Table 4 Predictive factors for hepatocellular development in hepatitis C virus group

Factor	Category	Risk ratio	95% CI	*P*-value
Univariate analysis				
Histological findings	1: stage 1 2: stage 2 3: stage 3 4: stage 4	2.56	2.34–2.79	<0.0001
Laparoscopic findings	1: smooth 2: irregular 3: nodular	4.31	3.74–4.97	<0.0001
Multivariate analysis				
Histological findings	1: stage 1 2: stage 2 3: stage 3 4: stage 4	1.63	1.39–1.92	<0.0001
Laparoscopic findings	1: smooth 2: irregular 3: nodular	2.31	1.78–2.99	<0.0001

Risk ratio, relative risk based on every progression of one rank in histological and laparoscopic findings. CI, confidence intervals.

that clinical backgrounds of the HCV- and HBsAg-positive patients were significantly different in many factors and the physicians had different experiences on the diagnosis of chronic liver disease. The macroscopic classification by the use of the laparoscopic examination may be subjective, which introduces a further bias; this heterogeneity makes it slightly difficult to interpret the results of the study.

However, the features of the present study are the large study population and prolonged observation study. Moreover, based on irregularities of the liver surface, the laparoscopic findings were classified into only three groups to minimize the subjective bias. The present study shows several findings with regard to laparoscopic and histological examinations in HCV- or HBV-positive patients. First, some patients with a nodular liver surface

Table 5 Predictive factors for hepatocellular development in hepatitis B virus group

Factor	Category	Risk ratio	95% CI	*P*-value
Univariate analysis				
Histological findings	1: stage 1 2: stage 2 3: stage 3 4: stage 4	1.72	1.43–2.07	<0.0001
Laparoscopic findings	1: smooth 2: irregular 3: nodular	2.29	1.79–2.92	<0.0001
Multivariate analysis				
Histological findings	1: stage 1 2: stage 2 3: stage 3 4: stage 4	1.13	0.85–1.49	0.403
Laparoscopic	1: smooth 2: irregular 3: nodular	2.12	1.44–3.14	<0.0001

Risk ratio, relative risk based on every progression of one rank in histological and laparoscopic findings. CI, confidence intervals.

were not diagnosed as having liver cirrhosis when only histological samples were used; in the HBV patients, approximately 45% of patients with a nodular liver surface were not diagnosed as having liver cirrhosis when only histological samples were used. These results suggest that HBV-positive patients with a nodular liver surface tend to have a sampling error compared to HCV-positive patients. A typical liver biopsy represents approximately 1/50 000 of the entire liver surface.[16] Based on this information, we presume that a significant sampling error will be found when diagnosis is based on a single, blind liver biopsy as reported previously.[12–15] Seven patients with a nodular surface were diagnosed as having stage 1, histologically. These seven patients had macro nodular liver surface laparoscopically and showed a part of septa of liver cirrhosis, histologically.

Second, cumulative HCC appearance rates based on the difference of liver surface were more accurate than those based on the difference of the histological findings; this was particularly the case for the HBV patients. In HBV-positive patients with the same histological findings of stage 1, 2, or 3, the HCC development rates differed due to differences in the laparoscopic findings. Using the Cox proportional hazard model, laparoscopic findings were important predictors for HCC development compared to histological findings in the HBV group. On the other hand, both laparoscopic and histological findings were important predictors for HCC development in the HCV group.

Third, there were no complications of bleeding at the biopsy site in our series of 4106 laparoscopies. On the other hand, five of approximately 4100 patients who received US-guided liver biopsy between 1985 and 2000 had bleeding at the biopsy site. The reason for good hemostasis in laparoscopy-guided biopsies is as follows; (i) operators could use gelatin sponge (Gelform) placement to the biopsy site; and (ii) operators could directly check the hemostasis. Laparoscopy offers the advantage of visualization of the liver surface, which leads to greater success in the diagnosis of chronic liver disease than biopsy alone. Although diagnostic laparoscopy is carried out to make an accurate diagnosis of chronic liver disease, the use of laparoscopy accompanies some severe complications. Thus laparoscopy should be examined under the experienced physicians given many training. Moreover, when patients have contraindications, the physician in charge should avoid doing laparoscopy-guided liver biopsy.

The present study suggests that laparoscopic findings are superior to histological findings for predicting HCC development in HBV patients. Based on our results, we recommend that laparoscopy should be considered in the evaluation of chronic liver disease for patients with HBV. When the patients with HBV or HCV are diagnosed without laparoscopic examination, the physician in charge should constantly consider the possibility of cirrhosis and HCC appearance.

In conclusion, our data indicate that laparoscopic findings of the liver are dominant predictors for HCC development compared to histological findings in patients with HBV.

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Prolonged-Efficacy of Bisphosphonate in Postmenopausal Women With Osteoporosis and Chronic Liver Disease

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Osteoporosis is present often in postmenopausal women. The aim of this retrospective cohort study is to assess the cumulative appearance incidence and predictive factors for bone fracture in postmenopausal women with osteoporosis and chronic liver disease. The patients were 80 postmenopausal women with osteoporosis and chronic liver disease due to hepatitis virus B or C. These patients were given cyclic etidronate therapy within 3 months after diagnosis of osteoporosis (etidronate-group). Another 400 postmenopausal women with osteoporosis and chronic liver disease were selected as controls (control group). Patients in control group were matched 1:5 with etidronate-group for age. Patients in control group were not given any drugs after diagnosis of postmenopausal osteoporosis. The mean observation period was 8.1 years. Four patients in the etidronate-group and 46 in control group developed bone fracture. The 10th year cumulative appearance rates of bone fracture were 4.9% in etidronate-group and 13.8% in control group. Cox regression model showed that the appearance rate of bone fracture decreased with statistical significance in the following cases: (1) patients < 65 years ($P < 0.001$), (2) patients with serum albumin level of ≥ 3.5 g/dl ($P = 0.003$), and (3) patients treated with etidronate ($P = 0.020$). The cumulative survival rate after bone fracture was 82.2% at the second year, and 57.6% at the fifth year. The present study suggests that a serum albumin level of ≥ 3.5 g/dl and cyclic etidronate treatment reduce the appearance of bone fracture. ***J. Med. Virol. 80:1302–1307, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: chronic hepatitis; osteoporosis; bisphosphonate; bone fracture

BACKGROUND

Hepatitis C virus (HCV) or hepatitis B virus (HBV) is one of the more common causes of chronic liver disease in world. Chronic hepatitis C or B is an insidiously progressive form of liver disease that relentlessly but progresses silently to cirrhosis and/or hepatocellular carcinoma (HCC) over a period of 10–30 years [Kiyosawa and Furuta, 1991; Alter et al., 1992; Ikeda et al., 1993; Tsukuma et al., 1993]. Additionally, chronic infection due to hepatitis virus has been associated with a variety of extrahepatic complications such as essential mixed cryoglobulinemia, membranoproliferative glomerulonephritis, autoimmune thyroiditis, and sialadenitis [Johnson et al., 1993; Gumber and Chopra, 1995; Pawlotsky et al., 1995].

Bone disease is one of the major complications of chronic liver disease. Bone fracture rate are increased in chronic liver disease, especially postmenopausal women [Rouillard and Lane, 2001]. Etidronate is an organic compound that inhibits osteoclast mediated bone resorption. Intermittent cyclical etidronate has been shown to be useful for the treatment of osteopenia [Storm et al., 1990; Watts et al., 1990; Miller et al., 1997; Emkey and Ettinger, 2006]. In addition, cyclical etidronate has been reported to be an effective means of increasing bone mineral density in patients with cirrhosis [Shiomi et al., 2002]. Bisphosphonates inhibition hold promise for the treatment of patients with hepatic osteodystrophy. However, there is little information on the yearly cumulative incidence and risk factors on the development rate of bone fracture in

Abbreviations used: ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

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patients with chronic liver disease during prolonged follow-up.

In Toranomon Hospital (Tokyo, Japan), a large number of patients with HCV or HBV-related hepatitis, were found often with hip fracture or vertebral fracture among elderly patients. With this background, the present retrospective cohort study was initiated to investigate the cumulative incidence and risk factors of bone fracture among postmenopausal women with osteoporosis and chronic liver disease.

MATERIALS AND METHODS

Patients

The number of patients who were diagnosed with chronic HCV or HBV infection between April 1994 and March 2004 in the Department of Hepatology, Toranomon Hospital, Tokyo, Japan was 15,400. Out of these, 80 postmenopausal women with osteoporosis and chronic liver disease were treated with cyclical etidronate. These 80 consecutive patients treated with cyclical etidronate were regarded as the etidronate-group. Inclusion criteria except etidronate administration were as follows: (1) 55–75 years; (2) postmenopausal osteoporosis; (3) features of chronic hepatitis or cirrhosis diagnosed by ultrasonography and/or computed tomography; (4) positive for anti-HCV and HCV-RNA or hepatitis B surface antigens (HBsAg); (5) negative for antinuclear antibodies, or antimitochondrial antibodies in serum, as determined by radioimmunoassay or spot hybridization; (6) no evidence of HCC nodules as shown by ultrasonography and/or computed tomography; (7) no underlying systemic disease, such as systemic lupus erythmatosus, rheumatic arthritis. Diagnosis of osteoporosis was based on bone mineral density (AP spine by dual-energy X-ray absorptionetry) less than 2 SD of young adult mean and/or X-ray evidence of vertical trabecular loss. Patients with either of the following criteria were excluded from the study: (1) malignant tumor, (2) advanced and decompensated stage of cirrhosis with encephalopathy, icterus, or refractory ascites, and (3) a short follow-up period of 6 months or less. The patients in the etidronate-group received 200 mg of cyclical etidronate orally once a day for 2 weeks, followed by a 10-week period without cyclical etidronate.

In the same period, 1,960 women with osteoporosis and chronic liver disease were not treated with bisphosphonate, steroids or hormone replacement therapy. These patients were followed after exercise and taking calcium-rich foods or drugs for osteoporosis. The 1,215 of these 1,960 patients were considered with seven inclusion criteria and three exclusion criteria described in ethidronate group. Four hundred subjects in the control group were selected from these 1,215 patients by matching 1:5 with etidronate-group for age. The differences of the cumulative appearance rate of bone fracture in the etidronate-group and control group were compared. Next, predictive factors for bone fracture in both groups were assessed. The physicians in charge explained the purpose and method of this clinical trial to each patient and/or the patients' family, who gave their informed consent for participation. This study had been approved by Institutional Review Board of Toranomon hospital.

Viral Markers of HCV and HBV

Diagnosis of HCV infection was based on detection of serum HCV antibody and RNA. Anti-HCV was detected using a second-generation enzyme-linked immunosorbent assay (ELISA II) (Abbott Laboratories, North Chicago, IL). HCV-RNA was determined by the Amplicor method (Cobas Amplicor HCV Monitor Test, v2.0, Roche Molecular Systems, Inc., NJ). HBsAg was tested by radioimmunoassay (Abbott Laboratories, Detroit, MI). The used serum samples were stored −80°C before the time of diagnosis of osteoporosis.

Follow-Up

Patients were followed-up monthly to tri-monthly after the diagnosis of osteoporosis in the Toranomon hospital. Physical examination and biochemical tests were conducted at each examination together with a regular check-up using abdominal ultrasonography and/or computed tomography imaging in each patient. When a patient had any symptoms in relation to bone fracture, the physicians in charge further explored the possibility that patient having bone fracture. Forty-seven patients were lost to follow-up. Because the appearance of bone fracture and death was not identified in these 47 patients, they considered as censored data in statistical analysis [Harrington and Fleming, 1983].

Statistical Analysis

Nonparametric procedures were employed for the analysis of background features of the patients, including the Mann–Whitney *U*-test. The cumulative appearance rate of bone fracture was calculated from the time of diagnosis of osteoporosis by using the Kaplan–Meier method. Differences in the development of bone fracture were tested using the log rank test. Independent factors associated with the incidence rate of bone fracture were analyzed by the Cox proportional hazard model. The following nine variables were analyzed for potential covariates for incidence of bone fracture at the time of diagnosis of osteoporosis at our hospital: age, state of liver disease (chronic hepatitis or liver cirrhosis), platelet count, albumin level, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), hepatitis virus, and etidronate therapy. A P value of less than 0.05 in two-tailed test was considered significant. Data analysis was performed using the computer program SPSS version 11.0.

RESULTS

Patients' Characteristics

Table I shows the characteristics of the 480 women with postmenopausal osteoporosis and chronic liver disease. There were no significant differences in clinical profiles between the etidronate- and the control group.

TABLE I. Clinical Characteristics at the Time of Diagnosis of Osteoporosis[a]

	Total	Etidronate-group	Control group	*P*-Value
N	500	80	400	
Age (years)	61.0 ± 6.6	61.0 ± 6.9	61.0 ± 6.6	1.0
Chronic hepatitis/liver cirrhosis	370/130	59/21	291/109	0.319
HBV/HCV	82/398	10/70	72/328	0.860
AST (IU/L)	88.6 ± 71.6	90.8 ± 93.1	85.5 ± 120.3	0.234
ALT (IU/L)	102.0 ± 90.8	108.9 ± 105.5	100.6 ± 120.3	0.272
Albumin (g/dl)	4.1 ± 0.4	3.9 ± 0.5	4.1 ± 0.5	0.680
γGTP (IU/L)	51.4 ± 49.6	52.4 ± 51.1	50.2 ± 49.2	0.989
Platelet count ($\times 10^4$ mm^{-3})	21.2 ± 18.5	19.5 ± 18.1	21.5 ± 18.6	0.556

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyl transpeptidase; HBV, hepatitis B virus; HCV, hepatitis C virus.
[a]Data are number of patients or mean ± SD.

Three patients treated with etidronate had gastrointestinal episodes. However, they could continue treatment using etidronate. The observation period (mean ± SD) was 8.1 ± 3.5 years.

Incidence of Bone Fracture

Fifty out of four hundred and eighty patients developed bone fracture. Thirty patients had vertebral fracture alone and nine patients had hip fracture alone. Three patients had both vertebral and hip fractures. Remaining eight patients had bone fractures except vertebral or hip fracture. The cumulative appearance rate of bone fracture was 3.7% at fifth year and 12.2% at 10th year in all the patients (Fig. 1). Four patients in etidronate-group and 46 in control group developed bone fracture. The 10th year cumulative appearance rates of bone fracture were 4.9% in etidronate-group and 13.8% in control group.

Determinants of Incidence of Bone Fracture

Table II shows the factors associated with the incidence of a total of bone fracture in all the 480 women

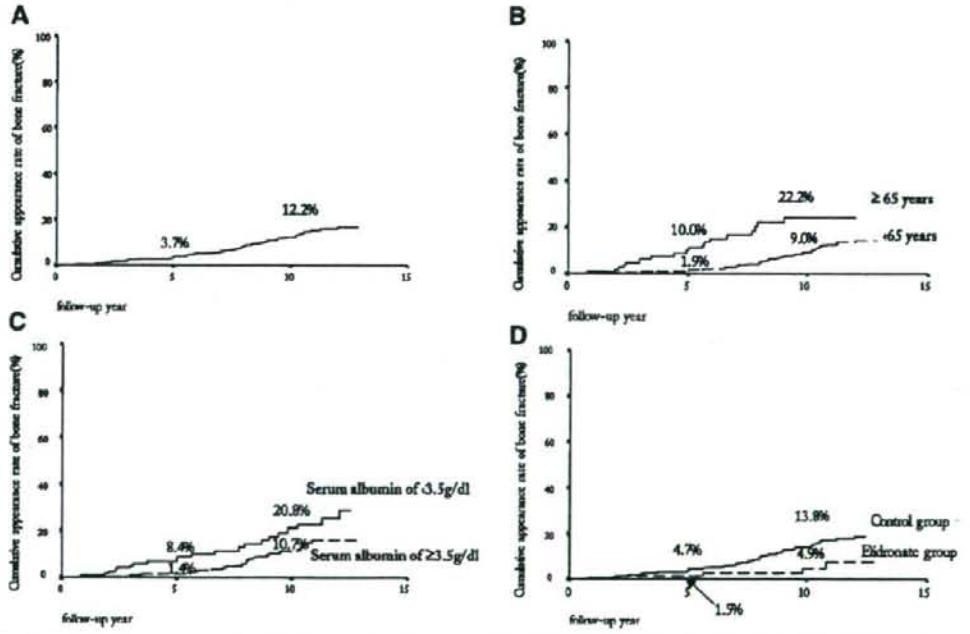

Fig. 1. Cumulative appearance rate of the bone fracture in women with osteoporosis and chronic liver disease. **Panel A**: Cumulative appearance rate of the bone fracture in a total of patients. **Panel B**: Cumulative appearance rate of the bone fracture based to difference of age (solid line, patients with ≥65 years; dotted line, patients with <65 years). **Panel C**: Cumulative appearance rate of the bone fracture based to difference of serum albumin level (solid line, patients with serum albumin level of <3.5 g/dl; dotted line, patients with serum albumin level of ≥3.5 g/dl). **Panel D**: Cumulative appearance rate of the bone fracture based to difference of treatment (solid line, controlled group; dotted line, etidronate-group).

TABLE II. Predictive Factors for a Total of Bone Fracture Development[a]

Factor	Category	Odds ratio	95% CI	*P*-Value
Univariate analysis				
Age (years)	<65/≥65	1/2.95	1.65–5.24	<0.001
Albumin (g/dl)	<3.5/≥3.5	1/0.49	0.27–0.88	0.016
Liver cirrhosis	−/+	1/1.86	1.04–3.32	0.036
Etidronate	−/+	1/0.61	0.37–1.02	0.057
AST (IU/L)	<76/≥76	1/0.49	0.22–1.08	0.076
Platelet ($\times 10^4$ mm^{-3})	<15/≥15	1/0.55	0.24–1.29	0.169
ALT (IU/L)	<100/≥100	1/0.63	0.29–1.38	0.250
Virus marker	HBV/HCV	1/1.51	0.38–5.96	0.560
γGTP (IU/L)	<110/≥110	1/0.78	0.19–3.28	0.734
Multivariate analysis				
Age (years)	<65/≥65	1/2.94	1.58–5.45	0.001
Albumin (g/dl)	<3.5/≥3.5	1/0.48	0.26–0.87	0.016
Etidronate	−/+	1/0.58	0.35–0.97	0.039

[a]ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; γ-GTP, γ-glutamyl transpeptidase; HBV, hepatitis B virus; HCV, hepatitis C virus.

with postmenopausal osteoporosis and chronic liver disease. Univariate analysis identified the following four factors that influenced incidence of bone fracture: age ($P < 0.001$), serum albumin level ($P = 0.004$), liver staging ($P = 0.036$), and etidronate ($P = 0.057$). These four parameters were entered into multivariate Cox proportional hazard analysis. The cumulative appearance rate of bone fracture decreased with statistical significance in the following cases: (1) patients <65 years ($P < 0.001$), (2) patients who had serum albumin level of ≥3.5 g/dl ($P = 0.003$), and (3) patients who were given cyclic etidronate therapy ($P = 0.020$).

In the case of vertebral fracture, the cumulative appearance rate of bone fracture decreased with statistical significance in the following cases: (1) patients <65 years, (2) patients who had serum albumin level of ≥3.5 g/dl, and (3) patients who were treated with cyclic etidronate therapy (Table III). In the case of hip fracture, the cumulative appearance rate of bone fracture decreased with statistically significant in the patients without liver cirrhosis by the multivariate Cox proportional hazard analysis (Table IV).

Mortality and Causes of Death After Bone Fracture

During the observation period after episode of bone fracture, 24 of the 50 patients died. Eight patients died of liver-related disease (HCC, decompensated liver cirrhosis, rupture of esophageal varices). On the other hand, 16 patients died of infection and aggravation of general condition. In these 24 died after bone fracture, liver-related death corresponded to 33.3% (8/24) of all deaths. The cumulative survival probability after bone fracture is shown in Figure 2. The cumulative survival probability after episode of bone fracture was 82.2% at the second year, and 57.6% at the fifth year in all.

DISCUSSION

The incidence of bone fracture in postmenopausal women with osteoporosis and chronic liver disease are described. The present study was limited by a retrospective cohort trial in postmenopausal women with osteoporosis and chronic liver disease. Postmenopausal women of 55–75 years with osteoporosis were selected. The reason was as follows: (1) onset of bone

TABLE III. Predictive Factors for Vertebral Bone Fracture Development[a]

Factor	Category	Odds ratio	95% CI	*P*-Value
Univariate analysis				
Age (years)	<65/≥65	1/3.01	1.47–6.18	0.003
Albumin (g/dl)	<3.5/≥3.5	1/0.37	0.18–0.78	0.009
Liver cirrhosis	−/+	1/1.86	1.04–3.32	0.036
Etidronate	−/+	1/0.37	0.14–1.01	0.051
Platelet ($\times 10^4$ mm^{-3})	<15/≥15	1/0.43	0.15–1.21	0.110
ALT (IU/L)	<100/≥100	1/0.55	0.19–1.53	0.247
AST (IU/L)	<76/≥76	1/0.63	0.42–1.95	0.288
γGTP (IU/L)	<110/≥110	1/0.81	0.19–4.86	0.670
Virus marker	HBV/HCV	1/1.10	0.14–5.64	0.930
Multivariate analysis				
Albumin (g/dl)	<3.5/≥3.5	1/0.38	0.18–0.81	0.012
Age (years)	<65/≥65	1/2.48	1.16–5.31	0.020
Etidronate	−/+	1/0.34	0.13–0.93	0.035

[a]ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; γ-GTP, γ-glutamyl transpeptidase; HBV, hepatitis B virus; HCV, hepatitis C virus.

TABLE IV. Predictive factors for hip bone fracture development[a]

Factor	Category	Odds ratio	95% CI	*P*-Value
Univariate analysis				
Liver cirrhosis	−/+	1/3.25	1.09–9.67	0.034
Age (years)	<65/≥65	1/2.75	0.95–7.98	0.063
Platelet ($\times 10^4$ mm^{-3})	<15/≥15	1/0.60	0.31–3.36	0.310
AST (IU/L)	<76/≥76	1/0.53	0.32–1.95	0.376
Albumin (g/dl)	<3.5/≥3.5	1/0.60	0.19–1.89	0.382
Etidronate	−/+	1/0.68	0.40–1.81	0.474
Virus marker	HBV/HCV	1/1.76	0.14–5.49	0.506
γGTP (IU/L)	<110/≥110	1/0.65	0.35–2.75	0.600
ALT (IU/L)	<100/≥100	1/0.66	0.13–3.43	0.623
Multivariate analysis				
Liver cirrhosis	−/+	1/3.25	1.09–9.67	0.034
Age (years)	<65/≥65	1/2.82	0.94–8.46	0.064

[a]ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; γ-GTP, γ-glutamyl transpeptidase; HBV, hepatitis B virus; HCV, hepatitis C virus

fracture based on osteoporosis is rare in young female with <55 years and/or male, (2) the rate of patients with >75 years at the time diagnosing osteoporosis is small. Other limitations are the followings: (1) the control patients were not matched with patients treated with etidronate by bone density measurement, (2) serum levels of vitamin D were not measured, and (3) bone density measurement were not followed.

However, there are several findings with regard to bone fracture in postmenopausal women with

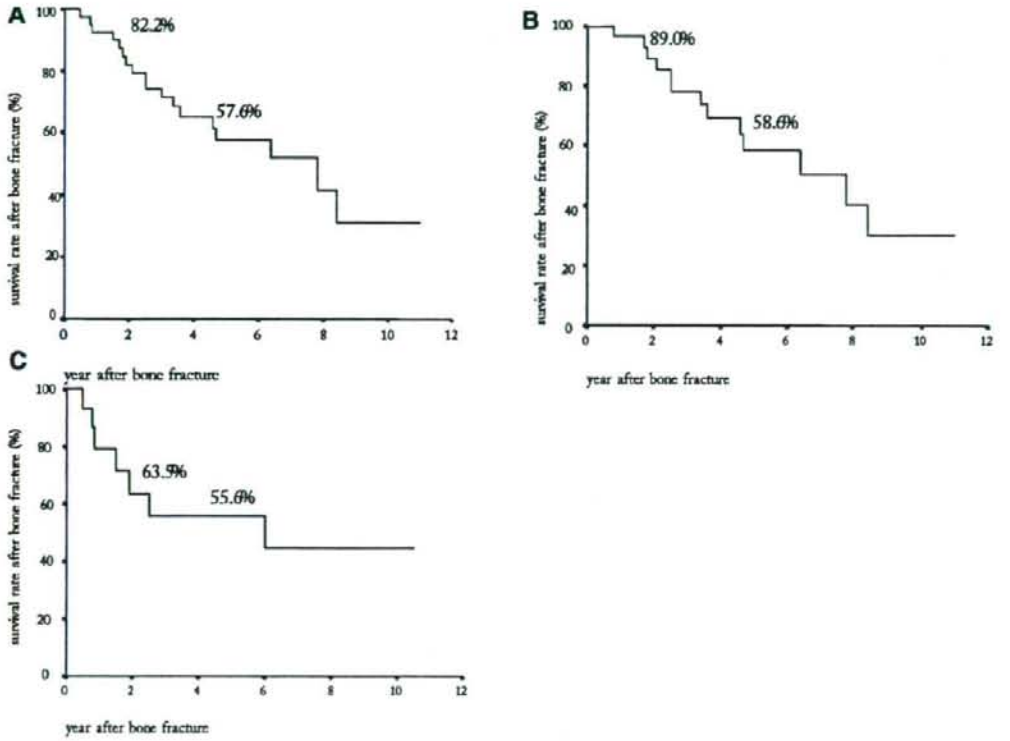

Fig. 2. Cumulative survival rate after the appearance of bone fracture in women with osteoporosis and chronic liver disease. **Panel A**: Cumulative survival rate after the appearance of bone fracture in a total of patients. **Panel B**: Cumulative survival rate after the appearance of hip bone fracture. **Panel C**: Cumulative survival rate after the appearance of vertebral bone fracture.

osteoporosis and chronic liver disease. First, the annual development rate of bone fracture among female patients with osteoporosis and chronic liver disease was one percent without treatment of osteoporosis.

Second, the appearance rate of bone fracture among postmenopausal women with osteoporosis and chronic liver disease was low with statistical significance in patients who had serum albumin of ≥3.5 g/dl and/or who were given intermittent cyclic therapy with etidronate. These results indicate that good nutrition and treatment using bisphosphonate for osteoporosis reduce the development of bone fracture in postmenopausal women with osteoporosis and chronic liver disease. In the case of vertebral fracture, good nutrition and etidronate therapy reduced the development of fractures. In the case of hip fracture, cirrhosis enhanced hip fracture. This result suggests that cyclical etidronate therapy could reduce significantly vertebral fracture compared to hip fracture.

Third, bone fracture reduced the survival rate. The survival rate after episodes of bone fracture was poor. About half of the postmenopausal women with osteoporosis died during the fifth year after the bone fracture. In patients who died, liver-related death corresponded to one-third. The remaining patients died of infection, aggravation of general conditions.

Recent studied have reported that osteodystrophy occurs not only in patients with alcoholic cirrhosis, but also in those with cirrhosis induced by hepatitis C or B virus. Due to improved treatment, patients with cirrhosis are living longer; an increasing proportion of such patients are found to have bone disease [Tsuneoka et al., 1996]. Intermittent cyclical therapy with etidronate has been reported to be increasing bone density and reducing the incidence of new vertebral fractures in postmenopausal women with osteoporosis [Fujita et al., 2007]. Although the potency of etidronate to inhibit bone resorption is relatively weak among some bisphosphonates, prolonged treatment with etidronate was reported to be effective, safe and well-tolerated. In the present study, none of the patients stopped the treatment due to adverse events.

In conclusion, the present retrospective study is the first to determine the annual incidence of bone fracture among postmenopausal women with osteoporosis and chronic liver disease in about 1% without treatment for osteoporosis. A serum albumin level of ≥3.5 g/dl and cyclic etidronate treatment reduce the development of bone fracture in postmenopausal women with osteoporosis and chronic liver disease.

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Low risk of adefovir resistance in lamivudine-resistant chronic hepatitis B patients treated with adefovir plus lamivudine combination therapy: Two-year follow-up☆

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***Background/Aims*: We studied the long-term efficacy (median follow-up of 28 months) of adefovir (ADV) in combination with lamivudine (LAM) in 132 LAM-resistant Japanese patients with chronic genotype C-dominant hepatitis B virus (HBV) infection.**

***Methods*: The viral response (undetectable HBV-DNA by PCR assay) and the predictor of viral response were evaluated. The emergence of ADV-resistant mutants was investigated during the combination therapy.**

***Results*: The cumulative probability of viral response was 69% at 12 months, and 81% at 24 months. Multivariate analysis identified baseline HBe antigen status ($P = 0.0001$), aspartate aminotransferase level (AST) ($P = 0.001$) and HBV-DNA level ($P = 0.002$) as determinants of viral response to treatment. At the beginning of ADV therapy, substitutions at rtA181 (rtA181T and rtA181S) were identified in 3 patients (2.3%). In the remaining 129 patients, the rtM204 mutants were identified at baseline, and two (1.6%) of the 129 patients developed new ADV-resistant mutants; one was rtA181S and another was rtA181T plus rtN236T mutation.**

***Conclusions*: Adefovir and lamivudine combination therapy effectively suppressed viral replication and maintained the efficacy well in LAM-resistant patients with chronic HBV infection. Genotypic analysis indicated that the emergence of ADV-resistant mutants is rare, at least over a period of 2 years, in patients with combination therapy.**

Keywords: **Adefovir dipivoxil; Lamivudine-resistant mutant; Hepatitis B virus; rtA181T; rtN236T; Combination therapy**

1. Introduction

Hepatitis B virus (HBV) is a small, enveloped DNA virus known to cause chronic hepatitis and often leads to cirrhosis and hepatocellular carcinoma [1,2]. To date, interferon and three nucleoside and nucleotide analogues (lamivudine [LAM], adefovir dipivoxil [ADV], and entecavir [ETV]) have been approved for the treatment of chronic HBV infection in Japan, while telbivudine is licensed in Europe and North America [3,4]. Nucleoside and nucleotide analogues

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Abbreviations: LAM, lamivudine; ADV, adefovir; rt, reverse transcriptase.

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suppress HBV replication in most patients and improve transaminase levels and liver histology [5–7]. However, prolonged therapy results in the emergence of drug-resistant mutants.

The rate of emergence of drug-resistant mutants is higher in patients treated with LAM than ADV and ETV, and the emergence of such mutants is followed by increases in viral load and re-elevation of transaminase levels [8–10]. Most LAM-resistant strains show amino acid substitutions in the YMDD (tyrosine-methionine-aspartate-aspartate) motif in the C domain of HBV polymerase. In addition to the emergence of the YMDD mutation, rtL180M and rtV173L mutations in the B domain of HBV polymerase are frequently observed [11,12]. Both experimental and clinical studies have shown that ADV and ETV could suppress not only wild-type but also LAM-resistant strains and have been confirmed as salvage therapy for LAM-refractory patients [13,14]. However, a few studies have already reported the emergence of resistant mutants to these drugs. ADV-resistant mutations are more common in LAM-resistant patients than in treatment-naïve patients during ADV monotherapy, and the selection of rtA181V/T or rtN236T mutant was associated with resistance to ADV [15,16]. However, a recent study reported that LAM-resistant HBeAg-negative patients treated with combination therapy of ADV with LAM did not develop resistance to ADV over a period of 3 years and the rate of undetectable HBV-DNA in combination therapy was higher than in the ADV monotherapy [14].

Recently, we reported the efficacy of ADV plus LAM combination therapy in patients with LAM-resistant chronic HBV infection [17]. However, the number of patients was limited and the virological analysis was inadequate in that study. In the present study, we analyzed the efficacy of ADV plus LAM combination therapy in 132 LAM-resistant patients with chronic hepatitis B over a period of 2 years. We also investigated the emergence of ADV-resistant mutants before and during the combination therapy.

2. Patients and methods

2.1. Patients

A total of 132 consecutive adult Japanese patients with chronic HBV infection were treated with adefovir dipivoxil at Toranomon Hospital, Tokyo, Japan, in addition to ongoing LAM treatment for more than 52 weeks starting in 2002. Enrolment in this study and the start of ADV treatment were determined by the following criteria: (1) Increase in serum HBV-DNA levels of $\geqslant$1 log copies/ml during LAM treatment on at least two consecutive occasions, compared with the nadir of initial antiviral efficacy. (2) Detection of mutations of the YMDD motif and/or other mutations related to LAM resistance before the start of ADV treatment, as diagnosed by the PCR-based method described later and/or direct sequence analysis. (3) No history of treatment with other nucleoside analogues such as famciclovir and entecavir. The exclusion criteria were as follows: (1) Serum creatinine levels $\geqslant$1.5 mg/dl. (2) Patients coinfected with hepatitis C, delta viruses, or HIV. (3) History of other liver diseases, such as autoimmune hepatitis, alcoholic liver disease, or metabolic liver disease.

2.2. Methods

Patients received a 10-mg once-daily dose of oral ADV, in addition to ongoing LAM treatment (100 mg/day). Blood samples were obtained once every month during the ADV + LAM combination therapy, and analyzed for virological markers, biochemical markers, together with liver function tests, renal function tests, and complete blood cell counts. The primary efficacy measures were undetectable HBV-DNA level by PCR assay (<2.6 log copies/ml) and normalization of ALT level (<50 IU/ml); the secondary efficacy measure was HBeAg seroconversion. The rate of each measure was evaluated 6, 12, 18 and 24 months after the start of ADV + LAM treatment.

2.3. Analysis of virological markers

HBsAg, HBeAg and antibody against HBeAg (anti-HBe) were determined by commercially available radioimmunoassay systems (Abbott Japan, Tokyo, Japan). HBV-DNA serum level was determined by using the Amplicor HBV monitor test (Roche Diagnostics, Tokyo, Japan). The measurement range of the assay is $10^{2.6}$–$10^{7.6}$ copies/ml (2.6–7.6 log copies/ml). The HBV genotype was determined by enzyme-linked immunosorbent assay (ELISA) (HBV Genotype EIA, Institute of Immunology, Tokyo) based on the method of Usuda et al. [18].

2.4. Detection of antiviral-resistant mutations

Substitution at rtM204 of the YMDD motif was identified at baseline by using the Enzyme-Linked Mini-sequence Assay with a commercial assay kit (PCR-ELMA; Genome Science). HBV-DNA was extracted from 100 μl of serum samples by SMITEST (Genome Science Laboratories, Tokyo) and dissolved in 20 μl H_2O. Detection of substitutions at rtA181 and rtN236 was achieved by PCR with restriction fragment length polymorphism (RFLP). For this purpose, HBV-DNA extracted from serum samples was amplified by PCR using primers 5′-GCCCGTTTGTCCTCTACTTCCA-3′ and 5′-ACCACTG AACAAATGGCACTAGTAAGCTGA -3′ for rtA181, and 5′-CCA CTTTTCTTTTGTCTTTGGGTATACATTTAA-3′ and 5′-GATCG GCAGAGGAGCCACAA -3′ for rtN236. The PCR products were digested with five units of restriction enzyme *Esp*I for rtA181, DraI for rtN236 and subjected to electrophoresis in 3.5% agarose gel. With regard to the sensitivity of the RFLP assay, when the mutant was mixed with 10-fold the amount of wild-type, the mutant ($\geqslant 10^2$ copies/ml) could be detected. The nucleotide and amino acid substitutions of the detected mutant samples were confirmed by direct sequence analysis.

2.5. Statistical analysis

All data were analyzed using the statistical package SPSS II (version 10.0, SPSS Inc, Chicago, IL). Non-parametric tests including the chi-squared test, Fisher's exact probability test, and the Mann–Whitney *U*-test were used to compare the background characteristics and efficacy. The cumulative rate of undetectability of HBV-DNA and HBeAg loss was calculated using the Kaplan–Meier method and differences between the curves were tested using the log-rank test. Univariate analyses were conducted using logistic regression analysis. All factors found to be at least marginally associated ($P < 0.15$) were entered into multivariate analysis using a stepwise Cox regression analysis. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Study population

The clinical and virological profiles of the 132 patients at the start of ADV + LAM treatment are shown in Table 1. At the commencement of ADV + LAM treatment, 41 patients (31.1%) had cirrhosis, and 79 patients (59.8%) were positive for HBeAg. Six of the 132 patients were treated with ADV at the time of virological breakthrough and the remaining 126 patients were treated at the time of breakthrough hepatitis.

3.2. Virological and biochemical response

The cumulative rates of undetectable serum HBV-DNA levels (<2.6 log copies/ml) were 56% at the end of 6 months, 69% at 12 months, 81% at 24 months and 87% at 36 months. The cumulative rates of normalized serum ALT levels were 73% at the end of 6 months, 85% at 12 months and 99% at 24 months. Of the 79 HBeAg-positive patients, the cumulative rates of HBeAg loss were 10% at 6 months, 16% at 12 months, 34% at 24 months and 39% at 36 months. The cumulative rates of HBeAg seroconversion were 7.5% at 6 months, 13% at 12 months, 24% at 24 months and 32% at 36 months.

3.3. Baseline parameters associated with virological response as determined by univariate and multivariate analyses

Univariate analysis identified six baseline parameters that influenced the undetectability of serum HBV-DNA during therapy: HBeAg status (negative; $P < 0.00001$), HBV-DNA (<7 log copies/ml; $P < 0.00001$), AST (>150 IU/L; $P < 0.00001$), ALT (>200 IU/L; $P = 0.0074$), fibrosis (liver cirrhosis; $P = 0.0057$) and T-Bil (>1 mg/dl; $P = 0.0535$). No association with other factors was noted: patient age, sex, serum albumin, serum creatinine, platelet count, YMDD mutant status and HBV genotype.

Multivariate analysis that included the above variables identified four parameters that independently influenced the virologic response: HBeAg status ($P = 0.0001$), AST ($P = 0.001$), HBV-DNA ($P = 0.002$), and fibrosis ($P = 0.015$) (Table 2). These results confirmed that HBeAg status is the most influential factor of undetectability of HBV-DNA. The time to undetectable HBV-DNA was significantly shorter in HBeAg-negative than in-positive patients ($P = 0.00001$). The time to normalization of ALT level was also shorter in HBeAg-negative than in-positive patients (Fig. 1a and b). The rates of undetectable HBV-DNA in the HBeAg-negative group were 94% at the end of 12 months and 100% at 24 months. On the other hand, the undetectability rates of HBV-DNA in the HBeAg-positive group were 47% at the end of 12 months, 68% at 24 months and 78% at 36 months (Fig. 1, Table 3). Therefore, we thought that it was important to investigate the predictive factor(s) of virologic response in HBeAg-positive patients. There were 21 non-responders (HBV-DNA ≥4.5 log copies/ml at 6 months of ADV + LAM), whose HBV-DNA level were all over 7 log copies/ml. Therefore we selected the responders (HBV-DNA <2.6 log copies/ml at 6 months of ADV + LAM) with high levels HBV-DNA (≥7 log copies/ml) at baseline and we found 15 patients who fulfilled the criteria. The 36 HBeAg-positive patients with high levels HBV-DNA underwent sequence analysis of the RT lesion in the polymerase gene. However, there were no differences in the RT lesion; i.e., rtH55, rtL80, rtV173, rtM180, rtI233, and rtN337, between responders (HBV-DNA <2.6 log copies/ml at 6 months of ADV + LAM) and non-responders (HBV-DNA >4.5 log copies/ml at 6 months of ADV + LAM).

Table 1
Baseline characteristics at commencement of adefovir dipivoxil ($n = 132$)

Age (years)*	47 (26–73)
Gender (Male:Female)	105:27
Prior LAM therapy (month)*	31 (8–110)
ADV treatment duration (month)*	28 (12–50)
Presence of cirrhosis (%)	41/132 (31.1)
HBV genotype (A:B:C:D)	7:5:119:1
HBeAg-positive (%)	79/132 (59.8)
HBV-DNA (log copies/ml)*	7.3 (3.3–>7.6)
rtM204 mutant (%)	130/132 (98.4)
I:V:I + V#	69:28:33
AST (IU/L)*	132 (31–1413)
ALT (IU/L)*	132 (24–1563)
T-Bil (mg/dl)*	0.8 (0.6–6.0)
Albumin (g/dl)*	3.9 (2.8–4.7)
Serum creatinine (mg/dl)*	0.8 (0.4–1.3)

* Data are median values (range).
# I → YIDD, V → YVDD, I + V → YIDD + YVDD mix.

3.4. Genotypic analysis of ADV- and LAM-resistant mutants

Genotypic resistance to ADV was looked for in PCR positive (HBV-DNA ≥ 2.6 log copies/ml) samples. Number of samples tested at baseline, 1 year and 2 years were 131 of 132 samples, 45 of 45 samples, 16 of 16 samples, respectively. The substitutions at rtA181 and rtN236 were assessed annually by RFLP method and direct sequence. At baseline, substitutions at rtA181 were identified in 3 patients (2.3%), whose genotypes were rtA181T without substitution at rt204, rtA181S without substitution at rt204 and rtA181T plus rtM204I double mutation (Fig. 2). On the other hand, substitution at rt236 was not identified at the start of ADV. In

Table 2
Multivariate analysis of baseline factors associated with virological response

Factors	Category	Hazard ratio	95% CI	P
HBeAg status	1: negative	1		
	2: positive	0.380	0.242–0.595	0.0001
AST (IU/L)	1: <150	1		
	2: ⩾150	2.115	1.357–3.296	0.001
HBV-DNA (log copies/ml)	1: <7	1		
	2: ⩾7	0.532	0.353–0.797	0.002
Cirrhosis	1: no cirrhosis	1		
	2: cirrhosis	1.683	1.107–2.559	0.015

Note. Virological response: undetectable serum HBV-DNA by amplicor monitor assay (<2.6 log copies/ml).

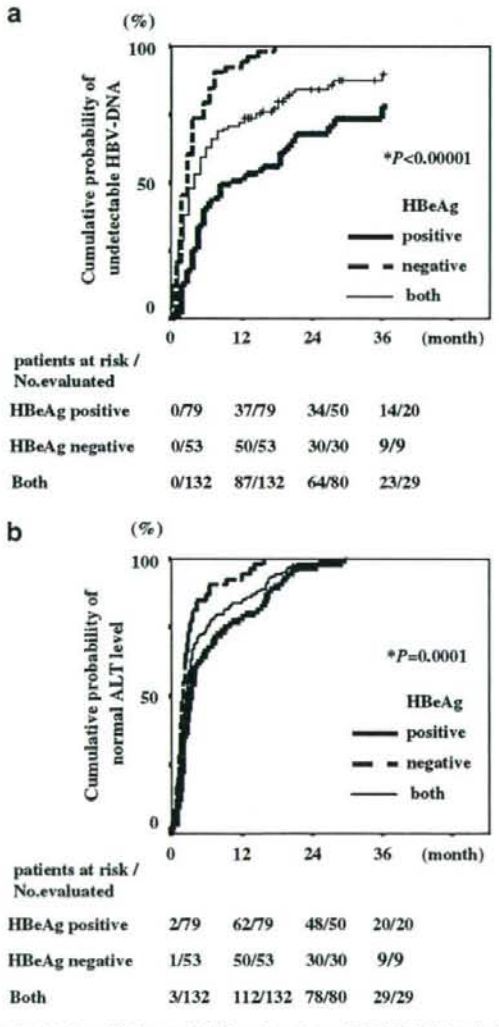

Fig. 1. (a) Cumulative probability of undetectable HBV-DNA during ADV + LAM combination therapy in patients with HBeAg-positive, -negative and both. (b) Cumulative probability of normal ALT during ADV + LAM combination therapy in patients HBeAg-positive, -negative and both. *P values between HBeAg-positive and -negative groups.

the remaining 129 patients, rtM204 mutations without substitutions at rt181 and rt236 were identified. Following ADV + LAM combination therapy, new ADV-resistant strains were identified in two patients (1.6%); one had rtA181S and the other had rtA181T plus rtN236T double mutation; they were the only two patients (among the 129 patients) who showed virological rebound during ADV + LAM therapy (Fig. 3). The cumulative rate of ADV-R was calculated every year; 1% of the first year, 1% of the second year, 1% of the third year and 8% of the fourth year. However, long follow-up studies of larger population samples are needed for a more accurate evaluation of the cumulative rate.

During combination therapy, 105 patients achieved virological response. Ninety-eight of 105 (93.3 %) patients maintained virological response. Only one patient was included according to our definition of virological breakthrough that was defined as increase in serum HBV-DNA levels of ⩾1 log copies/ml (3.6 log copies/ml) during combination therapy and also developed rtA181S mutation (Fig. 3b). However, the remaining 6 patients showed fluctuated HBV-DNA level of between <2.6 and 3.1 log copies/ml transiently, whose genotypes were wild-type at rtA181 and rtN236 during treatment.

3.5. Clinical course of patients who had developed rtA181 mutations at the start of ADV + LAM combination therapy

Three patients developed substitutions at rtA181 associated with LAM resistance. All patients were HBeAg-positive at the start of LAM. As shown in Fig. 2, two of the three patients developed rtA181T and rtA181S without YMDD mutation and the viral load did not respond sufficiently to ADV therapy. The patient with rtA181S continued to show HBV-DNA >7 log copies/ml after 2 years of ADV + LAM treatment (Fig. 2a). Subsequently, the patient was changed to 0.5 mg of ETV, which resulted in 2 log copies/ml reduction in viral load and improvement of ALT. The other patient developed rtA181T mutation mixed with wild strain (Fig. 2b). At the end of 6-month

Table 3
Undetectable rate of HBV-DNA by Amplicor monitor assay in HBeAg-negative and -positive patients

HBV-DNA (log copies/ml)	Baseline	6 months	12 months	18 months	24 months
HBeAg-negative					
<2.6	0 (0%)	40 (75%)	50 (94%)	47 (100%)	30 (100%)
2.6–<4.5	3 (6%)	13 (25%)	3 (6%)	0 (0%)	0 (0%)
≥4.5	50 (94%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total, *n* (%)	53 (100%)	53 (100%)	53 (100%)	47 (100%)	30 (100%)
HBeAg-positive					
<2.6	0 (0%)	32 (40%)	37 (47%)	34 (54%)	34 (68%)
2.6–<4.5	3 (4%)	26 (33%)	29 (37%)	21 (35%)	15 (30%)
≥4.5	76 (96%)	21 (27%)	13 (16%)	8 (13%)	1 (2%)
Total, *n* (%)	79 (100%)	79 (100%)	79 (100%)	63 (100%)	50 (100%)

ADV + LAM therapy, the HBV-DNA level diminished by 1.5 log copies/ml and ALT level improved to the normal range. At that time, only the mutant strain (rtA181T) was detected, suggesting that the viral reduction was due to the suppression of wild-type HBV strain. The HBV-DNA level was persistently above 5 log copies/ml even at the end of 1 year of ADV + LAM therapy. On the other hand, in the patients with rtA181T + rtM204I mutation, viral load rapidly decreased to the undetectable HBV-DNA level at the end of 6 months of ADV + LAM combination therapy (Fig. 2c).

3.6. Clinical course and clonal analysis in patients who developed ADV-related mutation during combination therapy

Fig. 3 shows the clinical course of patients with ADV-resistant mutants. The first ADV-resistant HBV strain was isolated from a 32-year-old Japanese man with genotype C (Fig. 3a). At 15 months after the start of LAM, viral and biochemical breakthroughs were observed. To suppress the viral HBV-DNA, ADV was added to LAM therapy. The mutant strain with rtA181T associated with ADV resistance appeared at 6 months of ADV + LAM therapy, while another rtN236T mutation appeared at 3 years of ADV therapy. Moreover, breakthrough hepatitis was observed after 3.5 years of ADV + LAM therapy (Table 4). Interestingly, the rtA181T at the end of 6 months of ADV therapy, due to single nucleotide substitution (TGG to TGA), resulted in early termination of overlapping HBs gene by creating a stop codon. On the other hand, at the end of 3 years of ADV therapy, all rtA181T mutant strains changed to double nucleotide substitutions (TGG to TTA), which induced amino acid substitutions in both polymerase (rtA181T) and HBs antigen (HBs W172L) developed.

Another mutant strain was detected in a 38-year-old Japanese man with genotype C-HBV infection (Fig. 3b). Following 46 months of ADV + LAM therapy when the viral load was increased, the rtA181S mutant strain without YMDD mutation was detected; however, the viral load diminished naturally to an undetectable level in a few months.

3.7. Clinical events

After the addition of ADV, 4 of the 132 (3%) patients elevated in serum creatinine >0.5 mg/dl above baseline and their ADV dose was reduced to 10 mg every other day.

Eight patients developed hepatocellular carcinoma (HCC) before the addition of ADV. After the addition of ADV, four patients developed HCC. Three of the four patients (75%) had cirrhosis at the start of ADV. The median duration from the start of ADV to the development of HCC was 14 months (range, 6–26 months). At the diagnosis of HCC, 3 of the 4 patients (75%) had undetectable HBV-DNA.

Of the 41 patients with cirrhosis, 5 patients had ascites and/or pleural effusion at the start of ADV. In 4 of the 5 patients, the fluid level diminished and disappeared during combination therapy. Only one patient with HCC showed worsened liver failure and died 22 months later. All patients without HCC and decompensation at the start of ADV therapy did not develop liver decompensation during follow-up.

4. Discussion

The efficacy of ADV combined with LAM has been reported in some studies; however, the rate of HBV-DNA undetectability under combination therapy was found to be the same as in patients treated with ADV alone [14,19]. We investigated whether combination therapy is characterized by a low risk of ADV resistance. In this study, we studied the long-term efficacy of ADV when added to LAM in 132 patients with chronic hepatitis B who developed LAM resistance. The results demonstrated that combination therapy

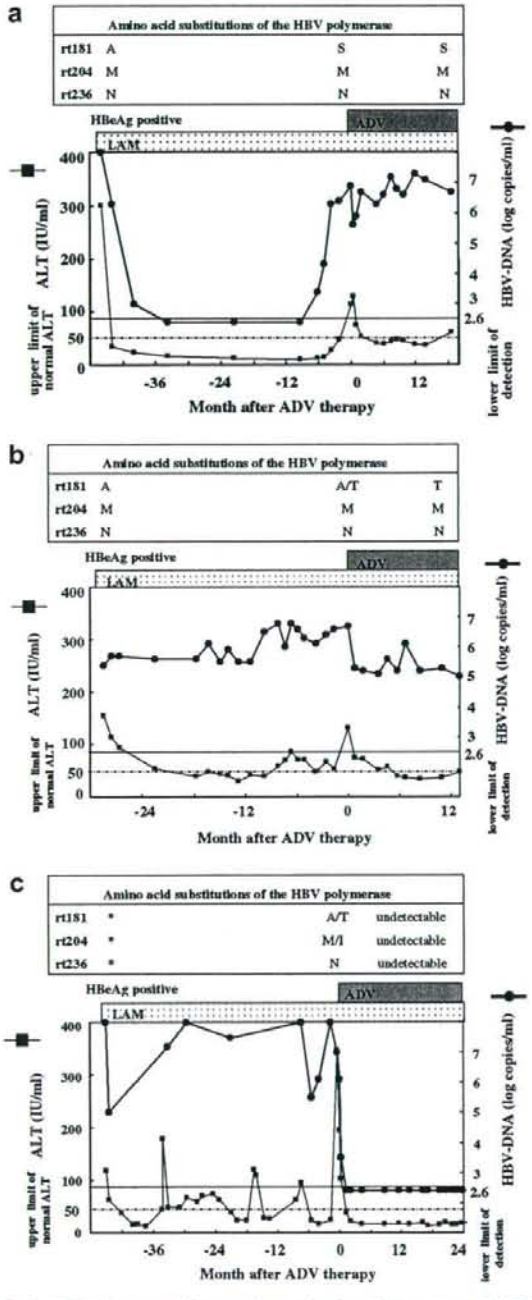

Fig. 2. Clinical course of three patients who showed emergence of ADV-resistant mutants at the commencement of ADV therapy. Amino acid substitutions in the HBV polymerase are displayed in the panel above each graph. (a) Clinical course of a patient who developed the rtA181S mutant. (b) Clinical course of a patient who developed the rtA181T mutant. (c) Clinical course of a patient who developed the rtA181T with rtm204I mutant. LAM, lamivudine; ADV, adefovir; rt, reverse transcriptase; *no data.

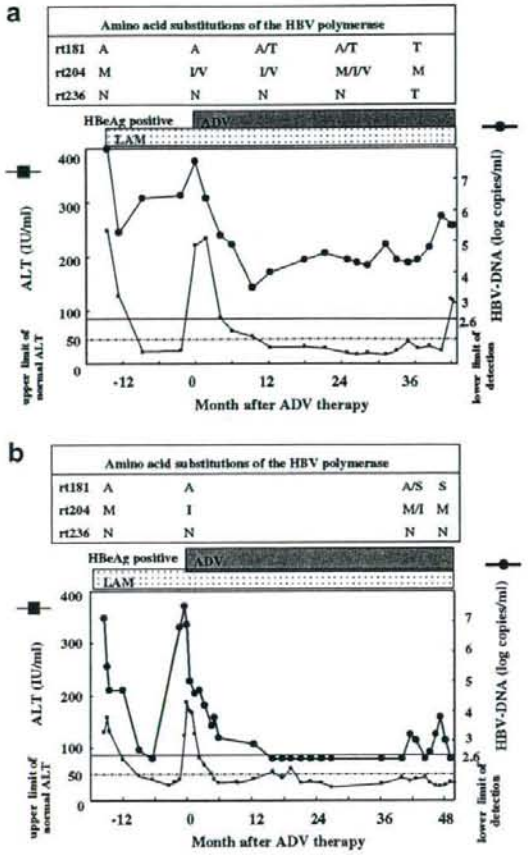

Fig. 3. Clinical course of two patients with LAM-resistant HBV who showed the emergence of an ADV-resistant mutant during combination therapy. Amino acid substitutions in the HBV polymerase are displayed in the panel above the graph. (a) Clinical course of a patient who developed the rtA181T + rtN236T mutant. (b) Clinical course of a patient who developed the rtA181S mutant. LAM, lamivudine; ADV, adefovir; rt, reverse transcriptase.

rapidly and consistently suppressed the HBV-DNA. Moreover, we demonstrated that the emergence of ADV-resistant mutants was rare during the combination therapy for up to 3 years. However, our virological analysis showed that substitutions at rt181, which were associated with both LAM and ADV resistance, need to be evaluated during combination therapy.

Multivariate analysis in this study revealed that the baseline HBeAg status, AST level and HBV-DNA level influenced the cumulative probability of undetectability of serum HBV-DNA. A number of previous studies also identified almost the same predictors of virological response during ADV alone or combination therapy [20,21]. In particular, the undetectable rate of HBV-

Table 4
Clonal analysis of samples from the patient who developed resistance to ADV + LAM combination therapy

	Relative rate (%) of clones (No. of clones/total)				
	Wild	rtM204I/V	rtA181T(1)	rtA181T(2)	rtA181T + rtN236T
rtA181	–	–	T(HBsAgstop)*	T(sW172L)#	T(sW172L)#
rtM204	–	I/ V	–	–	–
rtN236	–	–	–	–	N
(1) At the start of ADV + LAM	40 (4/10)	60 (6/10)	0	0	0
(2) 6 months after ADV + LAM	0	59 (13/22)	41 (9/22)	0	0
(3) 2 years after ADV + LAM	16 (4/25)	36 (9/25)	36 (9/25)	12 (3/25)	0
(4) 3 years after ADV + LAM	0	0	0	0	100 (20/20)

Note. rtM204I, methionine to isoleucine substitution at rt204; rtM204V, methionine to valine substitution at rt204; rtA181T, alanine to threonine substitution at rt181.

* The single nucleotide substitution (TGG to TGA) resulted in rtA181T mutation and early termination of overlapping HBs gene by creating a stop codon.

The double nucleotide substitution (TGG to TTA) resulted in amino acid substitutions in both polymerase (rtA181T) and HBs antigen (HBs W172L).

DNA was more frequent and faster in HBeAg-negative patients than in HBe-positive patients. At the end of 12 months of combination therapy, the rates of undetectability of HBV in HBeAg-negative and HBe-positive patients were 94% and 47%, respectively. However, in HBe-positive patients, the longer treatment course increased the virological response more frequently. Some patients achieved virological response after 2 more years of combination therapy (Fig. 1a). It was considered that the low risk of ADV resistance during combination therapy contributed to the longer effect of HBV suppression.

Our result is in agreement with the previous study that showed that ADV-resistant mutants are infrequent in combination therapy [14,19]; however, our study demonstrated that the ADV mutant could have emerged during combination therapy. We identified the emergence of rtA181T/S and/or rtN236T mutation in two of the 129 patients with YMDD mutant as an ADV-resistant strain during the ADV + LAM combination therapy. To our knowledge, this is the first report of emergence of ADV-resistant mutant followed by breakthrough hepatitis during combination therapy as shown in Fig. 3a. A previous open-label study in HBeAg-negative LAM-resistant patients demonstrated that combination therapy did not result in the development of resistance to ADV over a period of 3 years, in contrast to ADV monotherapy that was associated with the development of such resistance in 21% of the patients after the first year [14]. Although another recent study reported the appearance of ADV-resistant mutants in three patients during combination therapy, they were all initially switched to ADV monotherapy and later changed to the combination therapy after several months [20]. Another recent study reported an emergence rate of ADV resistance during ADV + LAM of 1% at 1 year and 4% at 3 years; however, no virological rebound was noted [21]. The patients in our study continued to show a viral load of up to 5.8 log copies/ml and developed breakthrough hepatitis. Some studies of ADV monotherapy reported that the rise in ALT after the emergence of rtA181 and/or rtN236 mutant is mild to moderate [9,14,24]. Several *in vitro* studies including our previous study [22–25] demonstrated that the rtA181T and rtN236T mutant leads to a minor reduction in the susceptibility to both LAM and ADV. However, one study of 998 naïve patients treated with ADV showed that the rtA181V + rtN236T mutation was significantly associated with virological breakthrough [26]. In our study, a similar phenomenon emerged; patients with ADV resistance developed breakthrough hepatitis after rtN236T mutation that appeared after rtA181T mutation.

Interestingly, clonal analyses of HBV in patients with ADV-resistant mutants in this study showed that such mutants were mixed with rtA181T mutants without substitutions at rt204, and rtM204I/V mutants without substitution at rt181. Moreover, we identified two types of rtA181T mutant strains; one was a single nucleotide substitution that induced prematurely terminated HBsAg and the other was a double nucleotide substitution that induced amino acid substitutions in the HBs antigen. The rtA181T mutant with prematurely terminated HBsAg cannot replicate and spread by itself because of the lack of HBs antigen. This type of strain is thought to replicate *in vivo* supplied HBs antigen from wild-type strains as helpers. Thus, the mutants changed themselves to the HBV with mature HBsAg by additional nucleotide substitution. Our previous study identified the rtA181T with mature HBsAg first; however, the mutant emerged during LAM therapy and it did not show a stepwise process [25].

We also demonstrated that the substitution at rt181 was associated with not only ADV resistance but also LAM resistance. At the commencement of ADV + LAM combination therapy, the substitutions at rtA181 as LAM resistance were identified in three patients (2.3%), who exhibited poor viral reduction

during the combination therapy. Of note, the rtA181S mutation is a novel LAM-resistant strain that has never been reported. There are a few reports of the rtA181 mutation associated with LAM resistance. A recent study reported the presence of rtA181T mutants in 3 of 57 (5.3%) LAM-resistant patients [15] and another study showed that 6 of 145 (4%) LAM-resistant patients developed rtA181T/V mutation [21]. If ADV therapy produces insufficient reduction of LAM-resistant HBV, it is important to suspect the emergence of ADV-related mutant at the commencement of ADV therapy and plan a new treatment strategy. However, there is no consensus at present on the management of patients with ADV + LAM-resistant mutant. Entecavir was the only agent reported to be effective both *in vitro* and *in vivo*. In our study, the patient with rtA181S mutation was switched to entecavir therapy; however, this did not produce a sufficient reduction in the viral load. On the other hand, recent studies reported the efficacy of tenofovir for patients with LAM-resistant mutants [27,28]. Further studies are needed to clear this issue.

In conclusion, ADV in combination with LAM effectively suppressed viral replication and was efficacious in LAM-resistant patients with chronic HBV infection. Genotypic analysis indicated that the emergence of ADV-resistant mutants was rare in patients on ADV + LAM combination therapy at least for 2 years. However, virological analysis showed that the substitution at rt181, which was associated with both LAM and ADV resistance, was needed for careful monitoring before and during combination therapy.

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529

Validating a Markov Model of Treatment for Hepatitis C Virus-related Hepatocellular Carcinoma

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Summary

Objective: We created and validated a Markov model to simulate the prognosis with treatment for HCV-related hepatocellular carcinoma (HCC) for assessment of cost-effectiveness for alternative treatments of HCC.

Method: Markov state incorporated into the model consisted of the treatment as a surrogate for HCC stage and underlying liver function. Retrospective data of 793 patients from three university hospitals were used to determine Kaplan-Meier survival curves for each treatment and transition probabilities were derived from them.

Results: There was substantial overlap in the 95% CIs of the Markov model predicted and the Kaplan-Meier survival curves for each therapy. The predicted survival curves were also similar with those from the nationwide survey data supporting the external validity of our model.

Conclusions: Our Markov model estimates for prognosis with HCC have both internal and external validity and should be considered applicable for estimating cost-effectiveness related to HCC.

Keywords

Hepatocellular carcinoma, prognosis, survival rate, Markov model, validation

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common forms of carcinoma and the incidence is increasing in Japan and other countries [1, 2]. Accordingly, the burden of HCC on health resources has risen considerably and the differences in the effect and cost of the specific treatments or periodic surveillance program for HCC has been given increasing attention.

HCCs in most cases originate from a fibrotic lesion in the liver and more than 70% of them are caused by hepatitis C virus (HCV), with some caused by hepatitis B virus (HBV) or other conditions, such as alcoholic liver injuries, in Japan [1]. Although several treatment standards for HCC have been proposed [2, 3], due to the absence of large randomized trials, the current treatment strategy for HCC remains a matter of choice, depending mostly on retrospective studies [4]. Treatments for HCC have been progressing using current technology, such as echo-guided needle insertion and catheterization into the hepatic artery. Thus, we need to establish a method to evaluate the survival benefit of each treatment. Moreover, another characteristic feature of HCC is its frequent recurrence, even after successful treatment, and the duration between a recurrence and subsequent recurrence tends to be short in the progression. These natures make it difficult to evaluate the superiority among treatments or appropriate one according to the conditions of HCC. To compensate for the lack of apparent evidence related to the treatment, a predicting prognostic computer simulation model could be a solution, but few such models for HCC that deal with the frequent recurrences and have been appropriately validated have been reported to date.

The aim of this study was to develop and validate a simulation model to predict the prognosis after initial treatments for HCV-related HCC using clinical data.

2. Method

2.1 Patients

We retrospectively studied the medical records of the patients admitted for the initial treatment of HCC between January 1994

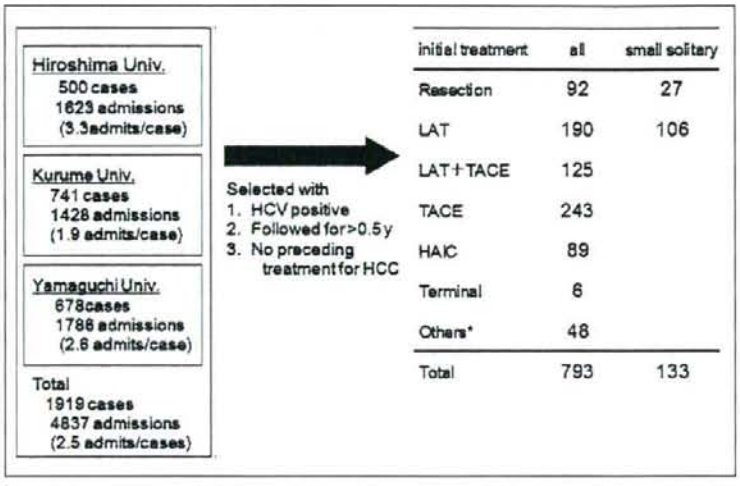

Fig. 1 Retrospective survey of HCC cases among three universities. * Others included combination of three treatment, combination of resection and LAT, and combination of resection and TACE.

and December 2003 at the three university hospitals; Yamaguchi, Hiroshima and Kurume. Total subjects were 1913 cases with 4837 admissions (Fig. 1).

The inclusion criteria were naïve HCV-related HCC cases with a follow-up period of more than half a year after the first admission. Total of 793 patients were eligible. They had an average 3.1 admissions (range: 1-17) and were followed for an average of 37 months (range: 181-3920 days). Among these patients, initial treatment by hepatic resection (HR), local ablation therapy (LAT), combination therapy of LAT and transcatheter arterial chemoembolization (TACE), TACE monotherapy, hepatic artery infusional chemotherapy (HAIC) or systemic chemotherapy and supportive treatment for terminal state were indicated for 92, 190, 125, 243, 89 and 6 patients respectively.

The average age of the patients was 64.7 years old (range: 40-83), male ratio was 0.62 and the proportion of liver cirrhosis was 0.89. The other characteristics were presented in Table 1. They were maximal size and number of tumors, the proportion of cases with previous history of interferon therapy, ratio of involvement with portal vein, hepatic artery or vein, or biliary duct, vascularity of tumor, total follow-up period, average number of admission and number of attained complete remission state and duration of it. Limiting the population to solitary and small tumors (less than 3cm in maximum diameter) as an early HCC state,

Table 1 Baseline characteristics of the cases

Initial treatment		HR (N = 92)		LAT (N = 190)		LAT + TACE (N = 125)		TACE (N = 243)		HAIC (N = 89)		Terminal (N = 6)		Others* (N = 48)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Age		63.4	8.3	66.2	8.3	66.5	7.7	65.8	7.4	65.6	6.3	62.7	11.9	66.4	8.0
Male ratio		0.83		0.64		0.67		0.70		0.81		0.67		0.75	
Tumor	Max. size	3.99	2.79	2.18	2.69	2.36	1.03	3.88	2.72	5.10	3.53	4.05	2.32	3.08	2.36
	Number**	1.71	1.35	1.52	0.89	2.31	1.91	3.04	2.91	6.16	6.08	2.83	1.60	2.83	2.21
IFN treatment before admission (rate)		0.23		0.14		0.11		0.06		0.05		0.00		0.16	
Involvement of vessels (rate)		0.23		0.05		0.02		0.14		0.27		0.33		0.19	
High vascularity of tumor (rate)		0.92		0.72		0.89		0.97		0.99		0.83		0.93	
Total FU*** (day)		1339	862	1255	866	1309	815	1026	770	682	529	404	295	1243	891
No. of admission		2.28	1.95	2.87	2.08	3.85	2.28	3.41	2.03	2.13	1.44	1.67	0.52	3.46	2.74
No. of CR#		1.27	0.76	1.29	0.98	1.08	1.14	0.67	0.94	0.37	0.77	0.00	0.00	0.58	0.85
CR duration (day)		922	836	709	691	501	598	359	626	142	351	0	0	287	488
CR	Rate/admission	0.71	0.36	0.56	0.38	0.32	0.33	0.22	0.31	0.16	0.31	0.00	0.00	0.20	0.31
	Duration/total FU	0.68	0.34	0.55	0.36	0.33	0.33	0.25	0.35	0.13	0.28	0.00	0.00	0.22	0.33

* include combination of three treatments, combination of resection and LAT, and combination of resection and TACE
** In case that more than ten nodules were seen, its number of tumors was treated as 11 nodules.
*** follow-up periods
# complete remission after treatment