Table 2 Association between weight status and NAFLD

Year	2004			2007		
	Lean-to-normal ($n = 202$)	Overweight ($n = 22$)	Obese ($n = 25$)	Lean-to-normal ($n = 237$)	Overweight ($n = 34$)	Obese ($n = 17$)
Hepatic steatosis						
Absent	199 (79.9)	22 (8.8)	17 (6.8)	234 (81.3)	30 (10.4)	11 (3.8)
Present (NAFLD)	3 (1.2)	0 (0)	8 (3.2)	3 (1.0)	4 (1.4)	6 (2.1)
Mild	3 (1.2)	0 (0)	6 (2.4)	3 (1.0)	4 (1.4)	2 (0.7)
Moderate-to-severe	0 (0)	0 (0)	2 (0.8)	0 (0)	0 (0)	4 (1.4)

Data are expressed as numbers (percentages of all students in each year)

NAFLD nonalcoholic fatty liver disease

Table 3 Comparison of clinical data

NAFLD	(−) ($n = 513$)	(+) ($n = 24$)	*P*
Male	258 (50.3)	17 (70.8)	**0.049**
Obesity (JSI ≥ 20%)	28 (5.7)	14 (58.3)	**≤0.001**
ALT ≥30 U/L	4 (0.8)	6 (25.0)	**≤0.001**
γGT ≥30 U/L	6 (1.2)	5 (20.8)	**≤0.001**
JSI (%)	−0.6 ± 12.9	27.0 ± 38.0	**0.002**
BMI (kg/m^2)	19.3 ± 2.7	23.2 ± 4.6	**≤0.001**
ALT (U/L)	13 ± 5	22 ± 15	**0.012**
γGT (U/L)	14 ± 4	20 ± 10	**0.006**
TG (mg/dL)	75 ± 42	99 ± 53	**0.037**
HDL-C (mg/dL)	64 ± 13	57 ± 10	**0.006**

Data were obtained from 249 junior high school students in 2004 and 288 students in 2007 and are expressed as numbers (percentages) or means ± SD. *P* values of less than 0.05 are in bold and underlined

NAFLD nonalcoholic fatty liver disease, *JSI* age-gender-adjusted Japanese standardized weight index for height, *ALT* alanine aminotransferase, *γGT* γ-glutamyltransferase, *BMI* body mass index, *TG* triglycerides, *HDL-C* high-density-lipoprotein cholesterol

15 ± 6 vs. 12 ± 6 U/L, $P < 0.001$ for ALT; and 16 ± 5 vs. 13 ± 3 U/L, $P < 0.001$ for γGT). The prevalence of elevated ALT and γGT levels tended to be higher in boys, though not significantly so (2.5% vs. 1.1% and 2.9% vs. 1.1%, respectively).

Comparison of lifestyle habits between students with and without NAFLD

To explore lifestyle habits associated with NAFLD development, questionnaires were distributed to all students in 2007 and results were compared between students with NAFLD ($n = 13$) and those without ($n = 275$). The clinical features of the students examined in 2007 are shown in Table 4. The ratios of students skipping breakfast (≥2 times/week) and always drinking more than half of the broth that comes with noodles were significantly higher in

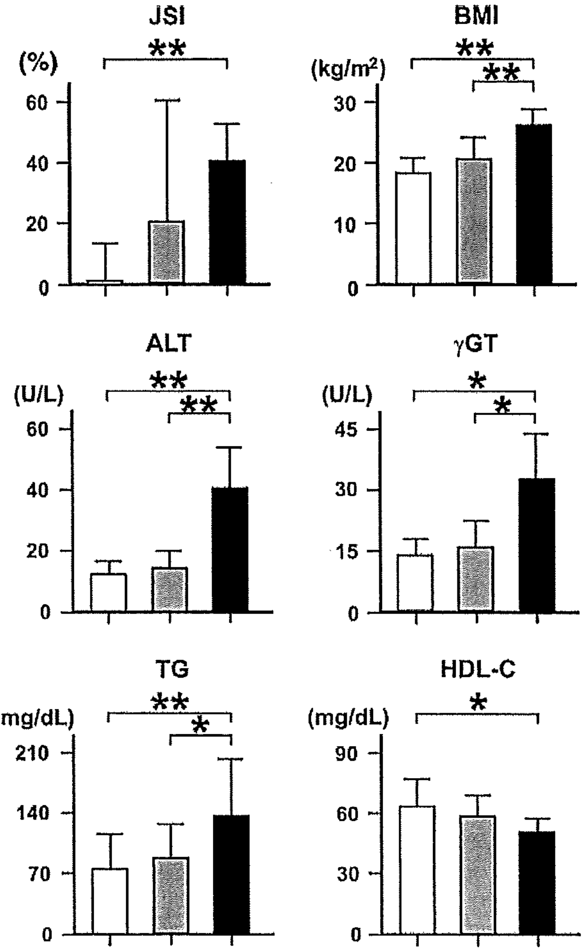

Fig. 1 Comparison of clinical parameters by degree of steatosis. Data were obtained in 2004 and 2007 and were compared among students without steatosis (*white bars*, $n = 513$), those with mild steatosis (*gray bars*, $n = 18$), and those with moderate-to-severe steatosis (*black bars*, $n = 6$). Data are expressed as means ± SD. Statistical analysis was conducted using Tukey's or Games–Howell's analysis. $^{**}P < 0.01$, $^{*}P < 0.05$; *JSI* age-gender-adjusted Japanese standardized weight index for height, *BMI* body mass index, *ALT* alanine aminotransferase, *γGT* γ-glutamyltransferase, *TG* triglycerides, *HDL-C* high-density-lipoprotein cholesterol

NAFLD students than in non-NAFLD ones (Table 5). The ratio of students often commuting to and from school by car was also significantly higher in NAFLD students (Table 5). As expected, the ratios of students liking exercise and doing sports outside of school were significantly lower in the NAFLD group (Table 5). There were no gender differences in the ratios of students having such lifestyle habits.

A similar comparison was performed between obese students with NAFLD ($n = 6$) and those without ($n = 11$). Although there were no remarkable differences in dietary habits, obese NAFLD students showed a higher tendency to commute to and from school by car (80.0% vs. 8.3%, $P = 0.010$) and a lower tendency to do sports outside of school (0 vs. 58.3%, $P = 0.041$) than obese non-NAFLD students. Furthermore, when lifestyle habits were compared between nonobese students with NAFLD ($n = 7$) and those without ($n = 264$), the former group had a higher ratio of drinking more than half of the broth that comes with noodles (87.5% vs. 50.6%, $P = 0.042$) and a lower ratio of doing sports outside of school (0 vs. 31.9%, $P = 0.048$).

Overall, the absence of outside-of-school sports was detected in all students with NAFLD, regardless of obesity, suggesting an important association between the development of NAFLD and reduced physical activity in junior high school students.

Table 4 Comparison of clinical data in 288 junior high school students examined in 2007

NAFLD	(−) ($n = 275$)	(+) ($n = 13$)	P
Male	147 (53.5)	8 (61.5)	0.568
Obesity (JSI ≥ 20%)	11 (4.0)	6 (46.2)	**≤0.001**
ALT ≥30 U/L	3 (1.1)	3 (23.1)	**0.001**
γGT ≥30 U/L	4 (1.5)	3 (23.1)	**0.002**
JSI (%)	−2.2 ± 12.2	26.8 ± 49.9	**0.048**
BMI (kg/m^2)	19.0 ± 2.5	22.5 ± 4.9	**0.025**
ALT (U/L)	14 ± 5	21 ± 14	**0.044**
γGT (U/L)	14 ± 4	20 ± 13	**0.032**
TG (mg/dL)	62 ± 29	83 ± 47	0.136
HDL-C (mg/dL)	68 ± 13	60 ± 8	**0.004**

Data are expressed as numbers (percentages) or means ± SD. P values of less than 0.05 are in bold and underlined. Abbreviations are the same as those in Table 3

Follow up of junior high school students with NAFLD

The 11 junior high school students with NAFLD found in 2004 were advised to come to our hospital for treatment and monitoring. Of these, only two obese students with severe steatosis and elevated ALT and γGT levels received further examination. They were negative for hepatitis B virus surface antigen, anti-hepatitis C virus antibody, and anti-nuclear antibody in sera, had normal levels of serum ceruloplasmin, and had no history of regular intake of drugs, which indicated that their liver dysfunction stemmed from NAFLD. One student reduced dietary calorie intake and started daily walking, and marked attenuation of hepatic steatosis and normalization of serum ALT levels were observed 1 year later. On the other hand, the other student continued to gain weight despite repeated lifestyle instructions, experienced worsened serum ALT levels, and was later diagnosed as having NASH by liver biopsy

Table 5 Lifestyle habits of 288 junior high school students examined in 2007

NAFLD	(−) ($n = 275$)	(+) ($n = 13$)	P
Skipping breakfast (≥2 times/week)	26 (9.5)	4 (30.8)	**0.036**
Eating quickly	118 (42.9)	7 (53.8)	0.444
Drinking more than half of the broth with noodles	140 (50.9)	11 (84.6)	**0.018**
Consuming sweetened drinks every day	131 (47.6)	6 (46.2)	0.907
Eating junk food (≥3 times/week)	209 (76.0)	8 (61.5)	0.186
Eating a midnight snack (≥3 times/week)	115 (41.8)	4 (30.8)	0.423
Watching TV during meals	207 (75.3)	12 (92.3)	0.144
Eating all of the fat around meat	160 (58.2)	9 (69.2)	0.438
Often consuming mayonnaise	152 (55.3)	6 (46.2)	0.509
Eating cakes/sweet rolls (≥3 times/week)	129 (46.9)	5 (38.5)	0.543
Eating convenience store lunches (≥once/week)	35 (12.7)	4 (30.8)	0.084
Often commuting to and from school by car	67 (24.4)	7 (53.8)	**0.026**
Playing computer games (≥1 h/day)	180 (65.5)	10 (76.9)	0.305
Liking exercise	217 (78.9)	8 (61.5)	**0.045**
Doing sports outside of school	82 (29.8)	0 (0)	**0.012**

Data are expressed as numbers (percentages). P values of less than 0.05 are in bold and underlined

NAFLD nonalcoholic fatty liver disease

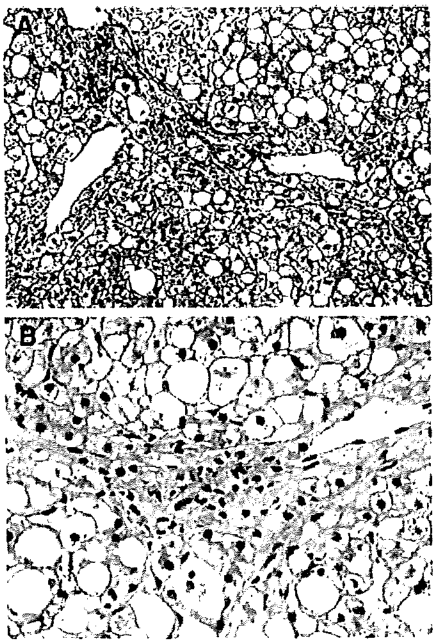

Fig. 2 Histological findings of a liver sample obtained from an obese student with nonalcoholic fatty liver disease (NAFLD). Severe macrovesicular steatosis, hepatocyte ballooning, and pericellular/perivenular fibrosis were evident. The NAFLD activity score was 7. **a** Azan-Mallory staining, ×140; **b** H&E staining, ×300

(Fig. 2). This student is now undergoing treatment for NASH.

Discussion

As far as we know, this is the first epidemiological study on the prevalence and clinical features of NAFLD in Japanese junior high school students. In addition, this study explored for the first time the relationship between the development of young adolescent NAFLD and lifestyle habits. Our results demonstrated that NAFLD in junior high school students occurs in approximately 1 out of 20 students and is primarily associated with obesity and decreased daily physical activity.

Very few population-based epidemiological studies have been conducted on pediatric NAFLD to date. Tominaga et al. [9] reported that the recent prevalence of NAFLD in Japanese children aged 11–15 years was 5.2%. Our findings are similar to these observations.

In the present study, the presence of obesity and an ALT level of 30 U/L or more were identified as independent predictors of NAFLD; anthropometric indices, such as the JSI and BMI, and serum ALT levels all increased with the degree of steatosis. Therefore, inclusion of serum ALT level determination in addition to anthropometric examination may be useful for detecting students with a risk of NAFLD/NASH in annual school-based health checkups. Alavian et al. [12] also described elevation of serum ALT levels to be a superior predictor of NAFLD over several metabolic variables, such as BMI, waist circumference, and serum concentrations of lipid profiles and insulin, in a cross-sectional study of 966 Iranian children aged 7–18 years. Further large-scale population-based prospective studies are needed to verify the usefulness of serum ALT measurement for the early detection of childhood NAFLD.

The most remarkable finding in the present study was that the ratios of students commuting to and from school by car and not doing sports outside of school were significantly higher in students with NAFLD compared with those without. Such tendencies were also detected in nonobese NAFLD students, suggesting that decreased daily physical activity contributes, at least in part, to the occurrence of NAFLD independently of the presence of obesity. This observation is supported by the evidence that regular aerobic exercise reduces hepatic lipids in obese individuals even in the absence of body weight reduction [13]. Thus, strategies to increase daily physical activity might prevent the development of NAFLD/NASH in junior high school students.

Concerning diet, we found a significant relationship between skipping breakfast and the presence of NAFLD. This observation is partially consistent with a previous study that showed skipping breakfast to be strongly associated with childhood obesity [14]. The dietary habit of always drinking most of the broth that comes with noodles might reflect an excess intake of salt, fat, and calories.

There were some NAFLD students without apparent obesity. Because all nonobese NAFLD students had normal concentrations of serum ALT and γGT and mild fatty deposition in the liver, it is inconclusive whether the clinical course of this type of NAFLD is similar to that of typical obesity-related NAFLD. Long-term longitudinal studies are required in such populations to address this issue.

It is well-known that gender differences exist in NAFLD [9, 15]. In the 537 students in our 2004 and 2007 surveys, the prevalence of NAFLD, JSI, and serum ALT and γGT levels were higher in boys. Such differences might result from the gender differences in susceptibility to fat accumulation in the body and insulin resistance [16]. Although gender differences in lifestyle habits could not be found in the present study, further large-scale assessment would enable us to clarify the relationship between gender differences in NAFLD prevalence and lifestyle habits.

Although the natural history of pediatric NAFLD has not been fully explored, some NAFLD cases in children can develop into advanced fibrosis and cirrhosis.

Considering that a high proportion of obese children are likely to become obese adults [17], early detection of pediatric NAFLD and the establishment of appropriate interventions may lead to a decreased incidence of adult NASH. To better predict pediatric NAFLD, we recommend the addition of serum ALT measurement to school-based health screening. More importantly, it remains difficult to convince NAFLD students to receive further examinations and follow up because of child and parent schedules and the lack of any apparent symptoms. To solve this problem, we believe that the establishment of a comprehensive community-based network system to screen for pediatric NAFLD and related lifestyle disorders [18] will be needed in the future.

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Journal of Medical Virology 81:452–458 (2009)

A Matched Case-Controlled Study of 48 and 72 Weeks of Peginterferon Plus Ribavirin Combination Therapy in Patients Infected With HCV Genotype 1b in Japan: Amino Acid Substitutions in HCV Core Region as Predictor of Sustained Virological Response

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Substitution of amino acid (aa) 70 and 91 in the core region of HCV genotype 1b is a useful pretreatment predictor of efficacy of 48-week peginterferon (PEG-IFN) plus ribavirin (RBV) therapy. Here, we determined the efficacy of 72-week PEG-IFN/RBV and the predictive factors to such therapy in a case–control study matched for sex, age, and periods from the start of treatment to initial point of HCV RNA-negative. We compared the treatment efficacy of 72-week regimen in 65 patients with that of 48-week in 130 patients, who were infected with HCV genotype 1b and treated with PEG-IFN/RBV. They consisted mainly of late virological responders (LVR) (HCV RNA-positive at 12 weeks and negative at 24 weeks after start of treatment). Sustained virological response (SVR) was achieved by 61.5% and 32.3% of patients of the 72- and 48-week groups, respectively, while non-virological response was noted in 9.2% and 29.2% of the respective groups. Multivariate analysis identified substitution of aa 70 and 91 (Arg70 and/or Leu91) and duration of treatment (72-week) as independent parameters that significantly influenced SVR. For Arg70 and/or Leu91 of core region, SVR rate was significantly higher in 72- (68.0%) than 48-week group (37.8%). For wild-type of ISDR, SVR rate was significantly higher in 72- (61.2%) than in 48-week group (29.3%). We conclude that 72-week PEG-IFN/RBV improves SVR rate for LVR, especially those with Arg70 and/or Leu91 of core region or wild-type of ISDR. Substitution of aa 70 and 91 is also a useful pretreatment predictor of response to 72-week PEG-IFN/RBV. ***J. Med. Virol. 81:452–458, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: HCV; core region; NS5A-ISDR; peginterferon; ribavirin; 72-week; case–control study; LVR

INTRODUCTION

Hepatitis C virus (HCV) usually causes chronic infection that can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [Dusheiko, 1998; Ikeda et al., 1998; Niederau et al., 1998; Kenny-Walsh, 1999; Akuta et al., 2001]. In patients with HCV-chronic hepatitis, treatment with interferon (IFN) can induce viral clearance and marked biochemical and histological improvement [Davis et al., 1989; Di Bisceglie et al., 1989]. Especially, peginterferon (PEG-IFN) plus ribavirin (RBV) combination therapy for 48 weeks can achieve a high sustained virological response (SVR) [Manns et al., 2001; Fried et al., 2002].

Although treatment of genotype 1-infected patients typically extends over 48 weeks, there has been interest in prolongation of therapy, particularly in late

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virological responders (LVR) (HCV RNA-positive at 12 weeks and negative at 24 weeks after the start of treatment), because high relapse rates in LVR may indicate that treatment was not administered for a sufficient duration [Ferenci et al., 2005]. Previous studies from Europe and United States have demonstrated that LVR improves SVR rates when treatment is extended to 72 weeks, compared with standard duration of therapy, largely as a result of reducing posttreatment relapse rates [Buti et al., 2003; Berg et al., 2006; Sánchez-Tapias et al., 2006; Pearlman et al., 2007]. Thus, prolongation of therapy in LVR may improve the virological response rate. However, it is not clear at present whether prolongation of treatment improves the SVR rate of treatment-resistant Japanese patients infected with HCV/genotype 1b [Akuta et al., 2007a,b,c].

Previous studies indicated that amino acid (aa) substitutions at position 70 and/or 91 in the HCV core region of genotype 1b were predictors of poor virological response to 48-week PEG-IFN plus RBV therapy [Akuta et al., 2005, 2006, 2007a,b,c; Donlin et al., 2007], and also risk factors for hepatocarcinogenesis [Akuta et al., 2007d, 2008a]. However, it is not clear at this stage whether aa substitutions in the core region can be used before therapy to predict the outcome of 72-week regimen.

The aims of the present study in HCV genotype 1b-infected Japanese adult patients, who received PEG-IFN plus RBV, were the following: (1) To conduct a case–control study matched for sex, age, and periods from the start of treatment to the initial point of HCV RNA-negative, to compare the treatment efficacy of 72-week regimen and 48-week regimen. (2) To identify the pretreatment factors that could predict treatment efficacy of the 72-week regimen, including pretreatment aa substitutions in the core region.

PATIENTS AND METHODS

Study Population

A total of 559 HCV genotype1b-infected Japanese adult patients were consecutively recruited into the study protocol of combination therapy with PEG-IFNα-2b plus RBV between 2001 and 2008 at Toranomon Hospital, Tokyo, Japan. They received PEG-IFNα-2b at a median dose of 1.4 μg/kg (range, 0.7–2.1 μg/kg) subcutaneously each week plus oral RBV at a median dose of 11.1 mg/kg (range, 3.4–16.0 mg/kg) daily. Among these, 383 patients, who could complete a total of 48 or 72 weeks of combination therapy, were enrolled in this retrospective study. The latter group consisted of 65 patients who extended combination therapy to 72-week (72-week group), and 318 patients who stopped combination therapy at the 48 weeks (48-week group). The decision to extend the combination therapy to 72 weeks was made by the patient. To compare the efficacy of the 72- and 48-week courses, all 65 patients of the 72-week group entered this study along with 130 patients of 48-week. The latter group was selected from among the 318 because they matched those patients of the 72-week group with respect to sex, age, and periods from the start of treatment to the initial point of HCV RNA-negative (matched case–control study). The treatment efficacy was evaluated by HCV-RNA positive based on qualitative PCR analysis at the end of treatment (non-virological response; NVR), and by HCV-RNA negative based on qualitative PCR analysis at 24 weeks after the completion of therapy (SVR). Furthermore, LVR was defined as HCV RNA-positive at 12 weeks and negative at 24 weeks after the start of treatment, based on qualitative PCR analysis. All patients fulfilled the following criteria: (1) Negativity for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positivity for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emerville, CA), and positivity for HCV RNA qualitative analysis with PCR (Amplicor, Roche Diagnostics, Manheim, Germany). (2) Infection with HCV genotype 1b only. (3) A high viral load ($\geq 100 \times 10^3$ IU/ml) by quantitative analysis of HCV RNA with PCR (AMPLICOR GT HCV Monitor v2.0 using the 10-fold dilution method, Roche Molecular Systems Inc., Pleasanton, CA) within the preceding 2 months of enrolment. (4) No hepatocellular carcinoma. (5) Body weight > 40 kg. (6) Lack of coinfection with human immunodeficiency virus. (7) No previous treatment with antiviral or immunosuppressive agents within the preceding 3 months of enrolment. (8) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg. (9) None had other forms of liver diseases, such as hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, or autoimmune liver disease. (10) None of the females was pregnant or a lactating mother. (11) All patients had completed a 24-week follow-up program after cessation of treatment, and SVR could be evaluated. (12) Each signed a consent form of the study protocol that had been approved by the human ethics review committee. The profile and laboratory data of 195 patients, who entered the matched case–control study, are summarized in Table I.

Laboratory Tests

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for alanine aminotransferase (ALT) and HCV-RNA levels. The serum samples were frozen at −80°C within 4 hr of collection and thawed at the time of measurement. HCV genotype was determined by PCR using a mixed primer set derived from the nucleotide sequences of NS5 region [Chayama et al., 1993]. HCV-RNA levels were measured by quantitative PCR (AMPLICOR GT HCV Monitor v2.0 using the 10-fold dilution method, Roche Molecular Systems Inc.) at least once every month before, during, and after therapy. The dynamic range of the assay was 5.0×10^3 to 5.0×10^6 IU/ml. Samples collected during and after therapy that showed undetectable levels of HCV-RNA ($<5.0 \times 10^3$ IU/ml) were also checked by qualitative PCR (AMPLICOR HCV v2.0, Roche Molecular Systems Inc.),

TABLE I. Patient Profile and Laboratory Data at Commencement of 48- and 72-Week Combination Therapy of Peginterferon Plus Ribavirin in Patients Infected With HCV Genotype 1b (Matched Case–Control Study)

	72-week group	48-week group	
Matching data			
Number of patients	65	130	
Sex (M/F)	28/37	57/73	Matched
Age (years)*	57 (22–70)	56 (25–68)	Matched
Periods to the initial point of HCV RNA-negative (weeks)*	17.4 (5.9–72.0)	19.7 (6.0–48.0)	Matched
Demographic data			
History of blood transfusion	18 (27.7%)	42 (32.3%)	NS
Family history of liver disease	21 (32.3%)	31 (23.8%)	NS
Body mass index (kg/m^2)*	22.6 (16.6–38.0)	22.2 (17.0–32.4)	NS
Laboratory data*			
Serum aspartate aminotransferase (IU/L)	49 (23–213)	51 (21–217)	NS
Serum alanine aminotransferase (IU/L)	64 (25–430)	68 (20–391)	NS
Serum albumin (g/dl)	3.9 (3.2–4.5)	3.8 (3.2–4.6)	NS
Gamma-glutamyl transpeptidase (IU/L)	40 (14–171)	38 (15–581)	NS
Leukocytes (/mm^3)	4,400 (2,300–8,800)	4,600 (1,200–9,400)	NS
Hemoglobin (g/dl)	14.0(11.3–17.8)	13.9 (10.6–18.1)	NS
Platelet count ($\times 10^4$/mm^3)	16.2 (8.2–30.7)	15.8 (6.4–31.6)	NS
ICG R15 (%)	13 (2–73)	15 (2–45)	NS
Level of viremia (KIU/ml)	2,650 (52->5,000)	1,850 (49->5,000)	0.013
Alfa-fetoprotein (μg/L)	6 (2–47)	6(2–110)	NS
Total cholesterol (mg/dl)	174(111–276)	175 (104–274)	NS
High density lipoprotein cholesterol (mg/dl)	45 (27–86)	51 (24–78)	NS
Low density lipoprotein cholesterol (mg/dl)	104 (49–204)	107 (50–182)	NS
Triglycerides (mg/dl)	91 (35–259)	94 (35–315)	NS
Uric acid (mg/dl)	5.3 (2.6–7.7)	5.0 (2.3–8.7)	NS
Fasting blood sugar (mg/dl)	95 (79–218)	98 (76–157)	NS
Histological findings			
Stage of fibrosis (F1/F2/F3/ND)	20/12/11/1/21	44/27/22/0/37	NS
Hepatocyte steatosis (none to mild/moderate to severe/ND)	40/2/23	78/8/44	NS
Treatment			
PEG-IFN α-2b dose (μg/kg)*	1.4 (0.8–2.1)	1.4 (0.7–1.9)	NS
Ribavirin dose (mg/kg)*	10.9 (6.6–16.0)	10.8 (3.7–14.2)	NS
Amino acid substitutions in the HCV			
Core aa 70 (arginine/glutamine (histidine)/ND)	37/23/5	11 47/6	NS
Core aa 91 (leucine/methionine/ND)	42/18/5	66/57/7	0.038
ISDR of NS5A (wild-type/mutant-type/ND)	49/5/11	99/17/14	NS

Data are number and percentages of patients, except those denoted by *, which represent the median (range) values. ND: not determined.

which has a higher sensitivity than quantitative analysis, and the results were expressed as positive or negative. The lower limit of the assay was 50 IU/ml.

Histopathological Examination of Liver Biopsies

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examinations contained six or more portal areas. Histopathological diagnosis was confirmed by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histopathological assessment according to the scoring system of Desmet et al. [1994]. Hepatocyte steatosis was graded as either none (absent), mild (less than 1/3 of hepatocytes involved), moderate (greater than 1/3 but less than 2/3 of hepatocytes involved), or severe (greater than 2/3 of hepatocytes involved) [D'Alessandro et al., 1991].

Detection of Amino Acid Substitutions in Core Region and NS5A Region

With the use of HCV-J (accession no. D90208) as a reference [Kato et al., 1990], the sequence of 1–191 aa in the core protein of genotype 1b was determined and then compared with the consensus sequence constructed on 50 clinical samples to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and aa 91 of leucine (Leu91) or methionine (Met91) [Akuta et al., 2005]. The sequence of 2209–2248 aa in the NS5A of genotype 1b (IFN-sensitivity determining region [ISDR]) reported by Enomoto et al. [1995, 1996] was also determined, and the numbers of aa substitutions in ISDR were defined as wild-type (≤ 1) or mutant-type (≥ 2).

In the present study, aa substitutions of the core region and NS5A-ISDR were analyzed by direct sequencing [Enomoto et al., 1995, 1996; Akuta et al., 2005]. HCV RNA was extracted from serum samples at the start of treatment and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo). Nucleic acids were amplified by PCR using the following primers: (a) Nucleotide sequences of the core region: The first-round PCR was performed with CC11 (sense, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (antisense, 5′-GGA GCA GTC CTT CGT GAC ATG-3′) primers, and the second-round PCR with CC9 (sense, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (antisense) primers. (b) Nucleotide sequences of NS5A-ISDR: The first-round PCR was performed with ISDR1 (sense, 5′-ATG CCC ATG CCA GGT TCC AG-3′) and ISDR2 (antisense, 5′-AGC TCC GCC AAG GCA GAA GA-3′) primers, and the second-round PCR with ISDR3 (sense, 5′-ACC GGA TGT GGC AGT GCT CA-3′) and ISDR4 (antisense, 5′-GTA ATC CGG GCG TGC CCA TA-3′) primers ([a] hemi-nested PCR; [b] nested PCR). All samples were initially denatured at 95°C for 15 min. The 35 cycles of amplification were set as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min at 55°C, and extension for 3 min at 72°C with an additional 7 min for extension. Then 1 µl of the first PCR product was transferred to the second PCR reaction. Other conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo, Japan) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan).

Statistical Analysis

Non-parametric tests (Mann–Whitney *U*-test, chi-squared test and Fisher's exact probability test) were used to compare the characteristics of the groups. Univariate and multivariate logistic regression analyses were used to determine those factors that significantly contributed to SVR. The odds ratios and 95% confidence intervals (95% CI) were also calculated. All *P* values less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P < 0.05$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. The potential pretreatment predictive factors associated with SVR included the following variables: sex, age, history of blood transfusion, familial history of liver disease, body mass index, aspartate aminotransferase (AST), ALT, albumin, gamma-glutamyl transpeptidase (γGTP), leukocyte count, hemoglobin, platelets, indocyanine green retention rate at 15 min (ICG R15), level of viremia, alfa-fetoprotein, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, uric acid, fasting blood sugar, hepatocyte steatosis, stage of fibrosis, PEG-IFN dose/body weight, RBV dose/body weight, duration of treatment, and amino acid substitution in the core and ISDR of NS5A. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL).

RESULTS

Comparison of Treatment Efficacy Between 48-Week Group and 72-Week Group

Figure 1 shows comparison of the treatment efficacy between 48- and 72-week groups. SVR was achieved by 42 of 130 patients (32.3%) and 40 of 65 (61.5%) in the 48- and 72-week groups, respectively. The proportion of SVR was significantly higher in 72-week group than in the 48-week group ($P < 0.001$). Furthermore, NVR was identified in 38 of 130 patients (29.2%) and 6 of 65 (9.2%) in the 48- and 72-week groups, respectively. The proportion of NVR was significantly lower in the 72-week group than in 48-week group ($P = 0.002$).

Predictive Factors Associated With SVR in Multivariate Analysis

Univariate analysis identified 13 parameters that influenced SVR either significantly or marginally: gender (female sex; $P = 0.002$), stage of fibrosis ($F_{1,2}$; $P = 0.008$), PEG-IFN dose/body weight (≥1.4 µg/kg; $P = 0.001$), RBV dose/body weight (≥11.0 mg/kg; $P = 0.029$), platelet count ($\geq 15.0 \times 10^4$/mm^3; $P = 0.002$), level of viremia (<1,000 KIU/ml; $P = 0.049$), γGTP (<50 IU/L; $P = 0.026$), ICG R15 (<15%; $P = 0.003$), triglycerides (<100 mg/dl; $P = 0.038$), high-density lipoprotein cholesterol (≥50 mg/dl; $P = 0.018$), α-fetoprotein (<20 µg/L; $P = 0.005$), substitution of aa 70 and 91 (Arg70 and/or Leu91; $P = 0.002$), and duration of treatment (72-week group; $P < 0.001$).

Multivariate analysis identified three independent parameters that either significantly influenced or tended to significantly influence SVR; substitution of aa 70 and 91 (Arg70 and/or Leu91; $P = 0.015$), duration of treatment (72-week group; $P = 0.014$), and high-density lipoprotein cholesterol (≥50 mg/dl; $P = 0.084$) (Table II).

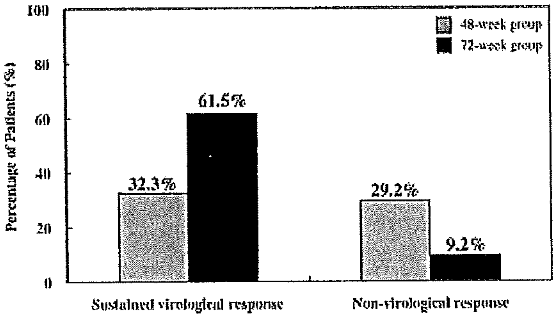

Fig. 1. Comparison of treatment efficacy between the 48-week group and 72-week group. The proportion of patients with sustained virological response in 72-week group was significantly higher than in 48-week group ($P < 0.001$). Furthermore, the proportion of patients with non-virological response in 72-week group was significantly lower than in 48-week group ($P = 0.002$).

TABLE II. Factors Associated With Sustained Virological Response to Combination Therapy of Peginterferon Plus Ribavirin in 195 Patients Infected With HCV Genotype1b, Identified by Multivariate Analysis

Factor	Category	Odds ratio (95% CI)	P
Substitution of aa 70 and 91	1: Gln70 (His70) and Met91	1	
	2: Arg70 and/or Leu91	5.46 (1.39–21.3)	0.015
Duration of treatment (weeks)	1: 48	1	
	2: 72	3.51 (1.28–9.62)	0.014
HDL-cholesterol (mg/dl)	1: <50	1	
	2: ≥50	2.42 (0.89–6.58)	0.084

*Only variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on multivariate logistic regression are shown.

Treatment Efficacy According to Amino Acid Substitutions in Core Region

Figure 2 shows comparison of the treatment efficacy according to aa substitutions in the core region. In Gln70 (His70) and Met91, SVR was achieved by 4 of 26 patients (15.4%) and 3 of 10 (30.0%) in the 48- and 72-week groups, respectively. The proportion of SVR in 72-week group was not significantly different than in 48-week group. In Arg70 and/or Leu91, SVR was achieved by 37 of 98 patients (37.8%) and 34 of 50 (68.0%) in the 48- and 72-week groups, respectively. The proportion of SVR in 72-week group was significantly higher than in 48-week group ($P = 0.001$).

Treatment Efficacy According to Amino Acid Substitutions in NS5A-ISDR

Figure 3 shows comparison of the treatment efficacy according to aa substitutions in NS5A-ISDR. In mutant-type, SVR was achieved by 9 of 17 patients (52.9%) and 3 of 5 (60.0%) in the 48- and 72-week groups, respectively. The proportion of SVR in 72-week group was not significantly different from that in 48-week group. In wild-type, SVR was achieved by 29 of 99 patients (29.3%) and 30 of 49 (61.2%) in the 48- and 72-week groups, respectively. The proportion of SVR in 72-week group was significantly higher than that in 48-week group ($P < 0.001$).

DISCUSSION

This matched case-controlled study of PEG-IFN plus RBV for LVR infected with HCV genotype 1b, showed that treatment extension to 72 weeks seems to improve SVR rates in Japanese patients. To our knowledge, the present study is the first to report that 72-week regimen of PEG-IFN plus RBV might be also useful in Asians. Especially, the 72-week regimen significantly improved the SVR rates in LVR with Arg70 and/or Leu91 of core or wild-type of ISDR. The present study based on patients, who could complete a total of 48 or 72 weeks of combination therapy, did not show the frequencies of patients, who could not complete by side effects. Patients, who dropped out by side effects between 48 and 72 week for therapy prolonged to 72 weeks, were only 3 of 559 HCV genotype1b-infected Japanese adult patients (data not shown), so the frequencies of side effects with 72-week regimen might be nearly equal to those with 48-week regimen. Large-scale prospective study based on the intention to treat analysis should be conducted to confirm the above finding in future.

NS5A-ISDR, reported as predictor of treatment efficacy with IFN monotherapy by Enomoto et al. [1995, 1996], is also useful as predictor of 48-week PEG-IFN plus RBV combination therapy [Murayama et al., 2007; Shirakawa et al., in press; Yen et al., 2008]. Furthermore, the present study also indicated that

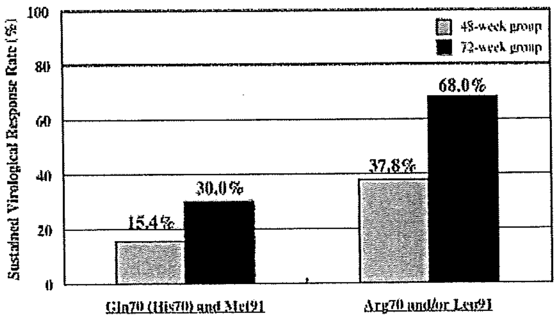

Fig. 2. Comparison of treatment efficacy according to amino acid substitutions in the core region. In Gln70 (His70) and Met91, the proportion of patients with sustained virological response in 72-week group was not significantly different from that in 48-week group. However, in Arg70 and/or Leu91, the proportion of patients with sustained virological response in 72-week group was significantly higher than in 48-week group ($P = 0.001$).

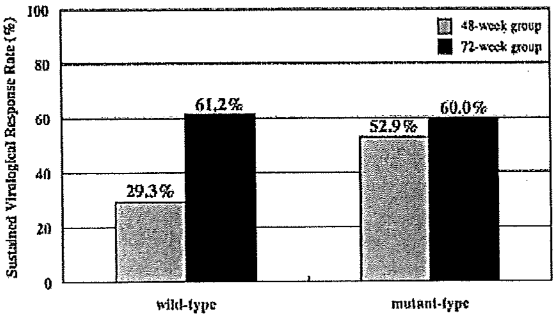

Fig. 3. Comparison of treatment efficacy according to amino acid substitutions in NS5A-ISDR. In mutant-type, the proportion of patients with sustained virological response in 72-week group was not significantly different from that in 48-week group. However, in wild-type, the proportion of patients with sustained virological response in 72-week group was significantly higher than in 48-week group ($P < 0.001$).

72-week regimen of PEG-IFN plus RBV significantly improved the SVR rate in LVR with wild-type of ISDR. Unfortunately, the 72-week regimen of PEG-IFN plus RBV did not improve the SVR rate in LVR with Gln70 (His70) and Met91 of the core region. Multivariate analysis also identified Gln70 (His70) and Met91 of the core region as independent parameter that significantly influenced non-SVR. PEG-IFN plus RBV carries potential serious side effects and is costly especially when used long enough to achieve higher SVR rates. For these reasons, we need to identify those patients who do not achieve SVR, to free them of unnecessary side effects and reduce costs, preferably before the start of the combination therapy. For patients unsuitable for PEG-IFN plus RBV, including LVR with Gln70 (His70) and Met91 of the core region, low-dose intermittent IFN monotherapy might be an efficacious therapeutic regimen, because it can lead to ALT normalization and thus reduce the risk of hepatocarcinogenesis [Akuta et al., 2008b].

One limitation of this study is that LVR could not be evaluated by the COBAS AmpliPrep/COBAS TaqMan HCV Test (the lower limit of this assay; 15 IU/ml), which has a higher sensitivity than AMPLICOR HCV v2.0 (the lower limit of this assay; 50 IU/ml) [Sizmann et al., 2007]. Rapid virological response (HCV RNA-negative at 4 weeks after the start of treatment) and early virological response (HCV RNA-positive at 4 weeks and negative at 12 weeks after the start of treatment) by AMPLICOR HCV v2.0 might be diagnosed as LVR by the COBAS AmpliPrep/COBAS TaqMan HCV Test. Further studies using highly sensitive real-time PCR assay should be performed to facilitate the development of more effective therapeutic regimens in future.

We previously reported that viral factors (e.g., aa substitutions in core region) and host factors (e.g., lipid metabolic factors, sex, and AFP) might be important predictors of treatment response to 48-week PEG-IFN plus RBV in Japanese patients infected with HCV genotype 1b, in addition to treatment-related factors (e.g., RBV dose) [Akuta et al., 2005, 2006, 2007a,b,c]. The present study also identified viral (aa substitutions in the core region), host (HDL-cholesterol), and treatment-related factors (duration of treatment) that can be useful as independent and significant pretreatment predictors of SVR. Thus, substitution of aa 70 and 91 is also useful as a pretreatment predictor of 72-week regimen. Further studies that examine the structural and functional impact of aa substitutions during combination therapy should be conducted to confirm the above finding.

Another limitation of our study was that we did not examine aa substitutions in areas other than the core region and NS5A-ISDR of HCV genome, such as the interferon/ribavirin resistance determining region (IRRDR), including V3 of NS5A region, although they should be investigated in future studies [El-Shamy et al., 2008; Muñoz de Rueda et al., 2008].

We conclude that treatment efficacy of 72-week PEG-IFN plus RBV seems to be based on a dynamic tripartite interaction of viral-, host-, and treatment-related factors. Further understanding of the complex interaction between these factors should facilitate the development of more effective therapeutic regimens.

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Virological Response and Hepatocarcinogenesis in Lamivudine-Resistant Hepatitis B Virus Genotype C Patients Treated with Lamivudine plus Adefovir Dipivoxil

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Key Words

Hepatitis B virus · Lamivudine · Adefovir dipivoxil · Hepatocellular carcinoma · Basic core promoter · Precore · Core region

Abstract

Aims: The long-term efficacy of adefovir dipivoxil in combination with lamivudine to chronic hepatitis B virus (HBV) infection is still unclear. ***Methods:*** Virological response and hepatocarcinogenesis during lamivudine + adefovir were investigated in 183 lamivudine-resistant Japanese patients with chronic genotype C-dominant HBV infection. As the predictors of virological response, an assessment of clinical parameters and a nucleotide (nt) sequence analysis of the negative regulatory element to core gene (nt 1611–2450) were performed at the start of adefovir. ***Results:*** The cumulative HBV-DNA non-detectable and ALT normalization rates were 93.6 and 97.6% at the end of 3 years, respectively. Multivariate analysis identified total bilirubin, AST, and nt substitutions (nt 1762, 1768, 1846, 1896, 2134, 2288, 2441) as determinants of early non-detectable HBV-DNA. The yearly incidence of hepatocellular carcinoma (HCC) during the first 3 years was 2.7%. At the diagnosis of HCC, ALT normalization, HBV-DNA non-detectable, and HBeAg-seronegative conversion rates were 75.0, 83.3, and 57.1%, respectively. Furthermore, the cumulative HBV-DNA non-detectable and ALT normalization rates were not significantly different according to the development of HCC or not. ***Conclusions:*** Lamivudine-resistant patients treated with lamivudine + adefovir could achieve the excellent virological response and biochemical response, but the low hepatitis activity was not enough to suppress hepatocarcinogenesis.

Introduction

Hepatitis B virus (HBV) is a small, enveloped DNA virus known to cause chronic hepatitis and often leads to liver cirrhosis and hepatocellular carcinoma (HCC) [1, 2]. To date, interferon and five nucleoside and nucleotide analogs (lamivudine, adefovir dipivoxil, entecavir, telbivudine, and tenofovir) have been approved for the treatment of chronic HBV infection. Nucleoside and nucleotide analogues suppress HBV replication in most patients and

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improve transaminase levels and liver histology [3–7]. Especially lamivudine monotherapy to naive patients for nucleoside analogues suppresses hepatocarcinogenesis [8, 9], but prolonged therapy results in the emergence of drug-resistant mutants.

Most lamivudine-resistant strains show amino acid substitutions in the YMDD (tyrosine-methionine-aspartate-aspartate) motif in the C domain of HBV polymerase [10, 11]. Both experimental and clinical studies have shown recently that adefovir and entecavir could suppress not only wild-type but also lamivudine-resistant strains and were confirmed as salvage therapy for lamivudine-refractory patients [12, 13]. Recently, Hosaka et al. [14] reported the efficacy of adefovir + lamivudine combination therapy in patients with lamivudine-resistant chronic HBV infection. However, the number of patients was limited and follow-up time was a short duration. Thus, the long-term efficacy in respect to viral response and suppression of hepatocarcinogenesis with lamivudine + adefovir is still unclear.

Virological predictors of viral response during the treatment of lamivudine + adefovir are insufficiently investigated. Negative regulatory element (NRE; nt 1611–1634), core upstream regulatory sequences (CURS; nt 1643–1742), basic core promoter (BCP; nt 1742–1849) are located mainly in the HBV X gene and play an important role in replication and hepatitis B core antigen/HBeAg formation [15–20]. Furthermore, in respect to the viral response to interferon, Erhardt et al. [21] reported that good response in HBeAg-positive patients was associated with a high number of mutations in the BCP and nt 1753–1766 as well as mutations at nt 1764, and that good response in HBeAg-negative patients correlated with a low number of mutations in the BCP and nt 1753–1766 and wild-type sequence at nt 1764. However, the significance of substitutions in NRE, CURS, BCP, precore, and core gene for viral response during the treatment of lamivudine + adefovir is still unknown.

The present study based on the long follow-up time included 183 lamivudine-resistant consecutive patients with chronic genotype C-dominant HBV infection treated with lamivudine + adefovir. The aims of the study were the following: (1) to evaluate the cumulative HBV-DNA non-detectable, alanine aminotransferase (ALT) normalization, and hepatocarcinogenesis rates during the treatment of lamivudine + adefovir, and (2) to analyze the predictive factors, including clinical parameters and a sequence analysis of the complete NRE, CURS, BCP, precore, and core gene, associated with early non-detectable HBV-DNA during the treatment of lamivudine + adefovir.

Table 1. Patient characteristics at the start of treatment with lamivudine + adefovir dipivoxil

Number	183
Male/female	150/33
Age, years[a]	47 (26–75)
Prior lamivudine therapy duration, years[a]	2.9 (0.6–10.8)
Lamivudine + adefovir treatment duration years[a]	2.2 (0.5–4.5)
HBeAg, number positive	109 (59.6%)
HBV-DNA, log copies/ml[a]	7.3 (3.3 to >7.6)
HBV genotype, number of A/B/C/D	7/7/168/1
Presence of cirrhosis	56 (30.6%)
Total bilirubin, mg/dl[a]	0.8 (0.2–6.0)
Aspartate aminotransferase, IU/l[a]	92 (18–1,413)
Alanine aminotransferase, IU/l[a]	130 (18–1,563)
γ-Glutamyl transpeptidase, IU/l[a]	58 (12–446)
Albumin, g/dl[a]	4.1 (2.3–4.7)
α-Fetoprotein, μg/l[a]	6 (2–282)
Creatinine, mg/dl[a]	0.8 (0.4–1.3)
Platelets, $\times 10^4/\text{mm}^3$ [a]	15.0 (3.1–38.8)
Mutant type of YMDD motif (YIDD/YVDD/YIDD+YVDD)	85/42/56

[a] Data are expressed as median (range).

Patients and Methods

Study Population

A total of 183 consecutive adult Japanese patients with chronic HBV infection were treated with adefovir at Toranomon Hospital, Tokyo, Japan, in addition to ongoing lamivudine treatment, for more than 24 weeks since 2002. Serum HBV-DNA and ALT levels re-increased despite the continuation of lamivudine, indicating breakthrough hepatitis, in all patients who then received adefovir along with the lamivudine. Enrolment in this study and the start of adefovir treatment were determined by the following criteria: (1) Increase in serum HBV DNA levels of ≥1 log copies/ml during lamivudine treatment on at least two consecutive occasions, compared with the nadir of initial antiviral efficacy. (2) Detection of mutations of the YMDD motif before the start of adefovir treatment 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) patients with HCC; (2) serum creatinine levels ≥1.5 mg/dl; (3) patients coinfected with hepatitis C, hepatitis delta virus, or HIV, and (4) history of other liver diseases, such as autoimmune hepatitis, alcoholic liver disease, or metabolic liver disease.

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and its subsequent amendments, and informed consent was obtained from every patient. This study was approved by the Local Ethics Committee of Toranomon Hospital.

Table 1 summarizes the profiles of the patients. They included 150 men and 33 women. The median duration of treatment with lamivudine + adefovir was 2.2 years (range 0.5–4.5). Patients received a 10-mg once-daily dose of oral adefovir, in addition to ongoing lamivudine treatment (100 mg/day). Blood samples were obtained at least once every month before, during, and after treatment with lamivudine + adefovir, and analyzed for virological markers, biochemical markers associated with liver function and renal function, and complete blood cell counts every visit. The diagnosis of cirrhosis was based on liver biopsy histology and/or on clinical criteria, including imaging studies and signs of portal hypertension. As the indicators of low hepatitis activity, non-detectable HBV-DNA level by PCR assay and normalization of ALT level were evaluated. Adverse reactions were monitored clinically by careful interview and medical examination at least once every month. Patient compliance with treatment was evaluated by questionnaire. Follow-up time represented the time from the start of the treatment with lamivudine + adefovir until the last visit.

Laboratory Tests

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, Japan) based on the method of Usuda et al. [22]. 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, Tokyo, Japan).

Nucleotide Sequencing of Negative Regulatory Element, Core Upstream Regulatory Sequences, Basic Core Promoter, Precore, and Core Gene

The sequences of nt 1611–2450, including the complete NRE (nt 1611–1634), CURS (nt 1643–1742), BCP (nt 1742–1849), precore (nt 1814–1901), and core gene (nt 1901–2450), were determined by the direct sequencing method using sera at the start of adefovir treatment. Nucleotide sequences of HBV were compared with the prototype sequences of the HBV genotype C (accession No. AB033550) [23]. In the present study, the PCR genotyping could be performed in 148 patients; the remaining 35 patients could not be analyzed due to the lack of adequate serum samples obtained at the start of adefovir treatment.

HBV DNA was extracted with a Smitest EX-R&D kit (Genome Science). Nucleic acids were amplified by PCR using the following primers: (a) *Sequences of nt 1588–2130:* the single-round PCR was performed with HBVPCCPseqF01 (sense, 5′-GCT TCA CCT CTG CAC GTC GCA TG-3′ [nt 1588–1610]) and HBVPCCPseqR03 (antisense, 5′-TCC AAA TTA CTT CCC ACC CAG GT-3′ [nt 2130–2108]) primers. (b) *Sequences of nt 2022–2529:* the single-round PCR was performed with HBVCOREseqF01 (sense, 5′-CCT TAG AGT CTC CGG AAC ATT G-3′ [nt 2022–2043]) and HBVCOREseqR02 (antisense, 5′-GCC ACT CAG GAT TAA AGA CAG G-3′ [nt 2529–2508]) primers. All samples were initially denatured at 95° for 2 min. 45 cycles of amplification were set as follows: denaturation for 30 s at 94°, annealing of primers for 30 s at 60°, and extension for 30 s at 68° with an additional 7 min for extension. The amplified PCR products were purified by the QIA Quick PCR purification kit (Qiagen, Tokyo, Japan) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing kit (PerkinElmer, Tokyo, Japan). To avoid false-positive results, the procedures recommended by Kwok and Higuchi [24] to prevent contamination were strictly applied to these PCR assays. No false-positive results were observed in this study.

Liver Histopathological Examination

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examination contained 6 or more portal areas. Histopathological diagnosis was made by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histopathological assessment according to the scoring system of Desmet et al. [25].

Diagnosis of Hepatocellular Carcinoma

Patients were examined for HCC by abdominal ultrasonography every 3–6 months. If HCC was suspected based on ultrasonographic results, additional procedures, such as computed tomography, magnetic resonance imaging, abdominal angiography, and ultrasonography-guided tumor biopsy if necessary, were used to confirm the diagnosis.

Statistical Analysis

The cumulative rates of non-detectable HBV-DNA and hepatocarcinogenesis were calculated using the Kaplan-Meier method and differences between the curves were tested using the log-rank test. Statistical analyses of non-detectable HBV-DNA and hepatocarcinogenesis were calculated using the period from start of treatment with lamivudine + adefovir. Stepwise Cox regression analysis was used to determine independent predictive factors that were associated with non-detectable HBV-DNA. The odds ratios and 95% confidence intervals (95% CI) were also calculated. Potential predictive factors associated with early HBV-DNA negativity included the following variables: age, sex, histological stage, HBV genotype, HBeAg, viremia level, mutant type of YMDD motif, total bilirubin, aspartate aminotransferase (AST), ALT, albumin, γ-glutamyl transpeptidase (GGT), α-fetoprotein (AFP), creatinine, platelets, nt substitutions in CURS to core gene. Each variable was transformed into categorical data consisting of two simple ordinal numbers for uni- and multivariate analyses. Variables that achieved statistical significance ($p < 0.05$) or marginal significance ($p < 0.10$) on univariate Cox proportional hazards model were tested by multivariate Cox proportional hazards model to identify significant independent factors. Statistical comparisons were performed using the SPSS software (SPSS Inc., Chicago, Ill., USA). All p values <0.05 by the two-tailed test were considered significant.

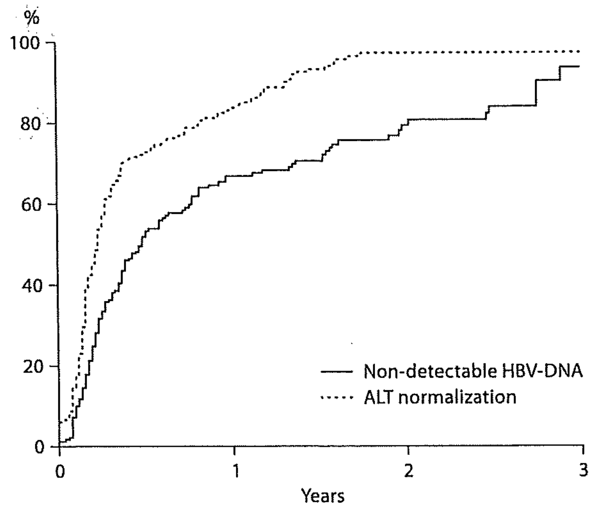

Fig. 1. Cumulative HBV-DNA non-detectable and ALT normalization rates. Patients treated lamivudine + adefovir dipivoxil could achieve the excellent virological response (non-detectable HBV-DNA) and biochemical response (ALT normalization) as an indicator of low hepatitis activity.

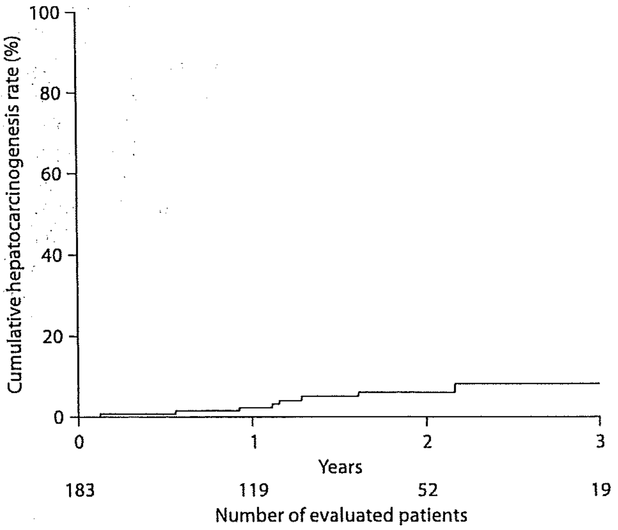

Fig. 2. Cumulative hepatocarcinogenesis rates during the treatment of lamivudine + adefovir dipivoxil. The yearly incidence of HCC during the first 3 years was 2.7%.

Results

Cumulative HBV-DNA Non-Detectable and ALT Normalization Rates

The cumulative HBV-DNA non-detectable rates were 48.4, 66.8, 79.4, and 93.6 at the end of 0.5, 1, 2, and 3 years, respectively. The cumulative ALT normalization rates were 72.4, 84.0, 97.6, and 97.6 at the end of 0.5, 1, 2, and 3 years, respectively. Thus, patients treated with lamivudine + adefovir could achieve the excellent virological response (non-detectable HBV-DNA) and biochemical response (ALT normalization) as an indicator of low hepatitis activity (fig. 1).

Predictive Factors Associated with Early Non-Detectable HBV-DNA by Uni- and Multivariate Analysis

The data for the whole population sample were analyzed to determine those factors that could predict early non-detectable HBV-DNA. Univariate analysis identified 21 parameters that tended to or significantly correlated with early non-detectable HBV-DNA. These included total bilirubin ($p = 0.027$), AST ($p = 0.004$), ALT ($p = 0.072$), HBV DNA ($p < 0.001$), HBeAg ($p < 0.001$), and nt substitutions [nt 1659 ($p = 0.073$), nt 1762 ($p = 0.040$), nt 1768 ($p = 0.084$), nt 1792 ($p = 0.077$), nt 1846 ($p < 0.001$), nt 1896 ($p < 0.001$), nt 1899 ($p = 0.031$), nt 1938 ($p = 0.019$), nt 2005 ($p = 0.058$), nt 2009 ($p < 0.001$), nt 2134 ($p = 0.074$), nt 2189 ($p = 0.017$), nt 2201 ($p = 0.031$), nt 2288 ($p = 0.038$), nt 2429 ($p = 0.042$), nt 2441 ($p < 0.001$)]. These factors were entered into multivariate analysis, which then identified 9 parameters that tended to or significantly influenced early non-detectable HBV-DNA independently; total bilirubin ($p = 0.002$), aspartate aminotransferase ($p = 0.077$), and nt substitutions [nt 1762 ($p = 0.092$), nt 1768 ($p = 0.001$), nt 1846 ($p = 0.034$), nt 1896 ($p = 0.001$), nt 2134 ($p = 0.034$), nt 2288 ($p = 0.016$), nt 2441 ($p = 0.019$)] (table 2).

Cumulative Hepatocarcinogenesis Rates and the Profiles of Patients Who Developed HCC

The cumulative hepatocarcinogenesis rates were 2.2, 5.9, and 8.1% at the end of 1, 2, and 3 years, respectively (fig. 2). The yearly incidence of HCC during the first 3 years was 2.7%. Table 3 summarizes the profiles of 12 patients who developed HCC during treatment with lamivudine + adefovir. They included 9 men and 3 women. The median age at the start of adefovir was 51 years (range 35–75). The median duration from the start of lamivudine to the diagnosis of HCC was 4.9 years (range 1.9–7.5), and the median duration from the start of adefovir

Table 2. Factors associated with early non-detectable HBV-DNA during the treatment with lamivudine + adefovir dipivoxil, identified by uni- and multivariate analysis

Factor	Category	Univariate Cox proportional hazards model		Multivariate Cox proportional hazards model	
		odds ratio (95% CI)	p	odds ratio (95% CI)	p
Total bilirubin, mg/dl	1: <1.0	1		1	
	2: ≥1.0	1.503 (1.047–2.159)	0.027	2.055 (1.289–3.279)	0.002
Aspartate aminotransferase, IU/l	1: <80	1		1	
	2: ≥80	1.695 (1.181–2.434)	0.004	1.506 (0.956–2.371)	0.077
Alanine aminotransferase, IU/l	1: <100	1		–	–
	2: ≥100	1.407 (0.970–2.041)	0.072	–	–
HBV DNA, log copies/ml	1: <7.0	1		–	–
	2: ≥7.0	0.488 (0.342–0.695)	<0.001	–	–
HBeAg	1: negative	1		–	–
	2: positive	0.428 (0.299–0.613)	<0.001	–	–
nt 1659	1: A	1		–	–
	2: not A	2.135 (0.931–4.895)	0.073	–	–
nt 1762	1: A	1		1	
	2: not A	1.988 (1.032–3.829)	0.040	1.987 (0.893–4.421)	0.092
nt 1768	1: T	1		1	
	2: not T	1.892 (0.917–3.903)	0.084	5.584 (2.096–14.88)	0.001
nt 1792	1: A	1		–	–
	2: not A	0.168 (0.023–1.211)	0.077	–	–
nt 1846	1: A	1		1	
	2: not A	2.080 (1.382–3.131)	<0.001	1.740 (1.043–2.902)	0.034
nt 1896	1: G	1		1	
	2: not G	2.207 (1.500–3.247)	<0.001	2.323 (1.430–3.775)	0.001
nt 1899	1: G	1		–	–
	2: not G	1.711 (1.049–2.789)	0.031	–	–
nt 1938	1: T	1		–	–
	2: not T	1.859 (1.107–3.124)	0.019	–	–
nt 2005	1: T	1		–	–
	2: not T	0.661 (0.431–1.014)	0.058	–	–
nt 2009	1: C	1		–	–
	2: not C	4.678 (2.191–9.986)	<0.001	–	–
nt 2134	1: C	1		1	
	2: not C	1.566 (0.957–2.561)	0.074	1.781 (1.044–3.038)	0.034
nt 2189	1: A	1		–	–
	2: not A	1.611 (1.087–2.385)	0.017	–	–
nt 2201	1: T	1		–	–
	2: not T	0.596 (0.373–0.953)	0.031	–	–
nt 2288	1: C	1		1	
	2: not C	1.518 (1.024–2.252)	0.038	1.733 (1.108–2.711)	0.016
nt 2429	1: C	1		–	–
	2: not C	2.573 (1.033–6.408)	0.042	–	–
nt 2441	1: T	1		1	
	2: not T	2.815 (1.656–4.783)	<0.001	2.001 (1.122–3.568)	0.019

Only variables that achieved statistical significance ($p < 0.05$) or marginal significance ($p < 0.10$) on uni- and multivariate Cox proportional hazards model are shown.

95% CI = 95% confidence interval.

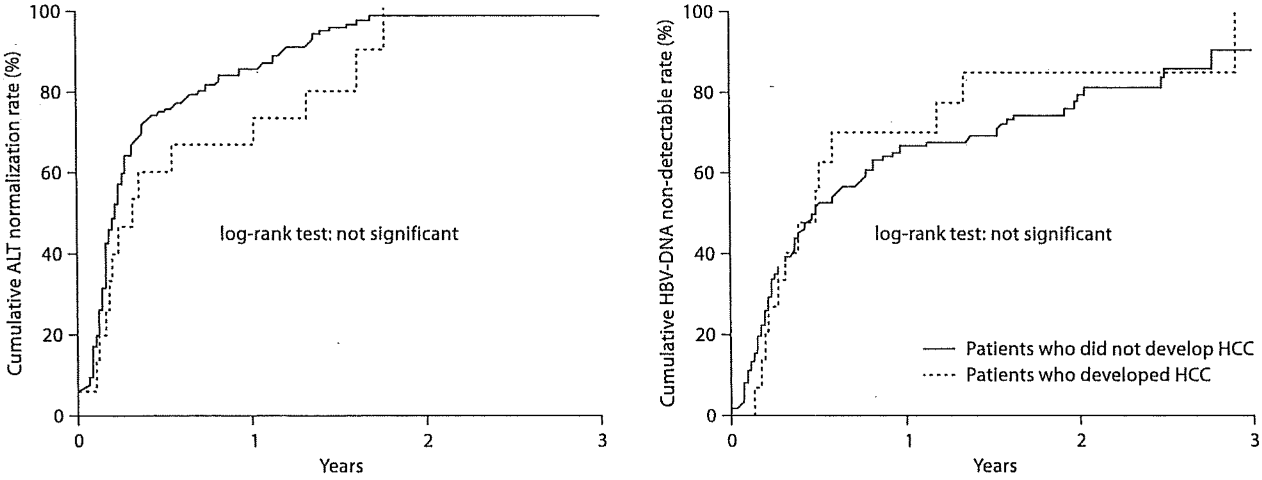

Fig. 3. Comparison of non-detectable HBV-DNA and ALT normalization in patients who developed HCC or not. The cumulative HBV-DNA non-detectable and ALT normalization rates were not significantly different according to the development of HCC or not. Low hepatitis activity during the treatment of lamivudine + adefovir dipivoxil was not enough to suppress hepatocarcinogenesis.

Table 3. Characteristics of 12 patients who developed HCC during treatment with lamivudine (LAM) + adefovir dipivoxil (ADV)

Case	Sex	Age years[a]	LAM to HCC years[b]	ADV to HCC years[c]	At the start of ADV			At the diagnosis of HCC		
					HBeAg	ALT IU/l	HBV-DNA log copies/ml	HBeAg	ALT IU/l	HBV-DNA log copies/ml
1	male	50	6.6	4.5	–	576	6.9	–	27	<2.6
2	male	40	4.9	3.8	–	124	6.3	–	24	<2.6
3	male	48	6.3	3.3	+	99	7.6	+	35	<2.6
4	female	58	7.4	3.3	+	214	4.4	–	13	<2.6
5	male	58	4.8	2.2	–	216	6.5	–	28	<2.6
6	male	35	7.5	1.6	+	164	7.5	–	35	<2.6
7	male	47	2.3	1.3	+	138	7.6	+	29	<2.6
8	male	50	2.5	1.1	+	272	7.6	–	72	<2.6
9	male	75	3.4	1.1	+	209	7.6	–	125	<2.6
10	female	51	1.9	0.9	–	130	5.3	–	73	<2.6
11	male	53	4.7	0.5	+	97	7.6	+	35	4.2
12	female	59	5.9	0.1	–	132	7.6	–	41	3.6

[a] Age at the start of adefovir dipivoxil.
[b] Duration from the start of lamivudine to the diagnosis of HCC.
[c] Duration from the start of adefovir dipivoxil to the diagnosis of HCC.

to the diagnosis of HCC was 1.5 years (range 0.1–4.5). At the diagnosis of HCC, 75.0% (9/12 patients) could achieve ALT normalization, and 83.3% (10/12) could achieve HBV-DNA non-detectable. 57.1% (4/7) of HBeAg-positive at the start of adefovir could achieve HBeAg-seronegative conversion at the diagnosis of HCC. Thus, they developed HCC in spite of the excellent virological response and biochemical response.

Comparison of Non-Detectable HBV-DNA and ALT Normalization in Patients Who Developed HCC or Not

The cumulative HBV-DNA non-detectable and ALT normalization rates were not significantly different according to the development of HCC or not (fig. 3). Thus, some of the patients during treatment with lamivudine + adefovir developed HCC in spite of the early non-detectable HBV-DNA and ALT normalization, and low hepatitis activity during treatment with lamivudine + adefovir was not enough to suppress hepatocarcinogenesis.

Discussion

This is the first report that investigates virological response and hepatocarcinogenesis during the treatment of lamivudine + adefovir in lamivudine-resistant patients with chronic genotype C-dominant HBV infection. Multivariate analysis identified total bilirubin, aspartate aminotransaminase, and nt substitutions (nt 1762, 1768, 1846, 1896, 2134, 2288, 2441) as determinants of early non-detectable HBV-DNA. Erhardt et al. [21] reported that the viral response to interferon was associated with a number of mutations in the BCP and nt 1753–1766 as well as mutation at nt 1764. As determinants of early non-detectable HBV-DNA, this study did not only identify nt substitutions in BCP (nt 1762, 1768), but also identified nt substitutions in precore (nt 1846, 1896), and core (nt 2134, 2288, 2441). This discrepancy between this results and previous findings may be explained by the difference of antiviral treatment, and design of this cohort study based on the only lamivudine-resistant patients during the treatment of lamivudine + adefovir. To our knowledge, the present study is the first to report that the precore, and core gene might influence viral response during lamivudine + adefovir. One limitation of the present study based on the small number of patients was that nt substitutions in areas other than the NRE, CURS, BCP, precore, and core gene of HBV genome, could not be examined. Further prospective studies based on the large numbers of patients, that examine the clinical impact of nt substitutions during lamivudine + adefovir (e.g., virological response and hepatocarcinogenesis) and the underlying mechanisms, should be conducted to confirm the above finding.

Lampertico et al. [26] recently reported the hepatocarcinogenesis during the treatment of lamivudine + adefovir in lamivudine-resistant patients with chronic genotype D-dominant HBV infection for a long-term follow-up period. To our knowledge, the present study is the first to report the hepatocarcinogenesis rates in patients with chronic genotype C-dominant HBV infection. Lamivudine-resistant patients treated with lamivudine + adefovir could achieve the excellent virological response and biochemical response, but the low hepatitis activity was not enough to suppress hepatocarcinogenesis. Kobayashi et al. [27] reported that the yearly incidence of HCC during the first 10 years was 3.3% in natural histories of patients with HBV genotype C-related-compensated cirrhosis without antiviral treatment, who have the higher risk for HCC development. This result showed that the yearly incidence of HCC during the first 3 years was 2.7% during the treatment of lamivudine + adefovir. Treatment of lamivudine + adefovir did not worsen natural histories of chronic HBV infection, but indicated the almost similar hepatocarcinogenesis rates in comparison to cirrhosis patients without antiviral therapy (namely, high-risk group for HCC development). Thus, lamivudine monotherapy to naive patients for nucleoside analogues without lamivudine-resistant HBV infection suppresses hepatocarcinogenesis [8, 9], but lamivudine-resistant chronic HBV patients might be also one of the high-risk groups for hepatocarcinogenesis. This study indicated the high cumulative hepatocarcinogenesis rates of 46.4% at the end of 4 years, and this reason is probably related to the small number of patients, in whom more than 4 years had elapsed since the induction of adefovir (data not shown). Further studies of a large group of patients for the longer-term follow-up period are required to clarify the true cumulative hepatocarcinogenesis rates during the treatment of lamivudine + adefovir.

Low hepatitis activity by suppression of viral replication was not enough to suppress hepatocarcinogenesis during the treatment of lamivudine + adefovir to lamivudine-refractory patients, in contrast to the suppression of hepatocarcinogenesis by lamivudine monotherapy to naive patients without lamivudine-resistant HBV infection [8, 9]. HBV DNA is often integrated into host chromosome in liver tumor tissue, possibly causing chromosomal instability [28–31]. Previous studies reported that antiviral treatment (e.g., lamivudine, adefovir, entecavir, peginterferon) also diminished the amount of intrahepatic covalently closed circular DNA (cccDNA) as an important intermediate in the life cycle of HBV [32, 33]. However, it is possible that any residual cccDNA in the hepatocytes may still have had integrative capacity at the HBV-DNA non-detectable state during lamivudine + adefovir, and that those may induce hepatocarcinogenesis. Further investigations should be performed whether cccDNA could influence hepatocarcinogenesis. In this study, it is regrettable that the associations of nt substitutions with the development of HCC could not be presented. This reason is related to the small number of HCC patients, who might provide misleading results (e.g., possible type II error). Further studies should be also conducted to investigate nt substitutions, which might affect the development of HCC during lamivudine + adefovir.

The hepatocarcinogenesis in many patients of this study might have started before the suppression of HBV replication under adefovir, since carcinogenesis begins several months or even years before HCC diagnosis. HCC was diagnosed from 0.1 to 1.6 years in 7 of 12 patients, so the potential beneficial effect of viral suppression might be expected to be seen after the first 2 years of adefovir therapy and only in patients who achieve HBV DNA nondetectable. Further studies should be performed to evaluate the HCC risk in patients who have remained at least 1 year in remission under adefovir to evaluate beneficial effect of viral suppression.

Previous studies reported that interferon monotherpy and lamivudine monotherapy to naive patients for nucleoside analogues without lamivudine-resistant HBV infection suppressed HBV-related hepatocarcinogenesis [8, 9, 34, 35]. In conclusion, lamivudine-resistant patients treated with lamivudine + adefovir could achieve the excellent virological response and biochemical response, but the low hepatitis activity by suppression of viral replication was not enough to suppress hepatocarcinogenesis. Further understanding including viral predictors should facilitate the development of more effective therapeutic regimens to reduce risk of hepatocarcinogenesis.

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