20 Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H: 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. J Med Virol 2009;81:452–458.
21 Nousbaum JB, Cadranel JF, Savary O, Legrand MC, Dumouchel P, Gouérou H: Sustained virological response after a short course of treatment with interferon and ribavirin in two chronic hepatitis C patients. J Hepatol 2003;39:655–656.
22 Dalgard O, Bjøro K, Hellum KB, Myrvang B, Ritland S, Skaug K, Raknerud N, Bell H: Treatment with pegylated interferon and ribavarin in HCV infection with genotype 2 or 3 for 14 weeks: a pilot study. Hepatology 2004;40:1260–1265.
23 Nagase Y, Yotsuyanagi H, Okuse C, Yasuda K, Kato T, Koike K, Suzuki M, Nishioka K, Iino S, Itoh F: Effect of treatment with interferon alpha-2b and ribavirin in patients infected with genotype 2 hepatitis C virus. Hepatol Res 2008;38:252–258.
24 Nomura H, Miyagi Y, Tanimoto H, Ishibashi H: Impact of early viral kinetics on pegylated interferon alpha 2b plus ribavirin therapy in Japanese patients with genotype 2 chronic hepatitis C. J Viral Hepat 2009;16:346–351.
25 Toyoda H, Kumada T, Kiriyama S, Sone Y, Tanikawa M, Hisanaga Y, Kanamori A, Atsumi H, Nakano S, Arakawa T: Eight-week regimen of antiviral combination therapy with peginterferon and ribavirin for patients with chronic hepatitis C with hepatitis C virus genotype 2 and a rapid virological response. Liver Int 2009;29:120–125.
26 Murakami T, Enomoto N, Kurosaki M, Izumi N, Marumo F, Sato C: Mutations in nonstructural protein 5A gene and response to interferon in hepatitis C virus genotype 2 infection. Hepatology 1999;30:1045–1053.
27 Akuta N, Suzuki F, Suzuki Y, Sezaki H, Hosaka T, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kobayashi M, Kumada H: Hepatocyte steatosis is an important predictor of response to interferon (IFN) monotherapy in Japanese patients infected with HCV genotype 2a: virological features of IFN-resistant cases with hepatocyte steatosis. J Med Virol 2005;75:550–558.

INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Efficacy and Safety of Combination Therapy of Natural Human Interferon β and Ribavirin in Chronic Hepatitis C Patients with Genotype 2 and High Virus Load

Yasuji Arase[1], Fumitaka Suzuki[1], Norio Akuta[1], Hitomi Sezaki[1], Yoshiyuki Suzuki[1], Yusuke Kawamura[1], Masahiro Kobayashi[1], Tetsuya Hosaka[1], Hiromi Yatsuji[1], Miharu Hirakawa[1], Naoki Matsumoto[1], Satoshi Saito[1], Kenji Ikeda[1], Mariko Kobayashi[1] and Hiromitsu Kumada[1]

Abstract

Objective The aim of this study was to evaluate the efficacy of combination therapy of natural human interferon-beta and ribavirin in patients infected with hepatitis C virus (HCV) genotype 2 and high virus load.

Methods Inclusion criteria were HCV-genotype 2, serum HCV RNA level of ≧100 KIU/mL before combination therapy. A total of 24 were enrolled in this retrospective cohort study. The treatment period of combination therapy was 24 weeks.

Results Of the 24 study patients, no patient stopped the treatment due to treatment-related adverse events. The dose of drugs were reduced in 8 patients. Twenty-one of 24 patients (87.5%) had sustained virological response (SVR) by the intention-to-treat analysis. The rate of negative HCV-RNA at 8 week after the initiation of treatment was 18/21 (86%) in patients with SVR and 1/3 (33%) in patients with non-SVR. Logistic regression analysis showed that SVR occurred when serum HCV RNA at 8 week after the initiation of combination therapy was negative (hazard ratio: 40.0; 95% confidence interval=1.75-914.78; p=0.021).

Conclusion The combination therapy of IFN-beta and ribavirin offers sufficient safety and efficacy in chronic hepatitis C patients with genotype 2 and high virus load.

Key words: chronic hepatitis C, natural interferon-beta, ribavirin, HCV genotype 2

(Inter Med 49: 965-970, 2010)
(DOI: 10.2169/internalmedicine.49.3299)

Introduction

Current evidence indicates that combination therapy of peginterferon and ribavirin for hepatitis C virus (HCV) is associated with a higher rate of sustained virological response (SVR) compared with interferon (IFN) alone (1-10). SVR in the patients with HCV genotype 2 treated with IFN monotherapy for 24 weeks was about 80% in group of low virus load and about 40-45% in high virus load (11). However, it has been reported that the SVR rate was about 80-90% in patients with genotype 2 and high virus load treated with peginterferon and ribavirin for 24 week (12-14). Hence, IFN-monotherapy has been recommended as a first choice for chronic hepatitis C patients with genotype 2 and low virus-load in Japan. On the other hand, combination therapy of peginterferon and ribavirin has been recommended as a first choice for chronic hepatitis C patients with genotype 2 and high virus-load. Thus, in the present study, we assessed the efficacy of the patients with genotype 2 and high virus load who showed low rate of SVR.

However, the dropout rates in patients treated with combination therapy of peginterferon and ribavirin are higher than those treated with IFN monotherapy (15-17). In particular,

Department of Hepatology, Toranomon Hospital, Tokyo and Hepatic Research Unit, Toranomon Hospital, Tokyo
Received for publication December 22, 2009; Accepted for publication February 10, 2010
Correspondence to Dr. Yasuji Arase, es9y-ars@asahi-net.or.jp

the adverse events due to combination therapy of IFN and ribavirin have a tendency to occur in elderly patients. Therefore, in the case of elderly patients, the physician in charge often avoids combination therapy of IFN and ribavirin due to side effects. However, recently, the life-span has been long in Japan. Thus, there is an ongoing need to refine treatment strategies with a strong effect and safety in HCV patients.

Festi et al reported that IFN-beta has sufficient tolerability (15). However, IFN-beta monotherapy does not result in a satisfactory outcome in patients with a high virus load (11). Enomoto et al have reported that IFN-beta plus ribavirin therapy might seem to have a strong effect and mild side effects originating from treatment (18, 19). However, to date there is little information regarding IFN-beta plus ribavirin therapy for chronic hepatitis C.

Thus, in the present study, we performed a retrospective study to examine the efficacy of combination therapy of IFN-beta and ribavirin in patients with genotype 2 and high virus load.

Materials and Methods

Patients

Eligibility criteria for entry into the study included the following: 1) HCV genotype 2a or 2b, 2) serum level of HCV RNA of ≥100 KIU/mL before combination therapy, 3) no corticosteroid, immunosuppressive agents, or antiviral agents used within 6 months; 4) no hepatitis B surface antigens (HBsAg), antinuclear antibodies (ANA), or antimitochondrial antibodies (AMA) detectable in serum, determined by radioimmunoassay; 5) leukocytes >2,000/mm^3, platelet count >80,000/mm^3, and bilirubin <2.0 mg/mL; 6) follow up for >6 months before treatment. We excluded from the study all of the patients with the following: 1) a history of alcohol abuse; 2) advanced liver cirrhosis of encephalopathy, bleeding esophageal varices, or ascites. The physician in charge explained the purpose and method of the combination therapy as well as the potential adverse reactions and informed consent was obtained from each patient.

From December 2004 to May 2008, 24 HCV patients were enrolled in this retrospective cohort study at the study hospital.

A SVR was defined as clearance of HCV RNA by commercial amplicor HCV qualitative assay (Amplicor HCV; Ver. 2.0, Roche Diagnostic Systems, Basel, Switzerland) at 6 months after the cessation of combination therapy (20).

Next, predictors of SVR in patients with undetectable HCV RNA in serum during treatment were assessed. Finally, SVR rate based on the attainment time of negativity of HCV RNA and continuance of negative HCV RNA during combination therapy were examined.

Combination therapy of IFN-beta and ribavirin

The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital and a signed consent form was obtained each patient. Treatment was provided for 24 weeks. IFN-beta (Feron, Toray Industries Inc., Tokyo, Japan) was given intravenously at a dose of 6 million units (MU) daily for 2-8 weeks initially, followed by three times a week for 16-22 weeks. Ribavirin (Rebetol, Schering-Plough, Osaka, Japan) were given at the dose described based on body weight. The ribavirin dose was adjusted according to body weight (600 mg for ≤60 kg, 800 mg for >60 kg and ≤80 kg, and 1000 mg for >80 kg). The period of daily administration in IFN-beta treatment was determined by the physician. The patients were divided into three groups based on the difference of period of daily administration of IFN-beta at the initial stage of treatment: a 2-week regimen, 10 patients; a 4-week regimen, 5 patients; and an 8-week regimen, 9 patients.

Blood samples were obtained just before and 6 month after combination therapy. The samples were stored at -80℃ until analyzed. Using these blood samples, HCV-RNA level before IFN therapy was analyzed by quantitative PCR assay (Amplicor GT-HCV Monitor Version 2.0, Roche Molecular Systems) (21). HCV-genotype was examined by polymerized chain reaction assay, using a mixture of primers for the six subtypes known to exist in Japan, as reported previously (22). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) concentrations, and HCV RNA were measured at least once per month during therapy. Negativity of serum HCV RNA was defined as clearance of serum HCV RNA by commercial amplicor HCV qualitative assay (20). Clinical evaluation and biochemical and hematological tests were performed at 4 weekly intervals.

Statistical analysis

Nonparametric procedures were employed for the analysis of background features of the patients with SVR and without SVR, including the Mann-Whitney U test, Fisher' exact test, Kruskal Wallis test, and/or logistic regression analysis. The following variables were evaluated as prognostic factors: sex, age, body mass index, a history of interferon therapy, a HCV RNA level, biochemical factors (AST, ALT, triglyceride, HDL-cholesterol, LDL-cholesterol), platelet count, HCV RNA 4, 8, 12 weeks after the initiation of IFN therapy, continuous negative period of HCV RNA during IFN therapy and period of IFN therapy. The SPSS software package (SPSS Inc., Chicago, IL) was used to perform statistical analysis. A p value of <0.05 was considered to indicate a significant difference.

Result

Clinical characteristics of the patients

A total of 24 patients were enrolled in the present study. Table 1 shows the characteristics of the patients who received combination therapy. Clinical profiles were as follows: mean age=55.9 years, male/female=11/13, and median

Table 1. **Clinical Backgrounds before Combination Therapy of Peginterferon and Ribavirin in Chronic Hepatitis C Patients**

Character	value
Patients, n	24
Sex, male (%)	11(45.8%)
Age (yrs)	55.9±10.2
BMI	23.0±2.5
A history of IFN (+)	12 (50.0%)
HCV RNA(KIU/mL)	870 (43-5000)
HCV genotype (2a, 2b)	14/10
AST (IU/L)	[illegible]
ALT (IU/L)	[illegible]
FPG (mg/dL)	[illegible]
Triglyceride (mg/dL)	[illegible]
HDL cholesterol (mg/dL)	52±19
LDL cholesterol (mg/dL)	117±31
Platelet (10^4/mm^3)	16.6±4.5
A regimen of daily administration of IFN-beta* (2 weeks/4 weeks/8 weeks)	10/5/9

Data are number of patients, percentage, mean ± standard deviation or median (range).

ALT: alanine aminotransferase, AST: aspartate aminotransferase, BMI: body mass index, FPG: fasting plasma glucose, HCV: hepatitis C virus, IFN: interferon.

*The patients were divided into three groups based on the difference of period of daily administration of IFN-beta at the initial stage of treatment: a 2-week regimen of daily administration of IFN-beta, 10 patients; a 4-week regimen, 5 patients; and an 8-week regimen, 9 patients.

(range) HCV-RNA=870(103-5,000) KIU/mL.

Safety and tolerance of IFN

Of the 24 patients included in this study, none of the patients discontinued combination therapy because of IFN-related adverse events. However, 7 out of 24 patients had dose reduction of interferon and/or ribavirin due to side effects. IFN-beta dose reduction was necessary in one case due to the development of neutropenia. RBV dose reduction was applied in 6 patients, due to anemia.

The leukocyte count was 4,700 ± 1,390/mm^3 and the platelet count was 166,000 ± 45,000/mm^3 before the initiation of IFN therapy, whereas the values were 3,020 ± 1,05/mm^3 and 134,000 ± 39,000/mm^3, respectively, two weeks after the initiation of the therapy.

Efficacy of treatment

Out of the 24 patients enrolled in the present study, 21 patients (87.5%) had SVR by the intention-to-treat analysis. Patients aged ≥65 years were five in total. Four out of five patients aged ≥65 years had SVR. Table 2 shows the differences in the clinical background between patients with SVR and those without SVR. The rate of negative HCV RNA at 8 weeks after the initiation of treatment was 18/21(86%) in patients with SVR and 1/3 (33%) in patients with non-SVR. Logistic regression analysis showed that SVR occurred when serum HCV RNA at 8 weeks after the initiation of combination therapy was negative (hazard ratio: 40.0; 95% confidence interval=1.75-914.78; P=0.021). Moreover, the SVR was not significantly different based on the difference of period of daily administration of IFN-beta at the initial stage of treatment.

Background of non-SVR cases

Three patients had negative HCV RNA at the end stage of treatment, but showed reappearance of HCV RNA after

Table 2. **The Difference of Clinical Backgrounds between Patients with SVR and Those without SVR**

	SVR (n=21)	Non-SVR (n=3)	p value*
Age (years old)	56.1 ± 9.1	57.0 ± 8.0	0.827
Sex (male/female)	12/9	2/1	0.449
BMI	22.9 ± 2.5	22.8 ± 2.6	1.000
a history of IFN (+/-)	11/10	1/2	0.759
HCV-load (KIU/mL)	794± 786	1545± 1797	0.759
AST (IU/L)	69 ± 47	44 ± 12	0.540
ALT (IU/L)	83 ± 39	70 ± 55	0.359
FPG (mg/dL)	96 ± 13	92 ± 3	0.813
Triglyceride (mg/dL)	112 ± 74	107 ± 57	0.614
HDL cholesterol (mg/dL)	51 ± 20	65 ± 17	0.297
LDL cholesterol (mg/dL)	113 ± 31	126 ± 15	0.509
Platelet (10^4/mm^3)	16.3 ± 4.7	17.7 ± 5.3	0.701
HCV RNA (+/-) 4W	9/12	2/1	0.576
HCV RNA (+/-) 8W	3/18	2/1	0.099, 0.021
HCV RNA (+/-) 12W	0/21	0/3	1.000
Period of daily administration of IFN* (2-week/4-week/8-week)	9/4/8	1/1/1	0.925

Data are number of patients (percentage) or mean ± standard deviation.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FPG, fasting plasma glucose; HCV, hepatitis C virus; IFN, interferon;

*IFN-beta was given intravenously at a dose of 6 million units (MU) daily for 2-8 weeks, followed by three times a week for 16-22 weeks. Figure of 2, 4, and 8 represents the (week) of daily administration of IFN-beta at the initial stage.

†Nonparametric procedures were employed for the analysis of background features of the patients with SVR and without SVR, including the Mann-Whitney U test, Fisher' exact test, Kruskal wallis test.

Logistic regression analysis showed that SVR occurred when serum HCV RNA at 8 week after the initiation of combination therapy was negative (hazard ratio: 40.0; 95% confidence interval =1.75-914.78; p = .021)

the termination of treatment. Clinical backgrounds of these three cases with relapse of HCV RNA after the termination of treatment are shown in Table 3. In case 1 and 2, the attainment time of negativity of serum HCV RNA was 12 weeks after the initiation of treatment. In case 3, the adherence of both drugs of IFN-beta and ribavirin was less than two-third compared to scheduled dose.

Discussion

We have described the efficacy of combination therapy of IFN-beta and ribavirin in patients infected with HCV genotype 2a or 2b. The present study was limited to small size with genotype 2 and HCV-load of ≥100 KIU/mL and high virus load before combination therapy. SVR in the patients with genotype 2 treated with IFN monotherapy for 24 weeks was about 80% in the group with a low virus load and about 40-45% with high virus load (11). Thus, in the present study, we assessed the efficacy of the patients with genotype 2 and a high virus load who showed low rate of SVR. Moreover, 7 of 24 patients did not have a histological examination of the liver within one year before combination

Table A. **Clinical Backgrounds of Patients with Non-SVR**

Case	Age/Sex	genotype	HCV RNA	AST/ALT (IU/L)	response*	Adherence (%)	
						IFN	RBV
1	53/M	2a	220	51/104	12W	104%	100%
2	67/M	2b	5000	30/27	12W	82%	84%
3	51/F	2a	103	50/51	4W	62%	68%

Data are number of patients (percentage) or mean±standard deviation.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; IFN, interferon; RBV, ribavirin

*Response of HCV RNA means attainment time of negativity of serum HCV RNA after the initiation of combination therapy

therapy. Another limitation is that the present study was not a randomized controlled study.

However, several findings from the present study have direct implications for combination therapy for chronic hepatitis C in the future. First, the present results suggest that drop-out rate due to side effects in combination therapy of IFN-beta and ribavirin is low. In the previous study, we have reported that the drop-out rate due to side effects in combination study of peginterferon and ribavirin was 8.1% in 0.5 year after the initiation of treatment and 14.9% in one year (12). In the present study, none of the patients discontinued combination therapy because of IFN-related adverse events.

Secondly, out of 24 patients given the combination therapy, 21 patients had SVR. This SVR rate is similar to that of the 24-week combination therapy of peginterferon and ribavirin reported previously (11-13).

Third, the patients with genotype 2 have the possibility of non-SVR in a regimen for 24-weeks when the attainment time of negativity of serum HCV RNA is longer than 8 weeks after the initiation of combination therapy. This indicates that patients with delayed undetectable HCV RNA should be treated to continue the negativity of serum HCV RNA for a prolonged period of >24 weeks to obtain a high rate of SVR.

IFN-beta should be given intravenously. The intravenous injection is not convenient for treatment compared to intramuscular or subcutaneous injection. However, IFN-beta related side effects are mild and few compared to combination therapy of IFN-alpha and ribavirin (18, 19). Moreover, IFN-beta induced mental disorders are milder than those induced by IFN-alpha (23). Thus, IFN-beta could be given in elderly patients of ≥65 years because of mild side effects (24).

In conclusion, the combination therapy of IFN-beta and ribavirin offers sufficient safety and efficacy in chronic hepatitis C patients with genotype 2 and a high virus load.

Acknowledgement

The present work was supported in part by grants-in-aid from the Japanese Ministry of Health, Labour and Welfare. The authors acknowledge the editorial assistance of Thomas Hughes.

References

1. Manns MP, McHutchison JG, Gordon SC, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet **358**: 958-965, 2001.
2. Fried MW, Shiffman ML, Reddy KR, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med **347**: 975-982, 2002.
3. Hadziyannis SJ, Sette H Jr, Morgan TR, et al; PEGASYS International Study Group. Peginterferon-alpha 2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med **140**: 346-355, 2004.
4. McHutchison JG, Manns M, Patel K, et al. Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. Gastroenterology **123**: 1061-1069, 2002.
5. Shiffman ML, Di Bisceglie AM, Lindsay KL, et al; Hepatitis C Antiviral Long-Term Treatment against Cirrhosis Trial Group. Peginterferon alfa-2a and ribavirin in patients with chronic hepatitis C who have failed prior treatment. Gastroenterology **126**: 1015-1023, 2004.
6. Shiffman ML, Ghany MG, Morgan TR, et al. Impact of reducing peginterferon alfa-2a and ribavirin dose during retreatment in patients with chronic hepatitis C. Gastroenterology **132**: 103-112, 2007.
7. Schalm SW, Weiland O, Hansen BE, et al; Eurohep Study Group on Viral Hepatitis. Interferon-ribavirin for chronic hepatitis C with and without cirrhosis: analysis of individual patient data of six controlled trials. Gastroenterology **117**: 408-413, 1999.
8. Bronowicki JP, Ouzan D, Asselah T, et al. Effect of ribavirin in genotype 1 patients with hepatitis C responding to pegylated interferon alfa-2a plus ribavirin. Gastroenterology **131**: 1040-1048,

2006.
9. Jensen DM, Morgan TR, Marcellin P, et al. Early identification of HCV genotype 1 patients responding to 24 weeks peginterferon alpha-2a (40 kd)/ribavirin therapy. Hepatology **43**: 954-960, 2006.
10. Bruno S, Cammà C, Di Marco V, et al. Peginterferon alfa-2b plus ribavirin for naïve patients with genotype 1 chronic hepatitis C: a randomized controlled trial. J Hepatol **41**: 474-481, 2004.
11. Mamori S, Suzuki F, Hosaka T, et al. Interferon monotherapy for patients with chronic hepatitis C and normal serum aminotransferase levels at commencement of treatment. J Gastroenterol **39**: 776-782, 2004.
12. Dalgard O, Bjøro K, Hellum KB, et al. Treatment with pegylated interferon and ribavarin in HCV infection with genotype 2 or 3 for 14 weeks: a pilot study. Hepatology **40**: 1260-1265, 2004.
13. Mangia A, Santoro R, Minerva N, et al. Peginterferon alfa-2b and ribavirin for 12 vs. 24 weeks in HCV genotype 2 or 3. N Engl J Med **352**: 2609-2617, 2005.
14. von Wagner M, Huber M, Berg T, et al. Peginterferon-alpha-2a (40 KD) and ribavirin for 16 or 24 weeks in patients with genotype 2 or 3 chronic hepatitis C. Gastroenterology **129**: 522-527, 2005.
15. Festi D, Sandri L, Mazzella G, et al. Safety of interferon beta treatment for chronic HCV hepatitis. World J Gastroenterol **10**: 12-16, 2004.
16. Iwasaki Y, Ikeda H, Araki Y, et al. Limitation of combination therapy of interferon and ribavirin for older patients with chronic hepatitis C. Hepatology **43**: 54-63, 2006.
17. Arase Y, Suzuki F, Suzuki Y, et al. Side effects of combination therapy of peginterferon and ribavirin for chronic hepatitis-C. Intern Med **46**: 1827-1832, 2007.
18. Kurosaki M, Enomoto N, Murakami T, et al. Analysis of genotypes and amino acid residues 2209 to 2248 of the NS5A region of hepatitis C virus in relation to the response to interferon-beta therapy. Hepatology **25**: 750-753, 1997.
19. Enomoto M, Tamori A, Kawada N, et al. Interferon-beta plus ribavirin for patients with hepatitis C virus genotype 1: a randomized pilot trial. Gut **55**: 139-140, 2006.
20. Doglio A, Laffont C, Caroli-Bosc FX, et al. Second generation of the automated Cobas Amplicor HCV assay improves sensitivity of hepatitis C virus RNA detection and yields results that are more clinically relevant. J Clin Microbiol **37**: 1567-1569, 1999.
21. Albadalejo J, Alonso R, Antinozzi R, et al. Multicenter evaluation of the COBAS AMPLICOR HCV assay, an integrated PCR system for rapid detection of hepatitis C virus RNA in the diagnostic laboratory. J Clin Microbiol **36**: 862-865, 1998.
22. Dusheiko G, Schmilovitz-Weiss H, Brown D, et al. Hepatitis C virus genotypes: an investigation of type-specific differences in geographic origin and disease. Hepatology **19**: 13-18, 1994.
23. Katamura Y, Suzuki F, Akuta N, et al. Natural human interferon beta plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus and a high viral load. Intern Med **47**: 1827-1834, 2008.
24. Arase Y, Suzuki F, Sezaki H, et al. Suitable treatment period in patients with virological response during combination therapy of peginterferon and ribavirin for chronic hepatitis C. Intern Med **47**: 1301-1307, 2008.

doi:10.1111/j.1440-1746.2009.06161.x

HEPATOLOGY

Efficacy of switching to entecavir monotherapy in Japanese lamivudine-pretreated patients

Fumitaka Suzuki,* Norio Akuta,* Yoshiyuki Suzuki,* Hiromi Yatsuji,* Hitomi Sezaki,* Yasuji Arase,* Miharu Hirakawa,* Yusuke Kawamura,* Tetsuya Hosaka,* Masahiro Kobayashi,* Satoshi Saitoh,* Kenji Ikeda,* Mariko Kobayashi,† Sachiyo Watahiki† and Hiromitsu Kumada*

*Department of Hepatology, Toranomon Hospital, Tokyo, and †Research Institute for Hepatology, Toranomon Branch Hospital, Kawasaki, Japan

Key words
entecavir, hepatitis B virus, lamivudine, viral resistance.

Accepted for publication 6 October 2009.

Correspondence
Dr Fumitaka Suzuki, Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan.
Email: fumitakas@toranomon.gr.jp

Abstract

Background and Aims: To assess the efficacy of switching Japanese chronic hepatitis B patients from lamivudine monotherapy to entecavir 0.5 mg/day.

Methods: A retrospective analysis was conducted on 134 patients switched to entecavir between September 2006 and February 2008 for 6 months or more. Patients were divided into three groups based on viral load at entecavir switching point (baseline < 2.6, 2.6–5.0 and > 5.0 $\log_{10}$ copies/mL).

Results: At baseline, detection of lamivudine-resistant virus was highest in patients with higher hepatitis B virus (HBV) DNA (76% *vs* 23% in ≥ 2.6 and < 2.6 $\log_{10}$ copies/mL, respectively), and in patients with longest previous exposure to lamivudine (52%, 28% and 24% for > 3 years, 1–3 years and < 1 year, respectively). Two years after entecavir switching, HBV DNA suppression to less than 2.6 $\log_{10}$ copies/mL was achieved in 100% (32/32), 92% (12/13) and 44% (4/9) of patients in the less than 2.6, 2.6–5.0 and more than 5.0 $\log_{10}$ copies/mL baseline groups, respectively. Alanine aminotransferase (ALT) normalization occurred in 76–96% and 90–100% of patients following 1 and 2 years of entecavir treatment, respectively. One patient (2.6–5.0 $\log_{10}$ copies/mL) with lamivudine-resistant mutants at baseline developed entecavir resistance at week 48 during follow up.

Conclusion: Switching to entecavir 0.5 mg/day achieves or maintains undetectable HBV DNA levels and ALT normalization over 2 years, especially in patients with viral load less than 5.0 $\log_{10}$ copies/mL.

Introduction

Hepatitis B virus (HBV) infection is a serious public health threat affecting 350–400 million people worldwide, the majority of whom live in the Asia–Pacific region.[1,2] Chronically-infected people are at risk of developing cirrhosis, liver failure and hepatocellular carcinoma. Studies have suggested that high serum HBV DNA is a key risk predictor of chronic hepatitis B (CHB) complications.[3,4] Therefore, the main purpose of CHB therapies is to permanently suppress viral replication and sustain viral suppression to prevent long-term liver damage.[2,5,6]

Lamivudine was the first nucleoside analog to be widely prescribed for CHB patients, mainly due to its antiviral efficacy and safety profile.[2] However, lamivudine's long-term efficacy is diminished by the emergence of drug-resistant substitutions, generally in the tyrosine–methionine–aspartate–aspartate (YMDD) motif of the reverse transcriptase (rt) polymerase gene.[7–9] Detection of lamivudine-resistant HBV substitutions occurs in 15–30% and 70% of patients after 1 and 5 years of treatment, respectively.[8] Continuing lamivudine monotherapy in the presence of lamivudine resistance is not recommended because it is no longer effective in suppressing viral replication.[2] Furthermore, the initial improvement in histology and clinical benefits may be reversed or decreased due to the emergence of lamivudine-resistant substitutions.

Antiviral efficacy of entecavir (0.5 mg/day) as first-line therapy was superior to lamivudine in treatment-naïve patients on all virological, biochemical and histological end-points after 48 weeks of treatment,[10–14] with very low rates of emergence of viral resistance (1.2% after 5 years of entecavir treatment).[15,16] Entecavir has a high genetic barrier to resistance,[17–19] requiring multiple substitutions (including YMDD mutations) to express viral resistance.[16–21] In agreement with this, entecavir-resistant mutants emerge more frequently in lamivudine-refractory patients.[22,23] In a study of hepatitis B e antigen (HBeAg)-positive lamivudine-refractory patients with high HBV DNA levels at baseline (mean > 9 $\log_{10}$ copies/mL), switching to entecavir 1 mg/day achieved HBV DNA suppression to undetectable levels (< 300 copies/mL; 40%, 96 weeks) and alanine aminotransferase (ALT) normalization (81%, 96 weeks) at higher proportions than continued lamivudine

monotherapy,[22] although response to therapy was less pronounced than in treatment-naïve patients with comparable baseline levels of HBV DNA.[10,13,14] The probability of achieving HBV DNA suppression to undetectable levels at 96 weeks with entecavir was 73% in patients whose baseline HBV DNA was less than 7 $\log_{10}$ copies/mL ($n = 11$), and none of these patients developed entecavir resistance.[22]

In a randomized controlled trial of lamivudine-refractory Japanese patients with mean HBV DNA at baseline of 7.6–7.7 $\log_{10}$ copies/mL, switching to entecavir (0.5 or 1 mg/day) for 48 weeks achieved HBV DNA suppression to below detectable levels in 33% of patients in the entecavir dose groups, and ALT normalization in 78–86%.[24] Switching to entecavir in patients with evidence of lamivudine-resistant substitutions and low viral load at switching point has not been prospectively investigated in Japanese patients. There are limited data concerning the efficacy of entecavir in lamivudine-pretreated patients who have not developed lamivudine resistance.

The objective of this study was to assess the efficacy of switching to entecavir 0.5 mg/day in Japanese lamivudine-pretreated patients whose HBV DNA levels at switching point (baseline) ranged from less than 2.6 to 7.6 $\log_{10}$ copies/mL, with or without lamivudine-resistant substitutions.

Methods

Design and setting

A retrospective analysis of a CHB patient population ($n = 134$) at Toranomon Hospital (Tokyo, Japan) was performed to identify patients switched from lamivudine 100 mg/day monotherapy to entecavir 0.5 mg/day between September 2006 and February 2008, and who had received entecavir for at least 6 months. Among all patients selected, only one had a history of adefovir add-on therapy prior to switching to entecavir (case report). Conserved serum from all patients was analyzed to determine baseline characteristics and study end-points.

Study end-points

Clinical efficacy of entecavir was assessed as the proportion of patients achieving HBV DNA suppression to undetectable levels (< 400 copies/mL or < 2.6 $\log_{10}$ copies/mL), and patients achieving ALT normalization (normal ALT levels: men 8–42 IU/L, women 6–27 IU/L). HBV DNA was measured using the polymerase chain reaction (PCR)-based Amplicor HBV Monitor assay (Roche Diagnostics, Indianapolis, IN, USA; lower limit of detection of < 2.6 $\log_{10}$ copies/mL).[25] HBeAg loss in patients who were HBeAg-positive at baseline was also analyzed. Measurements were made from conserved samples taken at baseline, and after 6 months, 1 and 2 years from entecavir treatment initiation.

Assessment of viral resistance

Conserved serum was used to detect the presence of viral lamivudine-resistant rtM204V/I substitutions in all patients at baseline, and following the entecavir switch in patients treated with entecavir for at least 6 months. Lamivudine-resistant virus (rtM204V/I or YMDD motif substitutions) was analyzed using a combination of the quantitative enzyme-linked immunosorbent assay standardized using a purified *Taenia solium* cysticerci fraction (PCR enzyme-linked immunosorbent assay) and the enriched PCR enzyme linked minisequence assay.[26] Direct sequencing of HBV DNA polymerase reverse transcriptase site was also performed.[27] Detection of entecavir-resistant virus was conducted using direct sequencing of HBV DNA polymerase reverse transcriptase site.[27]

Data analyses

Statistical comparisons between treatment groups were assessed using χ^2-test and Kruskal–Wallis test where appropriate. Calculations were performed using StatView software (ver. 4.5J; Abacus Concepts, Berkeley, CA, USA). A two-tailed P-value less than 0.05 was considered statistically significant.

To identify predictive factors of HBV DNA negativity (suppression to below detectable levels) after 6 months of the entecavir switch, univariate and multivariate logistic regression analyses were carried out. Potential predictive factors at baseline included: sex; age; levels of aspartate aminotransferase (AST), ALT, albumin, γ-glutamyl transpeptidase, total bilirubin and α-fetoprotein; platelet count; viral load; liver disease stage (cirrhosis or other); family history; HBV genotype; lamivudine treatment duration prior to entecavir switch; HBeAg status; and lamivudine resistance. Each variable was transformed into categorical data consisting of two simple ordinal numbers. All factors that were at least marginally associated with HBV DNA negativity ($P < 0.10$) were used in a multiple logistic regression analysis. To assess relative risk confidence, odds ratio (OR) and 95% confidence interval (CI) were calculated. All analyses were performed using SPSS II software ver. 11.0 (SPSS, Chicago, IL, USA).

Results

Patient characteristics before switching to entecavir

Lamivudine-pretreated patients switched to entecavir 0.5 mg/day ($n = 134$) were divided into three groups based on their HBV DNA level at the switching point: HBV DNA of less than 2.6 $\log_{10}$ copies/mL ($n = 92$), 2.6–5.0 $\log_{10}$ copies/mL ($n = 25$) and more than 5.0 $\log_{10}$ copies/mL ($n = 17$) (Table 1). Patients with HBV DNA levels of more than 5.0 $\log_{10}$ copies/mL had the highest AST/ALT levels and highest proportion of HBeAg-positive cases ($P < 0.05$). These patients had been treated with lamivudine for the shortest time period compared to patients from the two other groups ($P < 0.05$; Table 1).

Viral resistance to lamivudine at baseline

At baseline, lamivudine-resistant rtM204V/I mutant virus was detected in 23% of patients with HBV DNA of less than 2.6 $\log_{10}$ copies/mL, compared to 76% in each of the HBV DNA 2.6–5.0 $\log_{10}$ copies/mL and more than 5.0 $\log_{10}$ copies/mL groups (Table 2). In all treatment groups, a higher occurrence of resistant virus was observed with longer exposure to lamivudine, independent of viral DNA levels.

Table 1 Patient characteristics at point of switching to entecavir (baseline) and entecavir treatment duration

	All patients	Serum HBV DNA levels by baseline treatment group, log_{10} copies/mL			
		< 2.6	2.6–5.0	> 5.0	*P**
Patients, *n*	134	92	25	17	
Sex, *n* male/female	94/40	67/25	19/6	8/9	0.08
Age, years†	53 (23–83)	53 (27–83)	50 (32–77)	37 (23–77)	0.036
Bilirubin, mg/dL†	0.6 (0.2–3.4)	0.6 (0.2–3.4)	0.6 (0.3–1.8)	0.7 (0.3–1.2)	0.53
AST, IU/L†	24 (13–451)	23 (13–53)	23 (14–50)	37 (14–451)	0.0083
ALT, IU/L†	21 (8–1382)	21 (8–56)	20 (10–111)	46 (9–1382)	0.0002
Albumin, g/dL†	3.9 (2.7–4.8)	3.9 (2.7–4.4)	4.0 (3.3–4.8)	3.9 (3.6–4.6)	0.94
Histology, *n* CH/LC	89/45	56/36	19/6	14/3	0.11
HBeAg, *n* ±	30/104	11/81	5/20	14/3	< 0.0001
HBV DNA, log_{10} copies/mL†	< 2.6 (< 2.6–7.6)	< 2.6	3.9 (2.7–5.0)	6.5 (5.1–7.6)	–
Genotype, *n* A/B/C/unknown	3/9/115/7	2/6/78/6	1/2/22/0	0/1/15/1	0.87
Treatment duration, months†					
Lamivudine	36 (0.5–103)	36 (3–103)	70 (2–89)	17 (0.5–89)	0.009
Entecavir‡	21 (6–33)	20 (6–33)	24 (6–32)	27 (6–33)	0.034

*Comparison of the three patient subgroups using the Kruskal–Wallis test; $P < 0.05$ was considered statistically significant.
†Data are median (range).
‡Entecavir treatment duration is from point of switching.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; HBeAg, hepatitis B early antigen; HBV, hepatitis B virus; LC, liver cirrhosis.

Table 2 rtM204V/I mutant occurrence at baseline of switching to entecavir

	Duration of previous lamivudine treatment, years			All patients
	< 1	1–3	≥ 3	
Baseline treatment group				
< 2.6 log_{10} copies/mL	1/10 (10%)	4/35 (11%)	16/47 (34%)	23%
2.6–5.0 log_{10} copies/mL	1/5 (20%)	3/4 (75%)	15/16 (94%)	76%
> 5.0 log_{10} copies/mL	3/6 (50%)	6/7 (86%)	4/4 (100%)	76%
All patients	24%	28%	52%	–

Clinical efficacy of entecavir 0.5 mg/day

Switching to entecavir 0.5 mg/day for 1 year resulted in HBV DNA suppression to undetectable levels in the majority of patients with HBV DNA below 5.0 log_{10} copies/mL (100% and 96% for HBV DNA < 2.6 and 2.6–5.0 log_{10} copies/mL, respectively) (Table 3). This proportion was slightly decreased when previous lamivudine treatment duration exceeded 3 years in the 2.6–5.0 log_{10} copies/mL group. In the HBV DNA more than 5.0 log_{10} copies/mL group, approximately half (41%) of the patients achieved viral suppression after 1 year (Table 3); entecavir's efficacy seemed to decrease with prolonged previous exposure to lamivudine, with only 25% of patients having more than 3-year lamivudine treatment achieving undetectable viral load. Similarly, after 2 years, HBV DNA suppression was achieved by 100% and 92% of patients in the HBV DNA less than 2.6 and 2.6–5.0 groups, respectively, and by 44% of patients in the HBV DNA more than 5.0 log_{10} copies/mL group (Table 3).

Among those who failed to suppress viral load, only one case of virological breakthrough was found (2.6–5.0 log_{10} copies/mL group; described under case report). This patient had been previously exposed to lamivudine for more than 3 years.

Alanine aminotransferase levels were normalized in 76–96% and 90–100% of patients following 1 and 2 years of entecavir treatment, respectively (Table 3). HBeAg loss was observed in 27% (3/11), 20% (1/5) and 29% (4/14) of patients with HBV DNA of less than 2.6, 2.6–5.0 and more than 5.0 log_{10} copies/mL, respectively, in the first year.

Lamivudine-resistant substitutions in patients switched to entecavir

Of the 130 patients who received entecavir treatment for at least 1 year, 11 cases failed to suppress HBV DNA to below less than 2.6 log_{10} copies/mL and remained HBV DNA-positive in the first year (1 and 10 in the HBV DNA 2.6–5.0 and > 5.0 log_{10} copies/mL groups, respectively; Table 3). Serum HBV DNA analysis confirmed the presence of rtM204V/I substitutions in 10 of these patients, of which six were rtM204I and three were rtM204V substitutions (Table 4); the remaining patient (2.6–5.0 log_{10} copies/mL group; previous lamivudine exposure 5 years) carried a mixed type substitution, rtM204I plus rtM204V. The only HBV DNA-positive patient who did not

Table 3 Clinical efficacy of entecavir 0.5 mg/day in lamivudine-pretreated patients

End-point by baseline treatment group	Duration of entecavir treatment		
	6 months	1 year	2 years
HBV DNA suppression to undetectable levels, *n*/N (%)			
< 2.6 $\log_{10}$ copies/mL	90/92 (98%)	89/89 (100%)	32/32 (100%)
Previous lamivudine < 1 year	10/10 (100)	9/9 (100)	5/5 (100)
Previous lamivudine 1–3 years	35/35 (100)	35/35 (100)	14/14 (100)
Previous lamivudine > 3 years	45/47 (96)	45/45 (100)	13/13 (100)
2.6–5.0 $\log_{10}$ copies/mL	24/25 (96%)	23/24 (96%)	12/13 (92%)
Previous lamivudine < 1 year	5/5 (100)	5/5 (100)	3/3 (100)
Previous lamivudine 1–3 years	4/4 (100)	4/4 (100)	2/2 (100)
Previous lamivudine > 3 years	15/16 (94)	14/15 (93)	7/8 (88)
> 5.0 $\log_{10}$ copies/mL	5/17 (29%)	7/17 (41%)	4/9 (44%)
Previous lamivudine < 1 year	2/6 (33)	3/6 (50)	2/4 (50)
Previous lamivudine 1–3 years	2/7 (29)	3/7 (43)	2/4 (50)
Previous lamivudine > 3 years	1/4 (25)	1/4 (25)	0/1 (0)
ALT normalization, *n*/*n* (%)			
< 2.6 $\log_{10}$ copies/mL	88/92 (96%)	83/89 (93%)	32/32 (100%)
2.6–5.0 $\log_{10}$ copies/mL	24/25 (96%)	23/24 (96%)	12/13 (92%)
> 5.0 $\log_{10}$ copies/mL	14/17 (82%)	13/17 (76%)	9/10 (90%)

ALT, alanine aminotransferase; HBV, hepatitis B virus.

Table 4 HBV DNA positive rates in patients switched to entecavir 0.5 mg/day for at least 1 year

	HBeAg status	YMDD motif substitution	HBV DNA positive rate, *n*/N (%)	Duration of previous lamivudine treatment, years per patient
Baseline treatment group				
< 2.6 $\log_{10}$ copies/mL	Positive	Wild (or none)	0/10 (0%)	n/a
		YIDD	0/1 (0%)	n/a
	Negative	Wild (or none)	0/58 (0%)	n/a
		YIDD	0/15 (0%)	n/a
		YVDD	0/4 (0%)	n/a
		YIDD + YVDD	0/1 (0%)	n/a
2.6–5.0 $\log_{10}$ copies/mL	Positive	Wild (or none)	0/4 (0%)	
		YIDD + YVDD	1/1 (100%)†	5.0
	Negative	Wild (or none)	0/2 (0%)	n/a
		YIDD	0/10 (0%)	n/a
		YVDD	0/6 (0%)	n/a
		YIDD + YVDD	0/1 (0%)	n/a
> 5.0 $\log_{10}$ copies/mL	Positive	Wild (or none)	1/4 (25%)	0.2
		YIDD	6/9 (67%)	0.5; 1.3; 1.5; 2.7; 3.9; 7.4
		YVDD	1/1 (100%)	0.7
	Negative	YIDD	0/1 (0%)	n/a
		YVDD	2/2 (100%)	1.8; 4.5
All patients			11/130 (8%)	

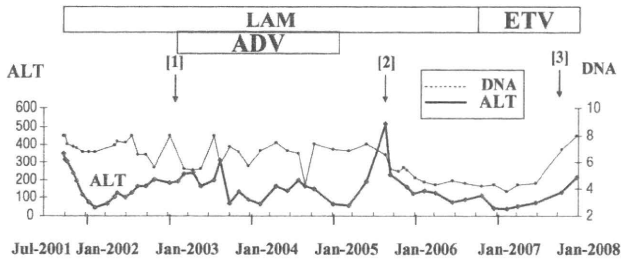
YMDD motif substitutions: wild, rt204M; YIDD, rt204I; YVDD, rt204V; YIDD + YVDD, rt204I + rt204V.
†Patient with lamivudine-resistant HBV who developed entecavir resistance.
HBeAg, hepatitis B early antigen; HBV, hepatitis B virus; n/a, not available.

carry any detectable lamivudine-resistant substitution had the shortest previous lamivudine exposure (< 6 months; Table 4).

Of the 10 patients carrying rtM204V/I substitutions, eight were HBeAg-positive; the other two patients were HBeAg-negative and carried a lamivudine-resistant rtM204V type substitution.

Emergence of entecavir-resistant mutant: case report

One patient (2.6–5.0 $\log_{10}$ copies/mL group) carrying a mixed substitution YIDD + YVDD (rtM204I + rtM204V) developed entecavir resistance with a recognized rtS202G substitution



Amino acid (reverse transcriptase)

	180	202 204	221 223 229
AB014394	SPFLLAQFTSAI	CLAFSYMDDVVLG	FTSVTNFLLS
[1] February 2003	----------	----------	----------
[2] August 2005	----M-------	------V------	----------
	----------	------I------	----------
[3] October 2007	----M-------	----G-V------	Y-A-----V-

Figure 1 Clinical course and evolution of viral polymerase reverse transcriptase gene sequence in a patient with confirmed rtM204V/I substitutions (YIDD + YVDD) and emerging entecavir resistance substitution (rtS202G). AB014394 was a strain reported by Takahashi *et al.*[33] Two kinds of strains emerged in August 2005 (rtL180M/rtM204V and rtM204I). In October 2007, an additional amino acid substitution (rtS202G) was detected. ADV, adefovir; ALT, alanine aminotransferase; ETV, entecavir; LAM, lamivudine. — DNA; — ALT.

(Table 4). Figure 1 describes the clinical course and evolution of viral DNA sequence. This 37-year-old Japanese man was found to be seropositive for hepatitis B surface antigen with mild ALT elevation in December 1998. He was diagnosed with CHB by peritoneoscopy and liver biopsy (mild hepatitis [A1] and mild fibrosis [F1]). HBeAg was positive; serum HBV DNA was more than 7.6 $\log_{10}$ copies/mL. Treatment with lamivudine 100 mg/day was initiated in October 2001, at which time serum HBV DNA was more than 7.6 $\log_{10}$ copies/mL and ALT was 314 IU/L. In February 2003, adefovir dipivoxil 10 mg/day was added-on to lamivudine, but failed to decrease HBV DNA load. In January 2005, adefovir was withdrawn; the patient remained on lamivudine monotherapy. Amino acid substitutions of the rt gene, rtL180M, rtM204V and rtM204I were detected in August 2005. In October 2006, the patient was switched directly from lamivudine to entecavir 0.5 mg/day without treatment interruption. In February 2007, ALT levels decreased to within normal values, and serum HBV DNA was less than 4 $\log_{10}$ copies /mL. However, shortly after, both ALT levels and HBV DNA began to rise again. In October 2007, amino acid substitutions rtL180M, rtM204V and rtS202G were detected.

Predictive factors of HBV DNA negativity

Univariate analyses identified six factors that correlated with HBV DNA suppression to undetectable levels after 6 months of the entecavir switch: viral load less than 5 $\log_{10}$ copies/mL ($P < 0.001$); HBeAg-negative status ($P < 0.001$); the absence of lamivudine resistance ($P < 0.001$); normal AST level (≤ 33 IU/L; $P = 0.008$); normal ALT level (men ≤ 42 IU/L, women ≤ 27 IU/L; $P < 0.001$); and chronic hepatitis stage of liver disease ($P = 0.069$). Multivariate analyses showed that viral load below 5 $\log_{10}$ copies/mL (OR = 69.03; 95% CI = 13.23–360.09; $P < 0.001$) and the absence of lamivudine resistance (OR = 8.17; 95% CI = 1.25–53.34; $P = 0.028$) each independently influenced entecavir's efficacy to suppress HBV DNA to undetectable levels after 6 months.

Discussion

Entecavir is recommended as a first-line CHB treatment by all major guidelines, due to its antiviral potency and high genetic barrier to resistance in nucleos(t)ide-naïve patients.[2,5,6] Conversely, in lamivudine-resistant patients, switching to entecavir is not a first-choice treatment, due to increased risk of emergence of entecavir resistance on a multiple substitution background.[22,23] However, in attempts to rescue those with suboptimal antiviral response and also to avoid the emergence of viral resistance in responsive patients during their treatment course, switching to entecavir is recommended by the Japanese Ministry of Health, Welfare and Labor for lamivudine-pretreated patients with undetectable viral load (< 2.6 $\log_{10}$ copies/mL), and for patients with detectable HBV DNA but without biochemical breakthrough and lamivudine resistance.[28] This study provides a unique opportunity to evaluate the efficacy of entecavir in a lamivudine-pretreated population with low viral load at switching point.

The majority of patients with HBV DNA at baseline of less than 5 $\log_{10}$ copies/mL maintained or achieved viral suppression 1 year after switching to entecavir, despite 23–76% of them carrying lamivudine-resistant substitutions. A similar trend was maintained during the second year. Conversely, viral suppression below detection limits was reported in less than half of patients with high viral load at baseline (HBV DNA 5.1–7.6 $\log_{10}$ copies/mL) carrying rtM204V/I substitutions (76% patients), in agreement with earlier studies showing diminished entecavir efficacy in lamivudine-refractory patients with elevated viral load.[22,23,29] In addition, multivariate analyses revealed that a viral load of less than 5 $\log_{10}$ copies/mL was an independent predictive factor of HBV DNA suppression to undetectable levels, after 6 months of entecavir therapy. Taken together, these data suggest that switching to entecavir is mostly efficacious in patients with low viral load regardless of the presence of rtM204V/I substitutions. This observation adds another perspective in predicting clinical response to entecavir in lamivudine-pretreated patients.

Another predictive factor of entecavir's efficacy in this retrospective cohort is the absence of lamivudine resistance. This is consistent with previous research suggesting decreased genetic barrier of entecavir to resistance in the presence of lamivudine-resistant substitutions.[22,23] The responsiveness of lamivudine-resistant patients with low viral load reported here could be explained by the ability of entecavir to clear low loads of rtM204V/I mutants. This is suggested by *in vitro* data showing maintained sensitivity of lamivudine-resistant mutants to entecavir, although at higher EC_{50}. Assessing the kinetics of rtM204V/I mutants in response to entecavir switching in patients with undetectable viral load is worth further characterization.

Previous studies have shown that developing entecavir resistance is higher in the presence of pre-existing lamivudine-resistant substitutions.[16–21,30] Despite the presence of lamivudine-resistant virus in 23%–76% of all patient groups, the emergence of entecavir resistance was rare, with only one confirmed case from the

HBV DNA 2.6–5.0 $\log_{10}$ copies/mL group. This patient's history is suggestive of a typical refractory case, with failure of multiple regimens including the combination of lamivudine plus adefovir (Fig. 1). The low entecavir resistance rate in this study may be due to the relatively short treatment period and small sample size. Further follow up will be required to monitor for subsequent emergence of entecavir resistance in these patients.

One could argue whether it is cost-effective to switch all lamivudine-treated patients with undetectable HBV DNA to entecavir. The GLOBE study demonstrated that although fewer lamivudine-treated patients with undetectable HBV DNA at week 24 developed viral resistance, resistance could still occur after 2 years of treatment (9% and 5% of HBeAg-positive and HBeAg-negative patients, respectively).[31] Moreover, Yuen and collaborators also reported that of lamivudine-treated patients who achieved HBV DNA suppression below 200 copies/mL at week 24, 8.3% developed resistance after 5 years.[32] In countries where medicine access is an issue, further studies are needed to evaluate the cost-effectiveness of entecavir switching of all patients with undetectable viral load, versus switching only those at risk of developing viral resistance. Comparative studies integrating the efficacy and safety of standard adefovir add-on versus switching to entecavir monotherapy are also warranted in these patients.

Study limitations should be considered. This is a retrospective analysis of CHB patients which, in the absence of matching controls, may introduce confounding errors and bias. Specifically, a control arm for the HBV PCR-negative group (< 2.6 $\log_{10}$ copies/mL; $n = 92$) would be required to strengthen study conclusions. Another limitation is the small sample size of the intermediate and high HBV DNA cohorts (25 patients with 2.6–5.0 $\log_{10}$ copies/mL, and 17 patients with > 5.0 $\log_{10}$ copies/mL, respectively); adding more patients to these samples as available would add weight to describing higher number entecavir response and resistance rates in these groups.

In conclusion, this study shows that the efficacy of switching from lamivudine to entecavir 0.5 mg/day is highest for Japanese patients with no rtM204V/I substitutions and a viral load of less than 5 $\log_{10}$ copies/mL, independent of their previous exposure to lamivudine. Efficacy is decreased for patients with rtM204V/I substitutions and low viral load, and is lowest for patients with rtM204V/I substitutions and high viral load. Viral resistance to entecavir after 48 weeks is rare in these patients. Multivariate analyses showed that viral load of less than 5 $\log_{10}$ copies/mL and the absence of lamivudine resistance are independent factors predicting entecavir's efficacy to reduce HBV DNA to undetectable levels after 6 months of treatment.

Acknowledgments

This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan. Editorial support for the manuscript was provided by BioMedCom Consultants.

References

1 Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J. Viral Hepat.* 2004; **11**: 97–107.
2 Liaw Y-F, Leung N, Kao J-H *et al.* Asian-Pacifc consensus statement on the management of chronic hepatitis B: a 2008 update. *Hepatol. Int.* 2008; **2**: 263–83.
3 Chen CJ, Yang HI, Su J *et al.* Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* 2006; **295**: 65–73.
4 Iloeje U, Yang HI, Su J, Jen CL, You SL, Chen CJ. Predicting liver cirrhosis risk based on the level of circulating hepatitis B viral load. *J. Gastroenterol.* 2006; 678–86.
5 Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507–39.
6 European Association for the Study of the Liver. EASL clinical practice guidelines: management of chronic hepatitis B. *J. Hepatol.* 2009; **50**: 227–42.
7 Locarnini S, Mason WS. Cellular and virological mechanisms of HBV drug resistance. *J. Hepatol.* 2006; **44**: 422–31.
8 Lok AS, Zoulim F, Locarnini S *et al.* Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. *Hepatology* 2007; **46**: 254–65.
9 Suzuki F, Tsubota A, Arase Y *et al.* Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. *Intervirology* 2003; **46**: 182–9.
10 Chang TT, Gish RG, de Man R *et al.* A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N. Engl. J. Med.* 2006; **354**: 1001–10.
11 Gish RG, Lok AS, Chang TT *et al.* Entecavir therapy for up to 96 weeks in patients with HBeAg-positive chronic hepatitis B. *Gastroenterology* 2007; **133**: 1437–44.
12 Lai CL, Shouval D, Lok AS *et al.* Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. *N. Engl. J. Med.* 2006; **354**: 1011–20.
13 Ren FY, Piao DM, Piao XX. A one-year trial of entecavir treatment in patients with HBeAg-positive chronic hepatitis B. *World J. Gastroenterol.* 2007; **13**: 4264–7.
14 Schiff E, Simsek H, Lee WM *et al.* Efficacy and safety of entecavir in patients with chronic hepatitis B and advanced hepatic fibrosis or cirrhosis. *Am. J. Gastroenterol.* 2008; **103**: 1–8.
15 Colonno RJ, Rose R, Baldick CJ *et al.* Entecavir resistance is rare in nucleoside naive patients with hepatitis B. *Hepatology* 2006; **44**: 1656–65.
16 Tenney DJ, Rose RE, Baldick CJ, Pokornowski K, Eggers BJ. Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naïve patients is rare through 5 years of therapy. *Hepatology* 2009; **49**: 1503–14.
17 Baldick CJ, Eggers BJ, Fang J *et al.* Hepatitis B virus quasispecies susceptibility to entecavir confirms the relationship between genotypic resistance and patient virologic response. *J. Hepatol.* 2008; **48**: 895–902.
18 Tenney DJ, Rose RE, Baldick CJ *et al.* Two-year assessment of entecavir resistance in Lamivudine-refractory hepatitis B virus patients reveals different clinical outcomes depending on the resistance substitutions present. *Antimicrob. Agents Chemother.* 2007; **51**: 902–11.
19 Tenney DJ, Levine SM, Rose RE *et al.* Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. *Antimicrob. Agents Chemother.* 2004; **48**: 3498–507.
20 Nagasaki F, Niitsuma H, Ueno Y *et al.* The high incidence of the emergence of entecavir-resistant mutants among patients infected with lamivudine-resistant hepatitis B virus. *Tohoku J. Exp. Med.* 2007; **213**: 181–6.
21 Villet S, Ollivet A, Pichoud C *et al.* Stepwise process for the development of entecavir resistance in a chronic hepatitis B virus infected patient. *J. Hepatol.* 2007; **46**: 531–8.

22 Sherman M, Yurdaydin C, Simsek H *et al.* Entecavir therapy for lamivudine-refractory chronic hepatitis B: improved virologic, biochemical, and serology outcomes through 96 weeks. *Hepatology* 2008; **48**: 99–108.
23 Sherman M, Yurdaydin C, Sollano J *et al.* Entecavir for treatment of lamivudine-refractory, HBeAg-positive chronic hepatitis B. *Gastroenterology* 2006; **130**: 2039–49.
24 Suzuki F, Toyoda J, Katano Y *et al.* Efficacy and safety of entecavir in lamivudine-refractory patients with chronic hepatitis B: randomized controlled trial in Japanese patients. *J. Gastroenterol. Hepatol.* 2008; **23**: 1320–6.
25 Matsuyama K, Hayashi K, Miura T. The quantitative assay for HBV-DNA and the detection of HBV-DNA point mutation by polymerase chain reaction—'AMPLICOR HBV MONITOR Test' and 'HBV pre Core/Core Promoter Mutation Detection kit. *Kan Tan Sui* 2000; **41**: 59–71.
26 Kobayashi S, Shimada K, Suzuki H, Tanikawa K, Sata M. Development of a new method for detecting a mutation in the gene encoding hepatitis B virus reverse transcriptase active site (YMDD motif). *Hepatol. Res.* 2000; **17**: 31–42.
27 Suzuki F, Kumada H, Nakamura H. Changes in viral loads of lamivudine-resistant mutants and evolution of HBV sequences during adefovir dipivoxil therapy. *J. Med. Virol.* 2006; **78**: 1025–34.
28 Kumada H. *Scientific Research Grant of Ministry of Health, Labour and Welfare. Research of hepatitis overcome urgent strategy. Research report of the standardization of viral hepatitis treatment including liver cirrhosis* (Japanese version). 2009.
29 Chang TT, Gish RG, Hadziyannis SJ *et al.* A dose-ranging study of the efficacy and tolerability of entecavir in Lamivudine-refractory chronic hepatitis B patients. *Gastroenterology* 2005; **129**: 1198–209.
30 Kobashi H, Fujioka S-I, Kumada H, Yokosuka O, Hayashi N, Suzuki K. Emergence of hepatitis B virus gene mutation related to entecavir-resistance in chronic hepatitis B patients participated in the phase 2 clinical studies of entecavir in Japan. *Hepatology* 2007; **46** (Suppl. 1): 666A.
31 Lai CL, Gane E, Liaw YF *et al.* Telbivudine versus lamivudine in patients with chronic hepatitis B. *Hepatology* 2006; **44**: 222A.
32 Yuen MF, Fong DY, Wong DK, Yuen JC, Fung J, Lai CL. Hepatitis B virus DNA levels at week 4 of lamivudine treatment predict the 5-year ideal response. *Hepatology* 2007; **46**: 1695–703.
33 Takahashi K, Akahane Y, Hino K, Ohta Y, Mishiro S. Hepatitis B virus genomic sequence in the circulation of hepatocellular carcinoma patients: comparative analysis of 40 full-length isolates. *Arch Virol* 1998; **143**: 2312–26.

Liver INTERNATIONAL
OFFICIAL JOURNAL OF THE INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER

Liver International ISSN 1478-3223

CLINICAL STUDIES

HBcrAg is a predictor of post-treatment recurrence of hepatocellular carcinoma during antiviral therapy

Tetsuya Hosaka[1], Fumitaka Suzuki[1], Masahiro Kobayashi[1], Miharu Hirakawa[1], Yusuke Kawamura[1], Hiromi Yatsuji[1], Hitomi Sezaki[1], Norio Akuta[1], Yoshiyuki Suzuki[1], Satoshi Saitoh[1], Yasuji Arase[1], Kenji Ikeda[1], Mariko Kobayashi[2] and Hiromitsu Kumada[1]

1 Department of Hepatology, Toranomon Hospital, Tokyo, Japan
2 Research Institute for Hepatology, Toranomon Hospital, Tokyo, Japan

Keywords
covalently closed circular DNA – HBcrAg – HCC recurrence nucleot(s)ide analogue – portal vein invasion

Correspondence
Tetsuya Hosaka, Department of Hepatology, Toranomon Hospital, 1-3-1 Kajigaya, Takatsu-ku, Kawasaki City 213 8587, Tokyo, Japan
Tel: +81 44 877 5111
Fax: +81 44 860 1623
e-mail: hosa-p@toranomon.gr.jp

Received 13 February 2010
Accepted 15 August 2010

DOI:10.1111/j.1478-3231.2010.02344.x

Abstract

Background/Aims: The recurrence rate of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) is high even in patients receiving curative therapy. In this study, we analysed the risk factors for tumour recurrence after curative therapy for HBV-related HCC while under treatment with nucleot(s)ide analogues (NAs) by measuring serum HBcrAg and intrahepatic covalently closed circular DNA (cccDNA) levels to elucidate the viral status associated with HCC recurrence. *Methods:* We enrolled 55 patients who developed HCC during NA therapy and underwent either curative resection or percutaneous ablation for HCC. *Results:* Hepatocellular carcinoma recurred in 21 (38%) of the patients over a period of 2.2 (range, 0.2–7.4) years. In multivariate analysis, serum HBcrAg levels $\geq$4.8log U/ml at the time of HCC diagnosis (hazard ratio, 8.96; 95% confidential interval, 1.94–41.4) and portal vein invasion (3.94, 1.25–12.4) were independent factors for HCC recurrence. The recurrence-free survival rates of the high cccDNA group were significantly lower than those of the low cccDNA group only in patients who underwent resection ($P = 0.0438$). A positive correlation ($P = 0.028$; $r = 0.479$) was observed between the intrahepatic cccDNA and the serum HBcrAg levels at the incidence of HCC. *Conclusion:* HBcrAg is a predictor of the post-treatment recurrence of HCC during antiviral therapy. Serum HBcrAg and intrahepatic cccDNA suppression by NAs may be important to prevent HCC recurrence.

Worldwide, an estimated 400 million people are infected with hepatitis B virus (HBV) persistently, and one million people die of decompensated cirrhosis and/or hepatocellular carcinoma (HCC) annually (1, 2). Recently, oral nucleot(s)ide analogues (NAs) have been used as the mainstay therapeutic strategy against chronic hepatitis B. Five such antiviral agents have been approved, and range in the profundity and rapidity of HBV DNA suppression, barrier to resistance and side-effect profile (3–10). Lamivudine (LAM) was the first NA to be approved for treating chronic hepatitis B, followed by adefovir dipivoxil (ADV) and entecavir (ETV), in Japan. However, a major problem with long-term LAM treatment is the potential development of drug resistance, mainly caused by mutation of the thyrosine–methionine–aspartic acid–aspartic acid (YMDD) motif of reverse transcriptase (11, 12). For preventing breakthrough hepatitis induced by LAM-resistant mutants, additional ADV administration has been recommended (13, 14).

The methods for monitoring the treatment response include measurements of the serum alanine transaminase (ALT) levels, HBV DNA levels, HBeAg and antibody levels, HBsAg and antibody levels and liver histology. Other serum markers have been reported to be useful for monitoring the effect of antiviral therapy (15, 16). Recently, a new assay was developed for detecting the HBcrAg, consisting of HBcAg, HBeAg and a 22 kDa precore protein coded with the precore/core gene (17, 18). Because NAs have no inhibiting action on the transcription and translation activities of viral mRNA, HBcAg- and HBeAg-related proteins continue to be produced for a certain period of time in spite of the achievement of adequate suppression of the viral DNA synthesis. Therefore, HBcrAg is a viral marker independent of HBV DNA for monitoring the antiviral effect of NAs (19). In addition, recent reports have indicated another interesting aspect of serum HBcrAg levels: these levels were found to be correlated with intrahepatic covalently closed circular DNA (cccDNA) levels and could be a surrogate marker of the intrahepatic cccDNA pool (20, 21). This phenomenon may be explained by the fact that the production of HBcrAg depends on the

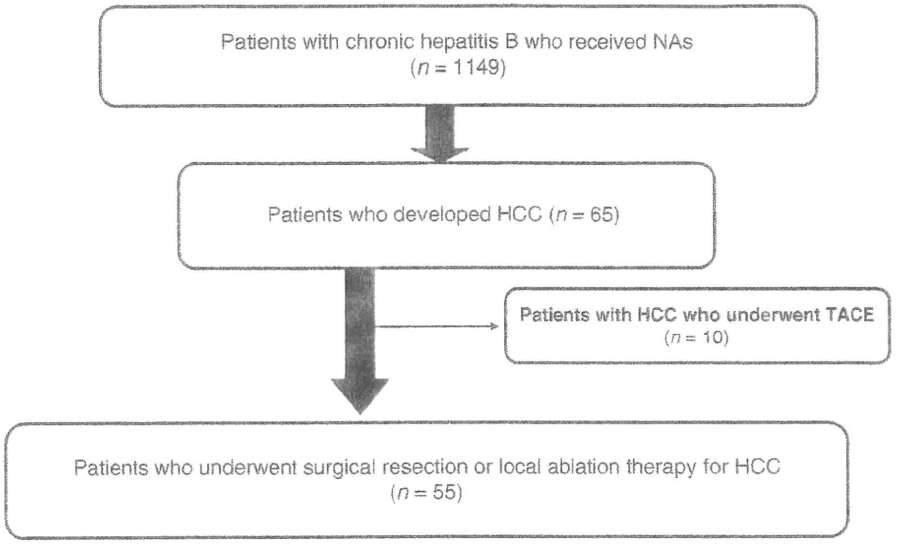


Fig. 1. The study protocol. HCC, hepatocellular carcinoma; NAs, nucleot(s)ide analogues; TACE, transcatheter arterial chemoembolization.

transcription of mRNA from cccDNA, and that cccDNA still remains in high levels during treatment with NAs.

Although patients with HBV-related cirrhosis have a significantly high risk of developing HCC, NA therapy can delay the progression of liver disease and reduce the risk of HCC in patients with cirrhosis by strong viral suppression (22, 23). Nevertheless, a few cases develop HCC during NA therapy at a constant rate (3–12%) (22, 24–26). The recurrence rate of HBV-related HCC after curative resection is estimated to be high, and is associated with viral factors, including HBeAg positivity and the viral load before surgery, besides host and tumour factors, but these findings were demonstrated in the absence of antiviral therapy (27–30). However, almost all patients, receiving NAs, showed negativity of serum HBV DNA. And so, we made the hypothesis that intrahepatic viral status, such as intrahepatic cccDNA and serum HBcrAg levels of its surrogate maker, might have an impact on tumour recurrence during NA therapy.

In this study, we examined the risk factors for tumour recurrence after curative resection and ablation for HBV-related HCC during NA therapy by measuring the serum HBcrAg and intrahepatic cccDNA levels with the aim to elucidate the viral status, persistent despite suppressive therapy, associated with HCC recurrence, in addition to the host and tumour factors reported in the past.

Patients and methods

Patients

Over a period of 13 years, from September 1995 to September 2008, 1149 patients with chronic hepatitis B received NA therapy, including LAM, ADV and ETV, at the Department of Hepatology, Toranomon Hospital, Metropolitan Tokyo. Of the 1149 patients, 65 developed HCC after the start of NA therapy from February 2001 to June 2009. Of the 65 consecutive patients, 55 underwent radical therapy, including either resection or percutaneous ablation as the initial therapy for HCC. These 55 patients were enrolled in this cohort study (Fig. 1). The median duration from the start of NA therapy to the development of HCC was 2.2 (range, 0.2–7.4) years. The exclusion criteria were (i) patients co-infected with hepatitis C, delta or human immunodeficiency virus and (ii) a history of other liver diseases such as autoimmune hepatitis, alcoholic liver disease or metabolic liver disease.

The diagnosis of HCC was predominantly based on imaging, including dynamic computed tomography, magnetic resonance imaging and/or digital subtraction angiography. When the hepatic nodule did not show the typical imaging features, fine needle aspiration biopsy was performed, followed by histological examination and diagnosis. The physicians and surgeons usually discussed the preferred choice of treatment for each patient. Hepatic resection was mainly performed for patients categorized as Child–Pugh grade A or B liver function, and had no serious complications. Percutaneous ablation was performed for patients with surgical contraindications or for those who did not prefer to undergo hepatic resection by using two different devices: the cool-tip system (Tyco Healthcare Group LP, Burlington, VT, USA) and the radiofrequency tumour coagulation system (RTC system; Boston-Scientific Japan Co., Tokyo, Japan). The term curative treatment was used to indicate that no tumours were left in the remnant liver, irrespective of the width of the margin around the tumour, confirmed using intra-operative ultrasonography, combined ultrasonography and dynamic computed tomography 1 month after the resection or ablation. Serum samples were collected from all patients before and after

the treatment for HCC and stored in −80 °C. Liver tissue from patients who underwent resection was collected, rapidly frozen and stored in −80 °C. Written informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in *a priori* approval by the institution's human research committee.

Antiviral therapy

Forty-seven patients received 100 mg LAM daily, and drug-resistant YMDD mutants developed in 26 (55%) of these patients, accompanied by an increase in HBV DNA ≥1log copies/ml. Seventeen of the 26 patients received 10 mg ADV in addition to LAM (100 mg) daily. The remaining nine continued to receive LAM monotherapy because of the lack of approval for ADV administration in Japan at the time, but received ADV with LAM after approval was obtained during the HCC post-treatment period. Eight NA-naïve patients received 0.5 mg ETV daily. These antiviral therapies were continued after the resection or percutaneous ablation.

Follow-up and HCC recurrence

The patients were followed for liver function and virological markers of HBV infection monthly, as well as blood counts and tumour makers including α-fetoprotein and des-γ-carboxylprothrombin. They also underwent ultrasonography or helical dynamic computed tomography every 3 months. Cirrhosis was diagnosed by laparoscopy or liver biopsy or by the clinical data, imaging modalities and portal hypertension. The median observation period after HCC treatment for the entire cohort was 2.7 years (range, 0.3–8.4 years). HCC recurrence was diagnosed by the typical hypervascular characteristics on angiography and/or histological examination with fine needle biopsy specimens, in addition to certain features on computed tomography and ultrasonography.

Markers of HBV infection

HBeAg was determined by enzyme-linked immunosorbent assay using a commercial kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). HBV DNA was quantitated using the Amplicor monitor assay (Roche Diagnostics, Tokyo, Japan) with a dynamic range over 2.6–7.6log copies/ml or COBAS TaqMan HBV v.2.0 (Roche Diagnostics) with a dynamic range over 2.1–9.0log copies/ml. Serum HBV DNA levels were measured using the Amplicor assay at both the start of NA therapy and the diagnosis of HCC and using the TaqMan assay at the diagnosis of HCC. For statistical analysis, the value of that HBV DNA was tentatively set at 2.1 if HBV DNA levels were under 2.1log copies/ml. HBV genotypes were determined serologically by the combination of epitopes expressed on the pre-S2 region product, which is specific for each of the seven major genotypes (A–G), using a commercial kit (HBV Genotype EIA; Institute of Immunology). YMDD mutants were determined by polymerase chain reaction-based enzyme-linked minisequence assay using a commercial kit (Genome Science Laboratories, Tokyo, Japan).

HBcrAg measurement

Serum HBcrAg levels were measured using a CLEIA HBcrAg assay kit (Fujirebio Inc., Tokyo, Japan) with a fully automated analyser system (Lumipulse System; Fujirebio Inc.) as described previously (21). In brief, 150 µl of serum was incubated with 150 µl of pretreatment solution containing 15% sodium dodecyl sulphate at 60 °C for 30 min. After heat treatment, 120 µl of pretreated specimen was added to a ferrite microparticle suspension in an assay cartridge. Ferrite particles were coated with monoclonal antibody mixture (HB44, HB61 and HB114) against denatured HBcAg, HBeAg and the 22 kDa precore protein. After 10 min of incubation at 37 °C and washing, further incubation was carried out for 10 min at 37 °C with alkaline phosphatase conjugated with two kinds of monoclonal antibodies (HB91 and HB110) against denatured HBcAg, HBeAg and the 22 kDa precore protein. After washing, 200 µl of substrate solution [3-(2′-spiroada-mantan)-4-methoxy-4-(3′-phosphoryloxy)pheny1-1,2-dioxetane disodium salt] (Applied Biosystems, Bedford, MA, USA) was added to the test cartridge, which was then incubated for 5 min at 37 °C. The relative chemiluminescence intensity was measured, and the HBcrAg concentration was calculated by a standard curve generated using a recombinant pro-HBeAg (amino acids −10 to 183 of the precore/core gene product). The HBcrAg concentration was expressed in U/ml, which is defined as the immunoreactivity of 10 fg/ml of recombinant pro-HBeAg. In this study, the HBcrAg values were expressed as log U/ml, and the cut-off value was set at 3.0 log U/ml. For the statistical analyses, HBcrAg-negative cases were calculated as 3.0log U/ml.

Intrahepatic cccDNA measurement

Intrahepatic cccDNA levels were analysed as described previously (21). In brief, liver specimens surrounding the tumour tissue were obtained and stored at −80 °C before DNA extraction. HBV DNA was extracted using a QIAamp DNA Mini Kit (Qiagen KK, Tokyo, Japan). The concentration of purified DNA was based on the absorbance at 260 nm. For this study, two oligonucleotide primers cccF2 (5′-cgtctgtgccttctcatctga-3′, nucleotides 1424–1444) and cccR4 (5′-gcacagcttggaggcttgaa-3′, nucleotides 1755–1737) and probe cccP2 (5′-VIC-accaatttat gcctacag-MGB-3′, nucleotides 1672–1655) were designed using PRIMER EXPRESS software (Applied Biosystems, Foster City, CA, USA) to flank the direct repeat region between the hepatitis B core and the polymerase gene. The use of cccF2 and cccR4, oligonucleotide primers spanning the direct repeat region of the HBV genome, allows the polymerase chain reaction of native viral DNA in the

Dane particle to block the amplification of products, because the partially double-stranded HBV DNA is disrupted in the direct repeat region. Twenty-five microlitres of extracted DNA (0.5 μg) was detected with the sequence detector system (ABI 7900HT; Applied Biosystems) in 50 μl of a PCR mixture containing TaqMan universal PCR Master Mix (Applied Biosystems), 300 nmol of each primer and 250 nmol of the probe. After initial activation of uracil-*N*-glycosylase at 50 °C for 2 min, AmpliTaq Gold (Applied Biosystems) was activated at 95 °C for 10 min. The subsequent PCR conditions consisted of 45 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 90 s per cycle (SRL Inc., Tokyo, Japan).

Statistical analyses

Standard statistical measures and procedures were used. Correlations between two variables were tested using Pearson's correlation analysis. Cox regression analysis was used to assess significant associations of the risk factors with tumour recurrence after HCC treatment. All factors found to be at least associated with recurrence ($P < 0.05$) were tested by multivariate analysis. Independent factors, associated with HCC recurrence, were calculated using stepwise Cox regression analysis. The cumulative recurrence-free survival rates after HCC treatment were analysed using the Kaplan–Meier method, and differences in the curves were tested using the log-rank test. A P value of < 0.05 in a two-tailed test was considered significant. Data analysis was performed with SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics at the start of NA therapy and HCC incidence

Table 1 presents a comparison of the patient characteristics at the start of NA therapy and the time of HCC diagnosis. Almost all the patients (93%) enrolled in this study had HBV genotype C. One patient had genotype B, and the genotypes of three patients could not be determined. The rate of HBV DNA disappearance from serum in all the patients was 64% (35/55; Amplicor monitor assay, < 2.6log copies/ml) and 51% (28/55; TaqMan assay, < 2.1log copies/ml), that of aspartate aminotransferase (AST) normalization (< 32 IU/L) was 56% (31/55) and that of ALT normalization (< 42 IU/L) was 71% (39/55) at the incidence of HCC. YMDD mutants were detected in 30 of 47 patients at the beginning of LAM monotherapy, and virological breakthrough (VBT), accompanied by an increase in HBV DNA (≥ 1log copies/ml), occurred in 26 patients with YMDD mutants by the diagnosis of HCC. Seventeen of these patients received ADV with LAM. No resistant mutation to ADV (rtA181T/S, rtN236T) occurred in patients receiving the combination therapy. Further, no drug-resistant mutant

Table 1. Patient characteristics at the start of nucleot(s)ide analogue therapy and the incidence of hepatocellular carcinoma

Characteristics	Start of NA therapy	Time of HCC Dx
Age (years)	51 (32–73)	54 (35–75)
Gender (male:female)	45:10	45:10
AST level (IU/L)	69 (27–195)	31 (16–207)
ALT level (IU/L)	78 (23–368)	29 (10–267)
Platelet count (10^5/mm^3)	11.4 (3.1–31.3)	12.9 (3.6–30.1)
Serum albumin level (g/dl)		3.8 (3.1–4.4)
Serum bilirubin level (mg/dl)		0.9 (0.4–2.4)
Prothrombin time (%)		90.8 (59–112)
Indocyanine green retention rate at 15 min (%)		14.5 (4–53)
Child–Pugh (A:B)		49:6
HBV genotype		
C	51 (93%)	51 (93%)
Others	4	4
HBeAg (+)	29 (53%)	23 (42%)
HBV DNA (log copies/ml)	7.1 (< 2.6 to > 7.6)	< 2.1 (< 2.1 to 8.5)
HBcrAg level (log U/ml)	6.6 (3.3 to > 6.8)	5.0 (< 3.0 to > 6.8)
Antiviral agents (LAM:LAM + ADV:ETV)	47:0:8	30:17:8
Duration of NA therapy before the incidence of HCC (years)		2.2 (0.2–7.4)
α-fetoprotein level (ng/dl)	6 (2–263)	4 (1–282)
Des-γ-carboxylprothrombin level (mAU/ml)		22 (< 10–933)
Tumour diameter (mm)		22 (7–60)
Tumour number (solitary:multiple)		50:5
Portal vein invasion (positive:negative)		49:6
TNM stage (I:II:III:IV)		25: 24: 5: 1
HCC treatment (resection:ablation)		37:18

Values are expressed as the median and range (parenthetically) or the number and percentage (parenthetically).
ADV, adefovir dipivoxil; ETV, entecavir; HBV DNA, hepatitis B virus DNA; HCC, hepatocellular carcinoma; LAM, lamivudine; NA, nucleot(s)ide analogues.

was detected in the NA-naïve patients receiving ETV monotherapy.

Correlation between serum HBcrAg and serum HBV DNA levels at the incidence of HCC

The median serum HBcrAg value was 6.6log U/ml (range, 3.3 to > 6.8) at the start of NA therapy and 5.0log U/ml (range, < 3.0 to > 6.8) at the time of HCC diagnosis. We observed a positive correlation ($P < 0.001$; $r = 0.610$) between the levels of HBcrAg and HBV DNA in serum at the time of HCC diagnosis (Fig. 2A).

HBcrAg was detectable in 23 (82%) of 28 patients with undetectable HBV DNA levels using TaqMan assay and was > 4.8log U/ml in eight (29%) of 28 patients. In contrast, serum HBV DNA was detectable in spite of undetected HBcrAg in only two patients. Then, we examined the correlation between the serum HBcrAg levels at the time of HCC diagnosis and the antiviral effect. The median duration of on-treatment undetected serum HBV DNA was 1.1 years (range, 0.1–4.8) before the first diagnosis of HCC. As shown in Figure 2B, we observed a significant negative correlation between the levels of HBcrAg in serum at the time of HCC diagnosis and the duration of undetected HBV DNA in serum just before the first diagnosis of HCC ($P < 0.001$; $r = -0.568$).

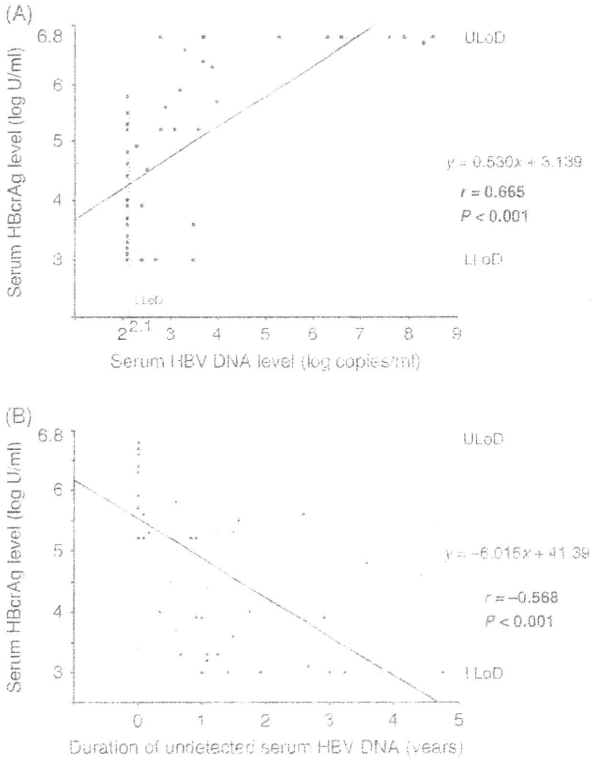


Fig. 2. (A) Correlation between serum HBcrAg and hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular carcinoma (HCC) diagnosis for each patient. (B) Correlation between serum HBcrAg levels at the time of HCC diagnosis and the duration of undetected serum HBV DNA (< 2.6log copies/ml).

Factors associated with HCC recurrence

Hepatocellular carcinoma recurred in 21 (38%) of the 55 patients, 17 (46%) of 37 patients who had undergone resection and four (22%) of 18 patients who had undergone ablation. Because a proportion of patients who had undergone resection with TNM Stage II or over (24 of 37 patients) was greater than ablation (six of 18), there were more patients who had HCC recurrence after resection than ablation. Eight factors were associated with the recurrence in univariate analysis: HBeAg positivity at the start of NA therapy, HBV DNA ≥ 2.1log copies/ml, HBcrAg level ≥ 4.8log U/ml, AST level ≥ 50 IU/L, ALT level ≥ 40 IU/L, tumour multiplicity, portal vein invasion at the time of HCC diagnosis and HCC treatment. In the multivariate analysis, HBcrAg level ≥ 4.8log U/ml and portal vein invasion were independent risk factors for the recurrence of HCC (Table 2). The cumulative recurrence-free survival rates in patients with ≥ 4.8log U/ml HBcrAg levels at the time of HCC diagnosis were 70% at 1 year, 35% at 3 years and 28% at 5 years. In contrast, the rates in patients with < 4.8log U/ml HBcrAg levels were 96% at 1 year, 89% at 3 years and 89% at 5 years. The recurrence-free survival rates of the high HBcrAg group (≥ 4.8log U/ml) were significantly lower than those of the low HBcrAg group (< 4.8log U/ml; $P < 0.001$), as shown in Figure 3A. Then, the cumulative recurrence-free survival rates in patients with ≥ 2.1log copies/ml HBV DNA levels at the time of HCC diagnosis were 70% at 1 year, 44% at 3 years and 39% at 5 years. In contrast, the rates in patients with < 2.1log copies/ml HBV DNA levels were 93% at 1 year, 76% at 3 years and 76% at 5 years. The recurrence-free survival rates of the positive HBV DNA group (≥ 2.1log copies/ml) were significantly lower than those of the negative HBV DNA group (< 2.1log copies/ml; $P = 0.007$), as shown in Figure 3B. The cumulative recurrence-free survival rates were 33% at 1 year and 33% at 2 years with portal vein invasion, and 87% at 1 year, 73% at 2 years and 64% at 3 years without invasion. Three of the six patients with portal vein invasion died of recurrent HCC.

Correlation between intrahepatic cccDNA and serum HBV DNA levels at the incidence of HCC

We measured intrahepatic cccDNA using liver specimens from 22 of 37 patients who underwent resection. The median intrahepatic cccDNA value was 4.2log copies/μg (range, 3.0–5.0). As shown in Figure 4A and B, we observed significant positive correlations between the levels of intrahepatic cccDNA and HBV DNA in serum ($P = 0.019$; $r = 0.486$) and between the levels of intrahepatic cccDNA and HBcrAg in serum at the time of HCC diagnosis ($P = 0.028$; $r = 0.479$). Twenty-eight patients who underwent resection had early- or intermediate-stage

9. Lai CL, Gane E, Liaw YF, *et al.* Telbivudine versus lamivudine in patients with chronic hepatitis B. *N Engl J Med* 2007; **357**: 2576–88.
10. **Marcellin P, Heathcote EJ, Buti M, *et al.* Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. *N Engl J Med* 2008; 359: 2442–55.**
11. Suzuki F, Suzuki Y, Tsubota A, *et al.* Mutations of polymerase, precore and core promoter gene in hepatitis B virus during 5-year lamivudine therapy. *J Hepatol* 2002; **37**: 824–30.
12. **Akuta N, Suzuki F, Kobayashi M, *et al.* Virological and biochemical relapse according to YMDD motif mutant type during long-term lamivudine monotherapy.** *J Med Virol* 2003; **71**: 504–10.
13. **Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; 45: 507–39.**
14. **Yatsuji H, Suzuki F, Sezaki H, *et al.* Low risk of adefovir** resistance in lamivudine-resistant chronic hepatitis B patients treated with adefovir plus lamivudine combination therapy: two-year follow-up. *J Hepatol* 2008; **48**: 923–31.
15. **Werle-Lapostolle B, Bowden S, Locarnini S, *et al.* Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy.** *Gastroenterology* 2004; **126**: 1750–8.
16. Wursthorn K, Lutgehetmann M, Dandri M, *et al.* Peginterferon alpha-2b plus adefovir induce strong cccDNA decline and HBsAg reduction in patients with chronic hepatitis B. *Hepatology* 2006; **44**: 675–84.
17. Kimura T, Rokuhara A, Sakamoto Y, *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; **40**: 439–45.
18. **Kimura T, Ohno N, Terada N, *et al.* Hepatitis B virus DNA-negative dane particles lack core protein but contain a** 22-kDa precore protein without C-terminal arginine-rich domain. *J Biol Chem* 2005; **280**: 21713–9.
19. Rokuhara A, Tanaka E, Matsumoto A, *et al.* Clinical **evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen: a marker distinct from viral DNA for monitoring lamivudine treatment.** *J Viral Hepat* 2003; **10**: 324–30.
20. Wong DK, Tanaka Y, Lai CL, *et al.* Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection. *J Clin Microbiol* 2007; **45**: 3942–7.
21. **Suzuki F, Miyakoshi H, Kobayashi M, Kumada H. Correlation** between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. *J Med Virol* 2009; **81**: 27–33.
22. **Liaw YF, Sung JJ, Chow WC, *et al.* Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med* 2004; 351: 1521–31.**
23. Matsumoto A, Tanaka E, Rokuhara A, *et al.* Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: a multicenter retrospective study of 2795 patients. *Hepatol Res* 2005; **32**: 173–84.
24. Di Marco V, Marzano A, Lampertico P, *et al.* Clinical outcome of HBeAg-negative chronic hepatitis B in relation to virological response to lamivudine. *Hepatology* 2004; **40**: 883–91.
25. **Lampertico P, Vigano M, Manenti E, *et al.* Low resistance to adefovir combined with lamivudine: a 3-year study of 145** lamivudine-resistant hepatitis B patients. *Gastroenterology* 2007; **133**: 1445–51.
26. Hosaka T, Suzuki F, Kobayashi M, *et al.* Development of **HCC in patients receiving adefovir dipivoxil for lamivudine-resistant hepatitis B virus mutants. *Hepatol Res* 2010; 40: 145–52.**
27. Kubo S, Hirohashi K, Tanaka H, *et al.* Effect of viral status on recurrence after liver resection for patients with hepatitis B virus-related hepatocellular carcinoma. *Cancer* 2000; **88**: 1016–24.
28. **Hung IF, Poon RT, Lai CL, *et al.* Recurrence of hepatitis** B-related hepatocellular carcinoma is associated with high viral load at the time of resection. *Am J Gastroenterol* 2008; **103**: 1663–73.
29. **Kim BK, Park JY, Kim do Y, *et al.* Persistent hepatitis B viral replication affects recurrence of hepatocellular carcinoma after curative resection.** *Liver Int* 2008; **28**: 393–401.
30. Wu JC, Huang YH, Chau GY, *et al.* Risk factors for early and late recurrence in hepatitis B-related hepatocellular carcinoma. *J Hepatol* 2009; **51**: 890–7.
31. **Jang JW, Choi JY, Bae SH, *et al.* The impact of hepatitis B viral load on recurrence after complete necrosis in patients** with hepatocellular carcinoma who receive transarterial chemolipiodolization: implications for viral suppression to reduce the risk of cancer recurrence. *Cancer* 2007; **110**: 1760–7.
32. **Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma.** *Lancet* 2003; **362**: 1907–17.
33. Wu TT, Coates L, Aldrich CE, Summers J, Mason WS. In hepatocytes infected with duck hepatitis B virus, the template for viral RNA synthesis is amplified by an **intracellular pathway. *Virology* 1990; 175: 255–61.**
34. **Newbold JE, Xin H, Tencza M, *et al.* The covalently closed duplex form of the hepadnavirus genome exists in situ** as a heterogeneous population of viral minichromosomes. *J Virol* 1995; **69**: 3350–7.
35. **Zoulim F. New insight on hepatitis B virus persistence from the study of intrahepatic viral cccDNA. *J Hepatol* 2005; 42: 302–8.**
36. Chan HL, Wong VW, Tse AM, *et al.* Serum hepatitis B surface antigen quantification can reflect hepatitis B virus in the liver and predict treatment response. *Clin Gastroenterol Hepatol* 2007; **5**: 1462–8.
37. **Brunetto MR, Noriconi F, Bonino F, *et al.* Hepatitis B virus surface antigen levels: a guide to sustained response to** peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. *Hepatology* 2007; **49**: 1141–50.