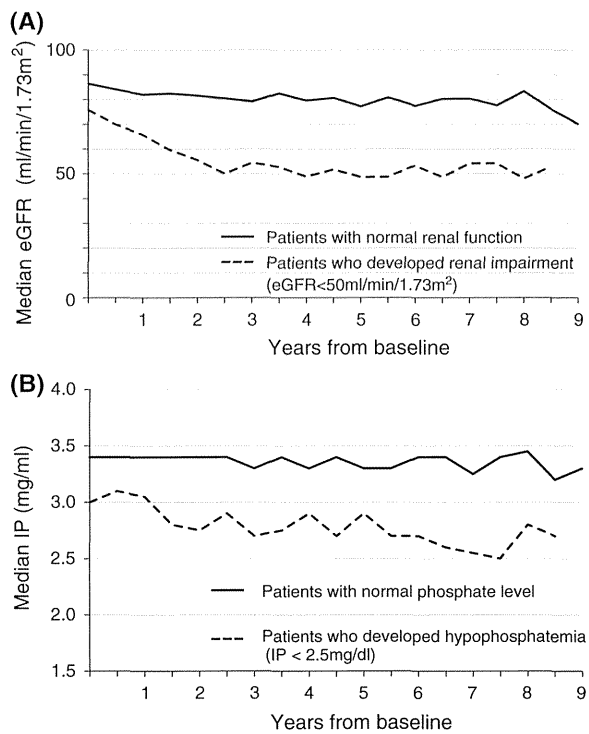

Fig. 2 Clinical course after the addition of ADV to LAM for treatment of chronic hepatitis B infection. **a** Changes in median eGFR level after the addition of ADV to LAM. *Solid line* patients with normal renal function, *broken line* patients who developed renal impairment (excluding patients who required reduction of the dose of ADV at the point). **b** Changes in the median level of serum phosphate after the addition of ADV to LAM. *Solid line* patients with normal phosphate level, broken line: patients who developed hypophosphatemia (excluding patients required reduction of the dose of ADV at the point)

Predictive factors for renal impairment

The results of univariate and multivariate analyses, including the hazard ratio for eGFR to < 50 ml/min/1.73 m^2, are shown in Table 2. Univariate analysis showed that old age (≥ 50 years, $P < 0.0001$), current cirrhosis ($P < 0.0001$), current and/or history of HCC ($P = 0.001$), history of hypertension ($P < 0.0001$), mild renal dysfunction at baseline (eGFR < 80 ml/min/1.73 m^2, $P = 0.001$), and thrombocytopenia (platelet count < 15 × 10^4/mm^3, $P = 0.003$) were associated with the development of nephrotoxicity. Multivariate analysis indicated that old age ($P = 0.006$), cirrhosis ($P = 0.011$), and history of hypertension ($P = 0.005$) were significant predictors of renal impairment.

Univariate and multivariate analyses were also performed for a fall in eGFR of ≥30 % relative to baseline. The results of univariate analysis showed that old age ($P < 0.0001$), female sex ($P = 0.007$), small body weight (< 60 kg, $P = 0.002$), history of diabetes mellitus ($P < 0.0001$), mild renal dysfunction at baseline ($P = 0.018$), hypo-albuminemia ($P = 0.010$), and thrombocytopenia ($P = 0.007$) were associated with decrease in eGFR of ≥30 % relative to baseline. On the other hand, multivariate analysis identified old age ($P < 0.001$), small body weight ($P = 0.015$), history of diabetes mellitus ($P = 0.020$), and mild renal dysfunction at baseline ($P < 0.0001$) as significant predictors of fall in eGFR of ≥30 % relative to baseline.

In either case, old age was a significant contributing factor of ADV-induced renal impairment. History of diabetes mellitus and arterial hypertension were also significant predictors.

Effect of modification of ADV dosing interval on renal impairment

Seventeen (5.8 %) patients required modification of the ADV dosing interval because of renal impairment. The ADV dosing interval was changed from 10 mg every day to 10 mg every other day when creatinine increased to ≥1.2 mg/dl. The clinical characteristics of the 17 patients could be summarized as follows: all were men with a median age of 54 years (35–63), 8 (47.1 %) patients had cirrhosis, 4 (23.5 %) patients had a history of HCC, baseline eGFR was 69.3 ml/min/1.73 m^2 (58.2–89.3), phosphate was 3.3 mg/dl (2.1–3.9), and the median time to modification of ADV dose was 48.5 months (20.7–70.0). Figure 3a shows changes in eGFR and Fig. 3b shows changes in serum phosphate after modification of the ADV dosing interval. The dose modification significantly improved eGFR and serum phosphate as measured at 6 months and 1 year after the modification. Analysis of the

Table 2 Determinants of renal impairment (eGFR less than 50 ml/min/1.73 m^2)

	Univariate analysis		Multivariate analysis	
	HR (95 % CI)	*P* value	HR (95 % CI)	*P* value
Age ≥50 years	7.661 (2.898–20.252)	<0.0001	4.280 (1.505–12.169)	0.006
Male sex	1.227 (0.464–3.236)	0.680		
Body weight < 60 (kg)	1.470 (0.687–3.145)	0.320		
Current cirrhosis	5.344 (2.479–11.518)	<0.0001	2.861 (1.279–6.401)	0.011
Current and/or history of HCC	3.855 (1.788–8.311)	0.001		
History of diabetes mellitus	2.841 (0.982–8.149)	0.054		
History of hypertension	5.116 (2.393–10.938)	<0.0001	3.087 (1.403–6.791)	0.005
Baseline eGFR < 80 (eGFR ≥50)	4.219 (1.786–10.00)	0.001		
Baseline IP < 3.2 mg/dl	1.634 (0.766–3.497)	0.204		
Platelet count < 15 × 10^4/mm^3	3.448 (1.511–7.874)	0.003		

CI confidence interval, *IP* inorganic phosphate, *HCC* hepatocellular carcinoma, *HR* hazard ratio

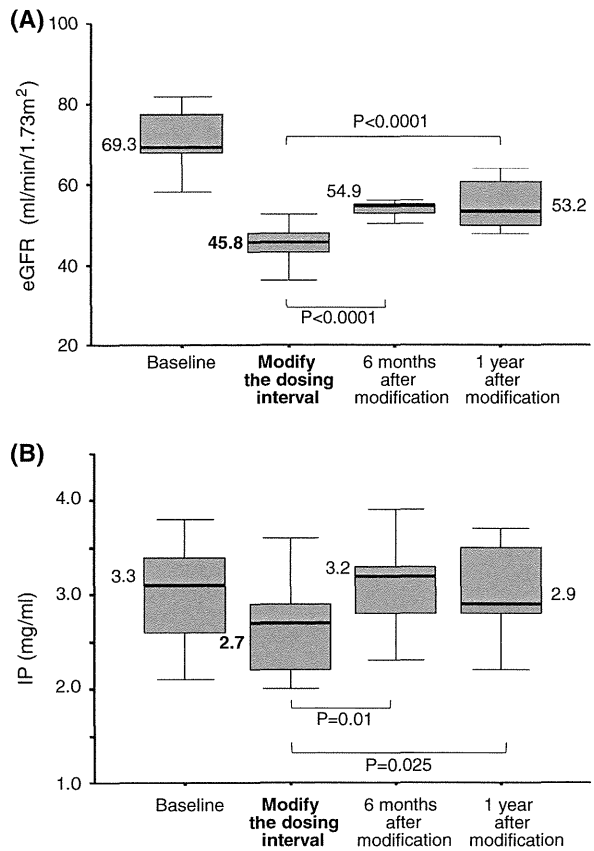

Fig. 3 Changes in eGFR and serum phosphate after modification of the ADV dosing interval. **a** Changes in eGFR. **b** Changes in serum phosphate level

long-term courses of eGFR and phosphate in these 17 patients after modification of ADV showed that the median eGFR after 1-, 2-, and 3- years of modification was 53.2, 56.7, 53.9 ml/min/1.73 m^2, respectively. eGFR remained > 50 ml/min/1.73 m^2 after modification, but never recovered to baseline level. None of the patients required discontinuation of ADV due to renal impairment.

ADV-induced hypophosphatemia

Frequency of hypophosphatemia

Seventy-three (27.1 %) of 269 patients who had normal phosphate at baseline developed hypophosphatemia during the course of the study. Fourteen (19.1 %) of the 73 patients who developed hypophosphatemia continued to show hypophosphatemia until the end of the study. On the other hand, the remaining 59 patients developed transient hypophosphatemia only. The cumulative incidence of hypophosphatemia is shown in Fig. 1c. The 1-, 3-, and 5-year cumulative incidence of hypophosphatemia was 6.8, 20.6, and 26.7 %, respectively. On the other hand, 23 patients had hypophosphatemia at baseline. Seven (30.4 %) of these 23 patients had chronic hypophosphatemia. The phosphate level of 4 (17.4 %) patients reverted spontaneously to normal, while serum phosphate level of the other 12 (52.2 %) patients fluctuated during the study.

Time-course of hypophosphatemia

Figure 2b shows changes in the median serum level of phosphate after the addition of ADV to LAM. We excluded from this analysis those patients in whom the dose of ADV was reduced at the point of modification. The median phosphate level decreased gradually after the addition of ADV in patients who subsequently developed hypophosphatemia.

Predictive factors for hypophosphatemia

Table 3 shows the results of univariate and multivariate analyses, including hazard ratio, of the factors associated

Table 3 Determinants of hypophosphatemia

	Univariate analysis		Multivariate analysis	
	HR (95 % CI)	*P* value	HR (95 % CI)	*P* value
Age ≥50 years	1.325 (0.836–2.100)	0.230		
Male sex	3.690 (1.600–8.475)	0.002	2.824 (1.212–6.759)	0.016
Body weight < 60 kg	1.417 (0.850–2.360)	0.181		
Current cirrhosis	1.854 (1.143–3.008)	0.012		
Current and/or history of HCC	1.824 (1.089–3.054)	0.022	1.871 (1.106–3.166)	0.020
History of diabetes mellitus	1.355 (0.546–3.362)	0.513		
History of hypertension	1.558 (0.870–2.791)	0.136		
Baseline eGFR < 80 (eGFR ≥50)	1.264 (0.788–2.029)	0.332		
Baseline IP < 3.2 mg/dl	3.155 (1.965–5.051)	<0.0001	2.833 (1.751–4.032)	<0.0001
Platelet count < $15 \times 10^4/mm^3$	1.472 (0.925–2.342)	0.103		

Abbreviations as in Table 2

with a fall in serum phosphate level to < 2.5 mg/dl. Patients with baseline serum phosphate of < 2.5 mg/dl ($n = 23$) were excluded from the analysis. Univariate analysis showed that male sex ($P = 0.002$), cirrhosis ($P = 0.012$), current and/or history of HCC ($P = 0.012$), and low baseline phosphate level ($P < 0.0001$) correlated with hypophosphatemia. On the other hand, multivariate analysis identified male sex ($P = 0.016$), current and/or history of HCC ($P = 0.020$), and low baseline serum phosphate level ($P < 0.0001$) as significant determinants of ADV-induced hypophosphatemia.

Further analysis showed that decreases in eGFR of more ≥30 % relative to the baseline value in 2.5 years correlated significantly with hypophosphatemia ($P = 0.007$).

Effect of modification of ADV dosing interval on hypophosphatemia and liver function

The median serum phosphate level after 1-, 2-, and 3- years of modification of ADV dose was 2.9, 3.1, and 3.0 mg/dl, respectively. Serum phosphate level fluctuated even after the dose modification. We also analyzed changes in serum ALT and HBV-DNA. After ADV dose modification, serum ALT level decreased to within the normal range (ALT < 40 IU/L) in 16 of 17 patients. Although serum ALT level of the remaining single case increased transiently after the modification, it normalized 1 year later. The HBV-DNA level was below the detection level at ADV dose modification in 14 of the 17

Table 4 Clinical features of patients with persistent ADV-induced hypophosphatemia

Case no.	Sex	Age (years)	BW (kg)	LC/CH/HCC	Baseline					Min. IP	Max. ALP	Min. UA	Max. Cr	Fall in eGFR (%)	Ostealgia
					IP	ALP	UA	Cr	eGFR						
1	F	63	64.6	LC/HCC	2.7	323	5.4	0.5	93.3	0.8	1081	1.2	0.9	47.9	+
2	F	73	57.2	CH	3.6	285	4.1	0.5	89.3	1.9	1102	2.2	0.8	41.1	+
3	M	35	61.4	CH	3.9	149	4.3	0.8	89.3	2.2	174	3.4	1.2	37.8	−
4	M	57	66.2	LC/HCC	2.9	361	2.8	0.8	77.7	2.2	742	1.7	1.2	37.1	−
5	F	40	60.4	CH	2.9	259	4.9	0.5	105.8	1.1	1012	2.5	0.7	33.1	−
6	M	47	57.4	CH	3.9	203	3.9	0.7	95.1	1.8	241	3.1	1.0	32.3	−
7	M	50	70.2	LC/HCC	3.4	300	5.4	0.6	110.2	1.1	351	5.3	0.8	29.3	−
8	M	41	80.3	LC/HCC	2.7	206	5.3	0.8	85.3	2.0	268	4.3	1.0	23.2	−
9	M	58	73.0	CH	2.6	259	2.9	0.9	67.8	2.2	378	2.2	1.1	20.5	−
10	M	31	89.0	LC	3.4	180	4.4	0.8	92.2	1.6	502	1.8	0.9	17.7	+
11	M	34	62.9	CH	2.7	111	6.4	0.6	123.7	2.2	179	4.6	0.7	16.2	−
12	M	49	83.0	CH	3.1	442	6.1	0.8	80.9	2.2	383	5.0	0.9	14.5	−
13	M	40	83.9	LC/HCC	3.7	216	6.9	0.9	75.4	1.9	383	6.0	1.0	10.9	−
14	M	39	66.0	CH	4.1	144	6.4	1.0	67.7	2.1	179	6.3	1.1	9.9	−

Fall in eGFR represents fall in eGFR relative to the baseline

BW body weight, *IP* inorganic phosphate, *ALP* alkaline phosphatase, *UA* uric acid, *Cr* creatinine, *LC* liver cirrhosis, *CH* chronic hepatitis, *HCC* hepatocellular carcinoma

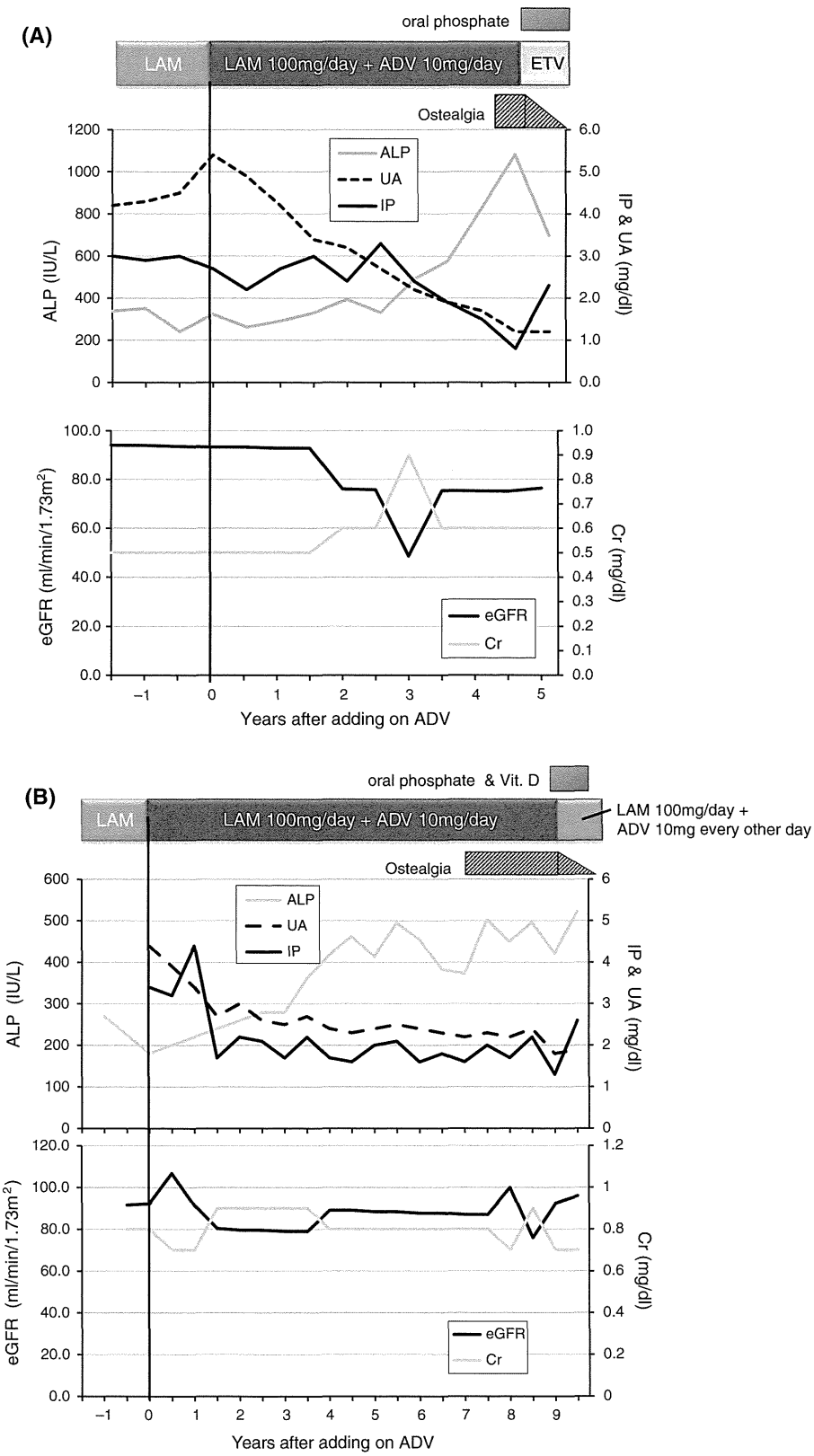

Fig. 4 Two cases who developed Fanconi's syndrome. **a** Case 1: a 63-year-old woman with HBeAg-positive liver cirrhosis. **b** Case 10: a 31 year-old man with HBeAg-positive liver cirrhosis

patients, and the level did not increase after the modification. The remaining three patients with detectable HBV-DNA at modification did not show any change in HBV-DNA.

Patients with persistent hypophosphatemia

Fourteen (5.2 %) patients developed persistent hypophosphatemia. There were no significant differences in clinical features and results of laboratory tests at baseline between patients with transient and persistent hypophosphatemia. Table 4 lists the clinical features of these patients. Three of these patients complained of bone pain during treatment. They had markedly elevated alkaline phosphatase (ALP) and low serum uric acid (UA) levels during the combination therapy. Their serum creatinine level remained normal, but their eGFR decreased relative to baseline. Figure 4 provides a summary of the clinical course of cases 1 and 10.

Case 1 was a 63-year-old woman with HBeAg-positive liver cirrhosis. She was first treated with LAM for chronic hepatitis, but ADV was added 17 months later due to the development of LAM resistance. The laboratory data (serum phosphate, ALP, UA and creatinine) were within normal ranges at baseline, and she had no other health problems. Continuous treatment with ADV for about 3 years resulted in increase in ALP level and decrease in UA and serum phosphate. After 4.5 years, she developed lumbago and right ankle pain. Blood tests showed ALP of 1102 IU/ml, UA of 1.2 mg/dl, and serum phosphate of 0.8 mg/dl. Other laboratory tests demonstrated metabolic acidosis, aminoaciduria, low tubular reabsorption of phosphate (34.8 %; normal value 85–98 %), and high fractional excretion of uric acid (47.6 %; normal value 4–14 %). These results indicated generalized dysfunction of the proximal renal tubules. A technetium bone scan showed increased uptake in bilateral ribs, carpal bones, lumbar spine, and bilateral calcaneus. She was diagnosed with acquired Fanconi's syndrome with hypophosphatemic osteomalacia associated with ADV therapy. ADV was discontinued and replaced with entecavir (ETV) while hypophosphatemia was treated with oral phosphate. Three months after cessation of ADV and oral phosphate supplementation, the patient reported symptomatic improvement and blood tests showed normalization of phosphate level and low ALP level.

Case 10 was a 31-year-old man with HBeAg-positive liver cirrhosis. He was also first treated with LAM, and ADV was added on 16 months later. The laboratory data were within the normal ranges at baseline. Treatment for 1.5 year with ADV resulted in decrease in serum phosphate and UA, and 4-year treatment increased ALP level. After 7 years, the right metatarsal bone broke in an accident. After 9 years of treatment, blood tests showed serum phosphate of 1.3 mg/dl. Detailed clinical examination was conducted at that stage. Other laboratory tests showed aminoaciduria, low tubular reabsorption of phosphate (65.5 %), and high fractional excretion of uric acid (19.1 %). A technetium bone scan showed increased uptake in bilateral ribs, bilateral ankles, tarsal bones, and right metatarsal. He was also diagnosed with acquired Fanconi's syndrome and hypophosphatemic osteomalacia associated with ADV therapy. ADV dosing interval was changed from 10 mg every day to 10 mg every other day, and oral phosphate supplementation and calcitriol were added to the treatment. Treatment for 2 months resulted in improvement of symptoms and normalization of phosphate level.

Discussion

Renal impairment is one of the most serious adverse effects of ADV. The following mechanism is considered to explain ADV-induced nephrotoxicity: the human organic anion transporter-1 (hOAT1) is a renal membrane protein expressed at the basolateral membrane of the proximal tubule cells. hOAT1 can efficiently transport cyclic nucleoside phosphonate, and thus contribute to ADV nephrotoxicity by accumulation of the drug in renal proximal tubules [18, 19].

Previous studies indicated that the ADV-related nephrotoxicity is dose-dependent [12]. In a large-scale clinical trial, 8 % of patients treated with 30 mg/day ADV for 48 weeks had high serum creatinine (≥0.5 mg/dl), relative to baseline. On the other hand, none of the patients treated with 10 mg/day ADV showed increase in creatinine (≥0.5 mg/dl), relative to baseline [20]. Thus, ADV at a dose of 10 mg/day has been used previously for the treatment of patients with CHBI. However, renal dysfunction has been reported even after the use of ADV at this dose, especially after long-term administration [13–15]. For example, in a study of the 10 mg ADV combined with LAM, serum creatinine increased in 38 % of patients following median treatment duration of 38 months [14]. In another retrospective study of 687 patients, during a median treatment period of 27 months, 10.5 % of patients developed renal impairment, which was defined as a decrease in eGFR of more than 20 % relative to the baseline [15]. In our study, 9.6 % of patients developed renal impairment during a median treatment duration of 64.3 months. Our results also showed that 20.2 % of the patients exhibited more than 30 % decrease in eGFR, and a much larger proportion (43.2 %) of the patients showed more than 20 % decrease in eGFR. These rates are higher than those reported previously. Furthermore, as shown in

Fig. 2a, patients with rapid falls in eGFR within the first 2 years of treatment should be carefully monitored for any renal dysfunction. Based on the results of our study, it seems that longer dosing period is associated with higher incidence of renal dysfunction.

We also analyzed the risk factors of renal impairment defined by a decrease in eGFR to less than 50 ml/min/1.73 m^2. Ha et al. [13] reported that age >50 years, mild renal impairment at baseline, hypertension and/or diabetes mellitus, and male sex were significant predictors of renal impairment characterized by decrease in eGFR of ≥20 % relative to baseline. Furthermore, Yu et al. [15] also reported that age ≥50 years was a significant predictor of renal dysfunction in those patients treated with ADV. In our study, age was also identified as a significant and independent determinant of the primary endpoint, together with liver cirrhosis and history of arterial hypertension. Considered together, these data indicate that care should be taken when ADV-based therapy is used for elderly patients with CHBI.

Cross-sectional studies have demonstrated a decline in GFR with age [21, 22]. Moreover, hypertension and diabetes mellitus are also reported to worsen the rate of decline of renal function [23–25]. Renal failure is common and often severe in patients with cirrhosis due to the activation of various vasoconstrictor systems, including the renin–angiotensin system and the sympathetic nervous system [26]. Taken together, eGFR is more likely to decrease during ADV therapy in patients with older age, hypertension, diabetes mellitus, cirrhosis, mild renal dysfunction at baseline.

ADV-induced proximal tubule failure can lead to hypophosphatemia. In a randomized clinical control trial using 120 mg/day ADV for treatment of patients with HIV, hypophosphatemia occurred in 50 % of patients after 48 weeks and in 61 % of patients after 72 weeks of ADV treatment [27]. On the other hand, in another study using 10 mg/day ADV for patients with CHBI, there was no overall change in serum phosphorus level during the 96-week study period [28]. However, in recent years, several reports have described the development of hypophosphatemia in patients treated with ADV at a daily dose of 10 mg [14, 29]. In our study, 27.1 % of patients developed hypophosphatemia during the combination therapy. Although 21.9 % of patients developed transient hypophosphatemia, 5.2 % of patients who had normal phosphate level at baseline developed persistent hypophosphatemia. In this regard, one previous study reported that approximately 2 % of hospitalized patients had hypophosphatemia [30]. Collectively, the above results and our findings indicate that ADV-based treatment is associated with a high incidence of hypophosphatemia. Tamori et al. [14] reported that serum phosphate level decreased to less than 2.5 mg/ml in 16.2 % of their patients during the 38-month combination therapy. Gara et al. [29] reported that 14 % of their patients treated with nucleotide analog therapy (10 mg/day ADV combined with 100 mg/day LAM, or 300 mg/day tenofovir monotherapy) developed persistent hypophosphatemia. Analysis of our data identified male sex, presence and/or history of HCC, and low serum phosphate level at baseline as significant determinants of hypophosphatemia. Furthermore, a decrease in eGFR by ≥30 % relative to baseline within 2.5 years was also associated with the development of hypophosphatemia.

Hepatic insufficiency is associated with impairment in 25-hydroxylation of vitamin D in the liver, which can lead to reduced synthesis of 1, 25 (OH) 2D3, with subsequent worsening of hypophosphatemia based on reduced intestinal absorption of phosphorus [31, 32]. In our study, 73 % of patients with HCC had liver cirrhosis, and the presence and/or history of HCC was a predictor of hypophosphatemia. Another mechanism of hypophosphatemia is protein and calorie malnutrition, which is a common feature of chronic liver disease. Furthermore, invasive treatment of HCC may itself cause hypophosphatemia. The present study also analyzed the relation between gender and hypophosphatemia. In a study that enrolled more than 4500 community-dwelling Italians of broadly diverse age, serum phosphorus levels were similar in males and females until the age of 45 years [33]. Interestingly, serum phosphate level increased in females aged between 45 and 54 years but fell after 55 years of age. The increase in serum phosphate level in females is probably related to menstrual status [33]. In the present study, serum phosphate level was higher in females than in males at baseline (3.51 vs. 3.18, $P < 0.0001$). Thus, male sex was a significant determinant of hypophosphatemia. These findings call for careful monitoring of serum phosphate level in patients treated with ADV, especially male patients, patients with HCC, and patients with renal dysfunction.

Several studies described the development of Fanconi's syndrome and subsequent hypophosphatemic osteomalacia in patients treated with 10 mg/day ADV [14, 16, 17]. Fanconi's syndrome is characterized by generalized transport defect in the proximal tubules, leading to renal losses of glucose, phosphate, uric acid, amino acids, bicarbonate, and other organic compounds [34]. Severe hypophosphatemia seems to cause inadequate mineralization of bone matrix, with subsequent osteomalacia [35, 36]. The electrolyte imbalance and osteomalacia cause symptoms of muscle weakness, fatigue, ostealgia, and bone fractures [37]. Acquired renal tubular defect resulting in Fanconi's syndrome have been described in association with many exogenous agents, including valproate, aminoglycosides, tetracycline, and acyclic nucleoside phosphonates [34].