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Accepted 18 April 2011; published online 21 October 2011

Hepatology Research

Hepatology Research 2012; 42: 139–149 doi: 10.1111/j.1872-034X.2011.00910.x

Original Article

Combination of hepatitis B viral antigens and DNA for prediction of relapse after discontinuation of nucleos(t)ide analogs in patients with chronic hepatitis B

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Aim: The factors associated with hepatitis recurrence after discontinuation of nucleos(t)ide analogs (NAs) in patients with chronic hepatitis B were analyzed to predict the risk of relapse more accurately.

Methods: A total of 126 patients who discontinued NA therapy were recruited retrospectively. The clinical conditions of a successful discontinuation were set as alanine aminotransferase (ALT) below 30 IU/L and serum hepatitis B virus (HBV) DNA below 4.0 log copies/mL.

Results: Relapse of hepatitis B were judged to occur when maximal serum ALT became higher than 79 IU/L or when maximal serum HBV DNA surpassed 5.7 log copies/mL following NA discontinuation since these values corresponded with mean values of ALT (30 IU/L) and HBV DNA (4.0 log copies/mL), respectively. At least 90% of patients with either detectable hepatitis B e antigen or serum HBV DNA higher than 3.0 log copies/mL at the time of NA discontinuation relapsed within one year. In the remaining patients, higher levels of both hepatitis B surface and core-related antigens at the time of discontinuation, as well as a shorter course of NA treatment, were significantly associated with relapse by multivariate analysis.

Conclusions: It appears that negative results for hepatitis B e antigen and serum HBV DNA lower than 3.0 log copies/mL are essential for successful NA discontinuation, which may be attained by a longer treatment period. Levels of hepatitis B surface and core-related antigens are also significant factors independently associated with relapse of hepatitis.

Key words: discontinuation, hepatitis B core-related antigen, hepatitis B surface antigen, nucleos(t)ide analogs, relapse of hepatitis

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major health concern that has an estimated 350 to 400 million carriers worldwide. Chronic infection with HBV can cause chronic hepatitis, and may eventually develop into liver cirrhosis and hepatocellular carcinoma.[1–3] Over the last decade, major advances in the treatment of chronic hepatitis B have been made with nucleos(t)ide

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Financial support
This research was supported in part by a research grant from the Ministry of Health, Labor and Welfare of Japan.
Received 7 August 2011; revision 31 August 2011; accepted 5 September 2011.

analogs (NAs) such as lamivudine (LVD), adefovir dipivoxil (ADV), and entecavir (ETV).[4] NAs are orally administered and are associated with low rates of adverse effects. Treatment with NAs shows strong suppression of HBV replication and consequently rapid improvement of elevated ALT levels. Furthermore, these drugs have been reported to lower the risk of complicating cirrhosis and hepatocellular carcinoma,[5–7] and so NAs are becoming widely used to treat patients with chronic hepatitis B. On the other hand, NAs carry the risk of developing drug-resistance;[8] drug-resistant viruses emerging during treatment may be associated with hepatitis flare-ups. Hepatitis B patients are also required to undergo prolonged treatment with NAs because early discontinuance often leads to relapse of hepatitis and ensuing hepatic failure following rises in alanine aminotransferase (ALT) level.[9,10]

Serum HBV DNA is normally used to monitor the antiviral effect of NAs. HBV DNA decreases rapidly and becomes undetectable in the majority of patients who are treated with NAs,[11–13] but relapse after discontinuation is not rare.[14–17] Since it is also true that favorable virological and biochemical responses to NAs may continue indefinitely in some patients,[9,15] reliable markers that can predict relapse of hepatitis after NA discontinuation are needed. Such markers would benefit not only patients who are considering discontinuation of NA treatment, but also clinicians, hospitals, and the medical economy.

In the present study, we assessed several factors associated with relapse of hepatitis after discontinuation of NAs in patients with chronic hepatitis B, including hepatitis B viral antigens, which have been reported as new and promising markers for monitoring the effect of antiviral agents, such as interferon and NAs.

METHODS

Patients

A TOTAL OF 126 patients with chronic hepatitis B who underwent and completed NA treatment between 2000 and 2010 were enrolled in this study. Patients were recruited retrospectively from 11 hospitals across Japan (Toranomon Hospital, Hokkaido University Hospital, Nagoya City University Hospital, Shinshu University Hospital, Hiroshima University Hospital, National Hospital Organization Nagasaki Medical Center, Chiba University Hospital, The Hospital of Hyogo College of Medicine, Japanese Red Cross Nagoya Daini Hospital, and Tokyo Women's Medical University Hospital, Sapporo Kosei General Hospital) and met the following conditions: (i) serum ALT higher than 30 IU/L and serum HBV DNA higher than 4.0 log copies/mL were observed at least twice within the 6 months prior to administration of NAs; (ii) stored serum samples at initiation and discontinuation of NAs were available for measurements of viral markers; (iii) clinical outcomes were followed for at least 6 months after the discontinuation of NAs; and (iv) tests for hepatitis C and human immunodeficiency virus antibodies were negative. Hepatitis B surface antigen (HBsAg) was confirmed to be positive on at least two occasions at least 6 months apart in all patients before treatment. Patients complicated with hepatocellular carcinoma or signs of hepatic failure at treatment discontinuation were excluded from the study. Our cohort consisted of 83 men and 43 women with a median age of 46 (range, 19 to 79) years when NA administration was discontinued. Hepatitis B e antigen (HBeAg) was positive in 64 patients (51%) at the initiation of treatment and in 24 patients (19%) at its discontinuation. HBV genotype was A in two (2%) patients, B in five (4%), C in 102 (81%), and undetermined in 17 (13%). Thirty-five of the 126 patients in this study were younger than 35 years old. Although not recommended as the first line treatment for this group by Japanese guidelines,[18] NA treatment was commenced since chronic active hepatitis had been persisting in all cases irrespective of their HBeAg status (26 positive and nine negative) at the initiation of treatment.

The decision to discontinue NAs was made by individual physicians using similar, but not uniform, conditions. Four patients who halted NAs for financial reasons were included. No patient underwent interferon treatment during or after NA treatment. The decision to recommence NA administration was also made by individual physicians, essentially when relapse of hepatitis became obvious. With few exceptions, patients were seen at least once a month during the first year after discontinuation of NAs, and at least once every several months afterwards. Stored serum samples were kept frozen at −20°C or below until assayed. This study was approved by the Ethics Committees of all participating institutions.

Hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg, and antibody to HBe (anti-HBe) were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd, Tokyo, Japan; Fujirebio Inc., Tokyo, Japan; and/or Sysmex Co., Kobe, Japan) at each hospital. Quantitative measurement of HBsAg[19] was done using a chemiluminescence enzyme immunoassay

(CLEIA)-based HISCL HBsAg assay manufactured by Sysmex Corporation (Kobe, Japan). The assay had a quantitative range of −1.5 to 3.3 log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range.

Serum concentration of HBV DNA was determined using an Amplicor HBV monitor kit (Roche, Tokyo, Japan),[20] which had a quantitative range of 2.6 to 7.6 log copies/mL. Serum HBV DNA was also determined using a COBAS TaqMan HBV kit (Roche, Tokyo, Japan)[21] with a quantitative range of 2.1 to 9.0 log copies/mL in 43 patients whose serum samples were available at the time of NA discontinuation. According to the manufacturer's instructions, detection of a positive signal below the quantitative range was described as a positive signal, and no signal detection was described as a negative signal. Six HBV genotypes (A–F) were evaluated according to the restriction patterns of DNA fragments from the method reported by Mizokami *et al.*[22]

Serum hepatitis B core-related antigen (HBcrAg) levels were measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio Inc., Tokyo, Japan) as described previously.[23,24] Briefly, 150 μL of serum was incubated with pretreatment solution and then added to a ferrite microparticle suspension in an assay cartridge. Ferrite particles were coated with a monoclonal antibody mixture against denatured HBcAg, HBeAg, and the 22 kDa precore protein. After incubation and washing, further incubation was carried out with alkaline phosphatase conjugated with two kinds of monoclonal antibodies against denatured HBcAg, HBeAg, and the 22 kDa precore protein. Following washing, a substrate solution was added to the test cartridge and then incubated. The relative chemiluminescence intensity was measured, and HBcrAg concentration was calculated by a standard curve generated using recombinant pro-HBeAg. The immunoreactivity of pro-HBeAg at 10 fg/mL was defined as 1 U/mL. We expressed HBcrAg in terms of log U/mL, with a quantitative range set at 3.0 to 6.8 log U/mL.

Statistical analyses

A linear regression model was used to examine for associations between mean and maximal values of both ALT and HBV DNA. Correlations between variables were calculated using the Spearman's rank correction correlation coefficient test. Each cut-off value was decided using receiver operating characteristic curve (ROC) analysis and results were evaluated by measuring the area under the curve (AUC). The Fisher's exact and Pearson's χ^2 tests were adopted to test for differences between subgroups of patients. To compare continuous data, the Mann–Whitney *U*-test was used. The Kaplan–Meier method was used to estimate rates of non-relapse observations, and the log-rank test was used to test hypotheses concerning differences in non-relapse observations between selected groups. Multivariate analyses were performed using the Cox regression model. Variables associated with a P-value < 0.2 in univariate analyses were included in a stepwise Cox regression analysis to identify independent factors associated with relapse of hepatitis after discontinuation of NAs. All tests were performed using the IBM SPSS Statistics Desktop for Japan ver. 19.0 (IBM Japan Inc., Tokyo, Japan). P-values of less than 0.05 were considered to be statistically significant.

RESULTS

Definition of hepatitis relapse after discontinuation of NAs

THE CLINICAL CONDITIONS of a successful discontinuation of NAs were set at serum HBV DNA below 4.0 log copies/mL and ALT below 30 IU/L according to the Japanese guidelines for the treatment of hepatitis B.[18] However, these criteria could not be directly applied to our cohort as post-therapy fluctuations in ALT and HBV DNA were difficult to evaluate consistently. In total, 26 (76%) of 34 patients with successful discontinuation of NAs showed transient abnormal levels of ALT and/or HBV DNA, especially during the early phase after cessation. We therefore used mean and maximal values of these markers to evaluate relapse of hepatitis B in this study; mean values were used to evaluate relapse of hepatitis as a whole, and maximal values were used to dynamically assess relapse during the follow-up period after NA discontinuation. Both ALT and HBV DNA were measured 11.0 times per year on average during the first year and 4.1 times per year on average thereafter.

The mean values of HBV DNA were significantly ($P < 0.001$) correlated with maximal values with a correlation coefficient of 0.853. Similarly, the mean values of ALT were significantly ($P < 0.001$) correlated with maximal values with a correlation coefficient of 0.940 (Fig. 1). The mean HBV DNA value of 4.0 log copies/mL corresponded to a maximal HBV DNA value of 5.7 by ROC analysis (AUC = 0.930, $P < 0.001$), and the mean ALT value of 30 IU/L corresponded to a maximal ALT value of 79 IU/L (AUC = 0.988, $P < 0.001$). These results suggested that patients having serum HBV DNA higher

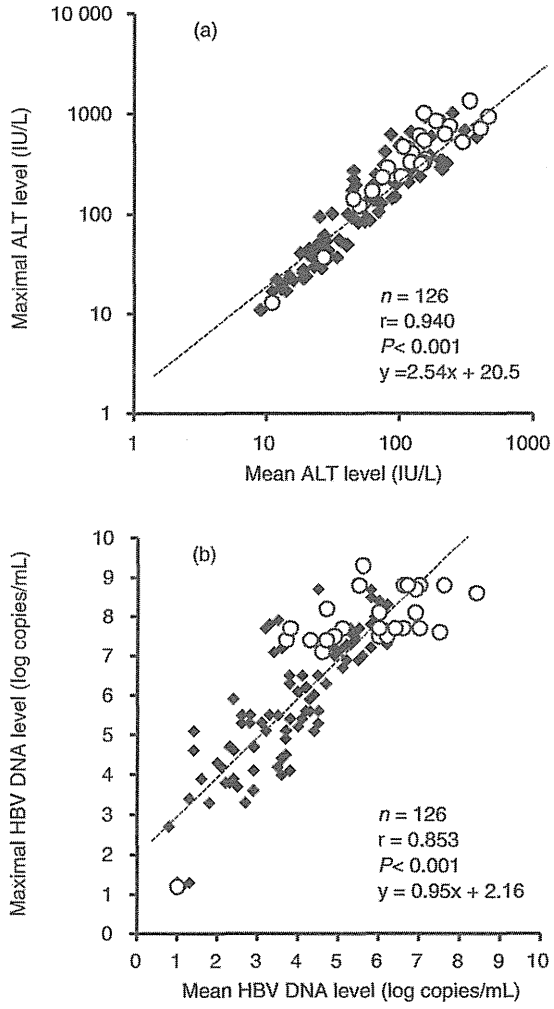

Figure 1 Correlation between maximal and mean levels of alanine aminotransferase (ALT) (a) and hepatitis B virus (HBV) DNA (b) after discontinuation of nucleos(t)ide analogs (NAs). Open circles indicate patients with detectable hepatitis B e antigen (HBeAg) and closed squares indicate patients without detectable HBeAg.

than 5.7 log copies/mL during the follow-up period after NA discontinuation were not likely to achieve the HBV DNA criterion of a successful discontinuation of below 4.0 log copies/mL. Similarly, it could be inferred that patients reaching ALT levels higher than 79 IU/L would also not likely achieve the ALT criterion of a successful discontinuation of below 30 IU/L.

Based on our findings, we judged that a relapse of hepatitis B occurred when serum ALT exceeded 79 IU/L or when serum HBV DNA exceeded 5.7 log copies/mL following NA discontinuation. Accordingly, 92 (73%) of the 126 patients enrolled in the present study showed a relapse. We set the follow-up period as discontinuation to relapse for relapse patients and as discontinuation to the last recorded examination for patients without relapse. Whereas re-administration of NAs due to relapse was commenced in 70% of relapse patients in the follow-up period, none was performed in non-relapse patients during that time.

Elimination of cases likely to show relapse of hepatitis

As it is generally believed that patients who are positive for HBeAg and/or have a higher level of HBV DNA at discontinuation of NAs are likely to relapse, these factors were assessed first. The progression of analyses in the present study and the population structure of each analysis are shown in Figure 2.

The non-relapse rate was compared using the Kaplan–Meier method between 31 patients with HBV DNA equal to or higher than 3.0 log copies/mL and 95 patients with levels lower than 3.0 log copies/mL when NAs were discontinued (Fig. 3). The revised cut-off value of 3.0 log copies/mL was determined by ROC analysis (AUC = 0.709, $P < 0.001$). Thirty (97%) of 31 patients with HBV DNA equal to or higher than 3.0 log copies/mL relapsed within one year of discontinuation. On the other hand, approximately 30% of patients with levels lower than 3.0 log copies/mL showed prolonged non-relapse. Thus, the 31 patients with high HBV DNA at the time of discontinuation were eliminated from the following analyses.

In the remaining 95 patients, the non-relapse rate was compared using the Kaplan–Meier method between 10 patients with detectable HBeAg and 85 patients without HBeAg when NAs were discontinued (Fig. 4). Ninety percent of patients with HBeAg experienced relapse within one year, which was significantly ($P = 0.005$) higher than in cases without HBeAg. In patients without HBeAg, the non-relapse rate decreased rapidly during the first year to approximately 45%, and then decreased relatively slowly over the following 3 years to nearly 30%. It is noteworthy that this subgroup did not relapse afterwards. Since the relapse rate was high among patients with detectable HBeAg, they were excluded from the following analyses as well.

Factors associated with relapse of hepatitis after discontinuation of NAs

Additional factors associated with relapse of hepatitis were analyzed in the remaining 85 patients who were

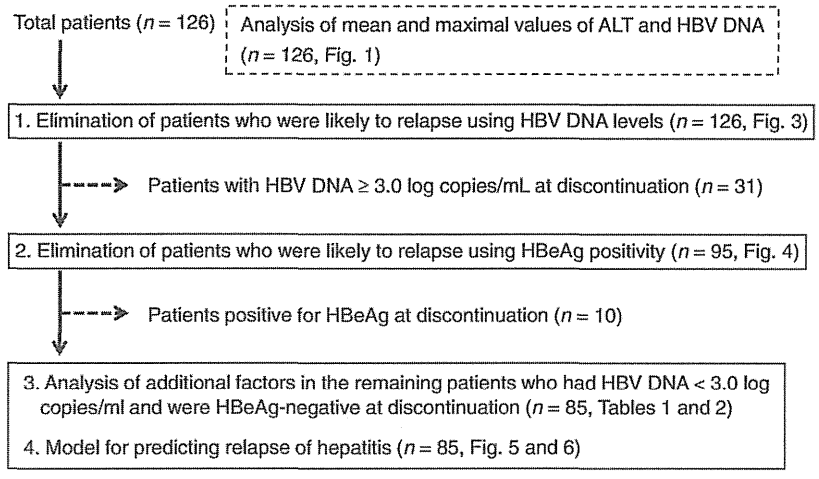

Figure 2 The progression of analyses in the present study and population structure of each analysis.

both negative for HBeAg and whose serum HBV DNA was lower than 3.0 log copies/mL at NA cessation. Table 1 shows the comparison of clinical and virological backgrounds between the 53 relapse and 32 non-relapse patients using univariate analysis. Age and gender distributions were similar between the groups. Approximately 75% of the 85 patients had HBV genotype C, but the distribution of genotypes did not differ between the groups. Approximately 90% of patients were being treated with LVD alone at the time of discontinuation, compared with 6% of patients being given ETV. The median duration of NA treatment was about two times longer in patients without relapse. Levels of both HBsAg and HBcrAg were significantly lower in non-relapse patients than in relapse patients at the time of NA discontinuation. The difference between serum HBsAg was also significant at the initiation of NAs, but not that of HBcrAg. As only patients with HBV DNA lower than 3.0 log copies/mL were analyzed, the majority of these cases showed levels below the 2.6 log copies/mL lower detection limit of the Amplicor assay at NA discontinuation. We therefore also tested HBV DNA with a TaqMan assay, which had a higher sensitivity than the Amplicor assay, in 43 patients whose serum samples were available. The prevalence of patients having a negative detection signal did not differ between the two groups. The number of

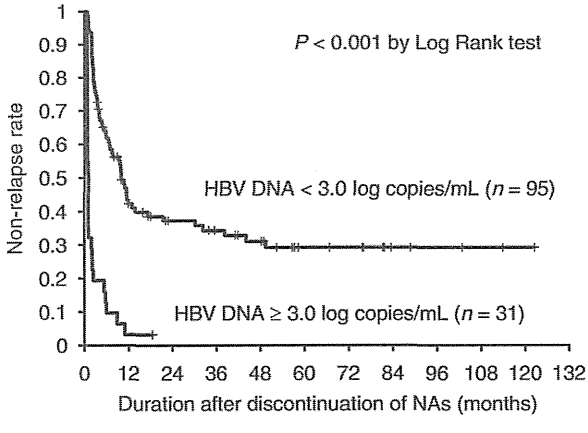

Figure 3 Comparison of non-relapse rates using the Kaplan–Meier method between 31 patients with serum hepatitis B virus (HBV) DNA equal to or higher than 3.0 log copies/mL and 95 patients with serum HBV DNA lower than 3.0 log copies/mL at the time of nucleos(t)ide analog (NA) discontinuation.

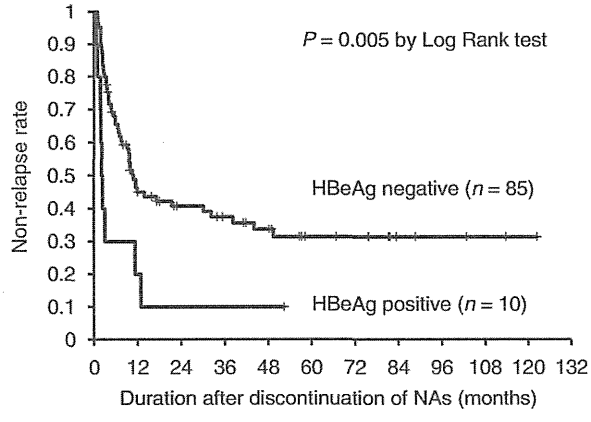

Figure 4 Comparison of non-relapse rates using the Kaplan–Meier method between 10 patients with detectable hepatitis B e antigen (HBeAg) and 85 patients without detectable HBeAg at the time of nucleos(t)ide analog (NA) discontinuation.

Table 1 Comparison of clinical and virological backgrounds between patients with and without relapse of hepatitis at initiation and discontinuation of nucleos(t)ide analogs (NAs)

Background	Non-relapse patients ($n = 32$)	Relapse patients ($n = 53$)	*P*-value
At initiation of NAs			
Age (years)†	47 (17–75)	48 (26–74)	>0.2
Gender (M : F)	23:9	32:21	>0.2
ALT (IU/L)†	183 (9–1182)	187 (20–2052)	>0.2
Genotype (A : B : C : UD)	1:2:21:8	0:3:44:6	0.193
HBeAg (positive)‡	11 (34%)	16 (30%)	>0.2
HBV DNA			
Amplicor assay (log copies/mL)†	6.2 (<2.6->7.6)	6.5 (<2.6->7.6)	0.099
HBsAg (log IU/mL)†	2.7 (0.1–4.3)	3.3 (1.6–3.9)	0.018
HBcrAg (log U/mL)†	5.2 (<3.0->6.8)	5.6 (<3.0->6.8)	>0.2
At discontinuation of NAs			
Age (years)†	50 (21–78)	49 (26–79)	>0.2
NAs (LVD : LVD+ADV : ETV : ADV)	28:1:3:0	50:0:2:1	>0.2
Duration of NA treatment (months)†	36 (4–129)	17 (4–84)	0.007
Follow-up period after discontinuation of NAs (months)†	45 (6–123)	12 (1–111)	0.002
ALT (IU/L)†	16 (7–38)	20 (9–65)	0.002
HBV DNA			
Amplicor assay (log copies/mL)†	<2.6 (<2.6–2.9)	<2.6 (<2.6–2.9)	>0.2
TaqMan assay (negative signal)‡	5 (23%) ($n = 22$)	3 (14%) ($n = 21$)	>0.2
TaqMan assay (negative or positive signal)‡	13 (59%) ($n = 22$)	13 (62%) ($n = 21$)	>0.2
HBsAg (log IU/ml)†	2.0 (<–1.5–4.3)	3.1 (0.6–4.0)	0.001
HBcrAg (log IU/mL)†	3.4 (<3.0–4.9)	4.3 (<3.0->6.8)	0.003

†Data are expressed as the median (range)
‡Data are expressed as a positive number (%)
ADV; adefovir dipivoxil; ALT, alanine aminotransferase; ETV, entecavir; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; LVD, lamivudine; UD, undetermined.

patients with a negative detection signal or a positive signal also did not vary significantly. The follow-up period after discontinuation of NAs was significantly shorter in patients with relapse than in those without because formal follow-up ended once patients relapsed. The median period of follow-up was 45 months in patients without relapse.

Multivariate analyses revealed that a shorter duration of NA treatment and higher levels of HBsAg and HBcrAg at discontinuation were significantly associated with the occurrence of hepatitis relapse (Table 2). The cut-off values that showed the highest significance by ROC analysis were 1.9 log IU/mL for HBsAg (AUC = 0.707, $P = 0.001$), 4.0 log U/mL for HBcrAg (AUC = 0.692, $P = 0.003$), and 16 months (AUC = 0.674, $P = 0.007$) for treatment duration.

Model for predicting relapse of hepatitis using levels of HBsAg and HBcrAg

The existence of a second cut-off value was suggested by ROC analysis for both of HBsAg (2.9 log IU/mL) and HBcrAg (3.0 log IU/mL) to discriminate between

Table 2 Multivariate analysis of factors associated with relapse of hepatitis after discontinuation of nucleos(t)ide analogs (NAs)

Factor	Hazard ratio	95%CI	*P*-value
HBsAg at discontinuation ≥ 1.9 log IU/mL	5.21	1.87–14.55	0.002
HBcrAg at discontinuation ≥ 4.0 log U/mL	2.20	1.25–3.87	0.006
Duration of NA treatment ≥ 16 months	0.54	0.31–0.93	0.027

CI, confidence interval; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen.

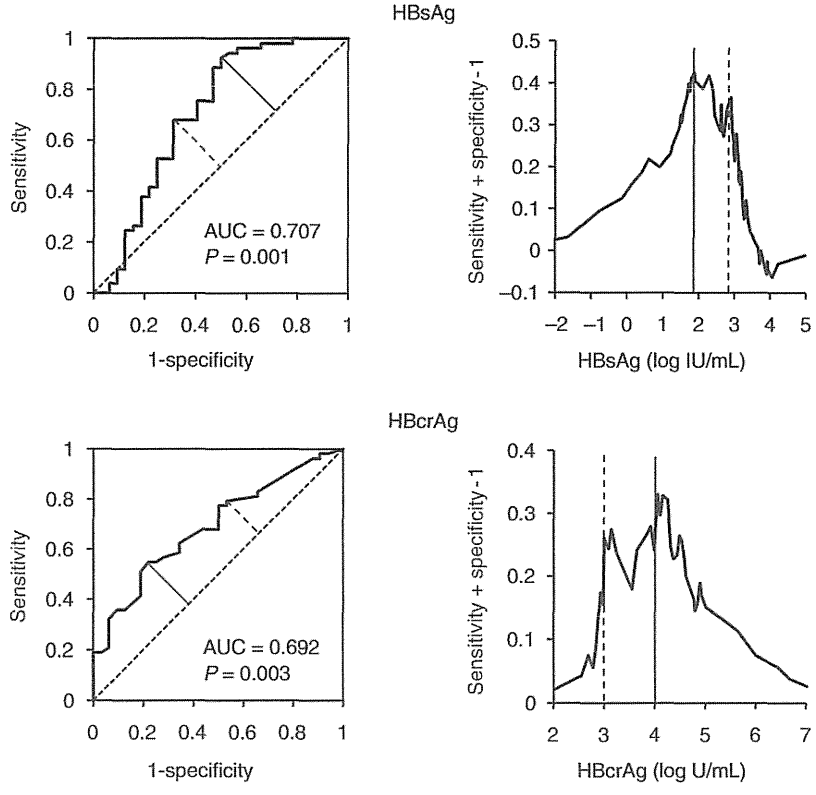

Figure 5 Receiver operating characteristic curve (ROC) analysis of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) to discriminate between patients with and without hepatitis relapse. The existence of two inflection points is suggested for both HBsAg and HBcrAg. Short diagonal lines indicate main inflection points and short broken diagonal lines indicate second inflection points. Vertical lines indicate actual values of antigens that correspond to the main inflection points and vertical broken lines indicate actual values of antigens that correspond to the second inflection points.

patients with and without relapse (Fig. 5). Thus, we set cut-off values as 1.9 and 2.9 log IU/mL for HBsAg and 3.0 and 4.0 log U/mL for HBcrAg in our model for predicting hepatitis relapse.

We tentatively defined three groups using the sum of the scores for HBsAg and HBcrAg levels at the time of NA discontinuation for our model. Conversions were made by assigning a score of 0 for an HBsAg level lower than 1.9 log IU/mL, 1 for a level from 1.9 to 2.8 log IU/mL, and 2 for a level equal to or higher than 2.9 log IU/mL. HBcrAg was scored as 0 for a level lower than 3.0 log U/mL, 1 for a level from 3.0 to 3.9 log U/mL, and 2 for a level equal to or higher than 4.0 log U/mL. Overall, group 1 consisted of patients with a total score of 0, group 2 of patients with a total score of 1 or 2, and group 3 of patients with a total score of 3 or 4.

Patients whose HBV DNA was lower than 3.0 log copies/mL and in whom HBeAg was negative at the time of NA discontinuation were assigned to one of the three groups. Figure 6 shows the comparison of non-relapse rates among the three groups using Kaplan–Meier analysis, which differed significantly. The non-relapse rate was approximately 90% in group 1, as low as 10% in group 3, and intermediate in group 2. When factors associated with relapse were analyzed in group 3 patients, an age of over 40 years at the time of discontinuation was calculated as a significant factor (hazard

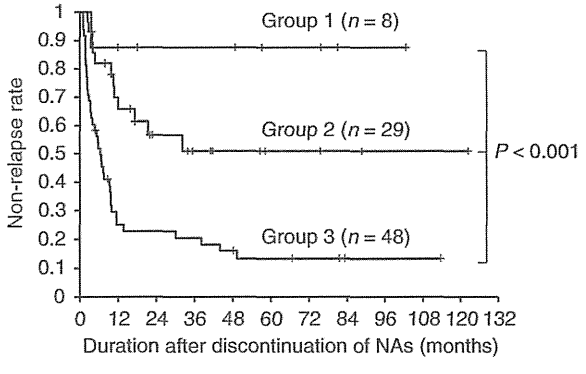

Figure 6 Comparison of non-relapse rates using the Kaplan–Meier method among three groups classified by the sum of the scores of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) levels at the time of nucleos(t)ide analog (NA) discontinuation.

ratio = 5.25, range 2.37–11.65, $P < 0.001$). No significant factors were associated with relapse in group 2 patients.

DISCUSSION

THE EUROPEAN ASSOCIATION for the Study of the Liver recommends continuation of NA treatment until HBsAg is cleared.[25] Liu *et al.* came to a similar conclusion in their study of chronic hepatitis B patients treated with LVD.[14] Indeed, the clearance of HBsAg is a reliable marker for the safe discontinuation of NAs, but the rate of patients who can clear HBsAg is relatively low (1–3%/year).[26–28] Thus, additional factors associated with relapse of hepatitis B after discontinuation of NAs were analyzed in the present study to better identify candidates who could achieve drug-free status. Such studies are relatively few, possibly because patients who discontinue NAs prematurely often experience severe complicating relapse and hepatic failure.[9] Although prospective studies are desirable to obtain accurate results, retrospective studies, such as ours, are also necessary to minimize the risk of adverse complications.

Since HBV cannot be completely eradicated in hosts, the primary goal in treating chronic hepatitis B is to convert symptomatic patients into inactive carriers in whom HBeAg is negative (usually anti-HBe-positive), serum HBV DNA is low, and serum ALT is normal.[1,2,18,29] Thus, we set the clinical conditions of a successful discontinuation of NAs as serum HBV DNA level below 4.0 log copies/mL and ALT below 30 IU/L following NA cessation. Patients who satisfy these conditions are not recommended for treatment by the Japanese guidelines for hepatitis B,[18] and it is also widely accepted that the risk of developing cirrhosis or complicating hepatocellular carcinoma is very low in such patients.[30,31] We used our cohort's mean and maximal values of HBV DNA and ALT for relapse analyses. Mean values were useful for evaluating relapse of hepatitis as a whole since parameter levels often fluctuated after discontinuation, and maximal values were used to evaluate relapse in a real-time fashion during the follow-up period. It is noteworthy that the mean and maximal values correlated very closely for both HBV DNA and ALT. The mean HBV DNA value of 4.0 log copies/mL corresponded to the maximal HBV DNA value of 5.7 by ROC analysis, and similarly the mean ALT value of 30 IU/L corresponded to the maximal ALT value of 79 IU/L. Thus, relapse of hepatitis B was judged to occur when serum ALT became higher than 79 IU/L or when serum HBV DNA surpassed 5.7 log copies/mL after the time of NA discontinuation. Such criteria may also be useful for physicians to detect relapse at an early phase and avoid the occurrence of severe reactivation or unnecessary discontinuation of NAs.

It is generally understood that patients with a higher level of HBV DNA at the time of NA discontinuation are likely to relapse, but this cut-off value has not been analyzed sufficiently. Our findings using ROC analysis showed that patients with levels lower than 3.0 log copies/mL have a good possibility to achieve successful discontinuation. The presence of HBeAg is also generally accepted as a reliable factor to predict relapse of hepatitis. Our study showed that patients with detectable HBeAg at the time of NA discontinuation were likely to relapse, even if their HBV DNA levels were lower than 3.0 log copies/mL. Therefore, we next analyzed additional factors associated with a relapse of hepatitis after discontinuation of NAs by selecting patients who met both of these criteria.

Nucleos(t)ide analog treatment produces a rapid decrease in serum HBV DNA by suppressing reverse transcription of pregenomic HBV RNA. However, the key intrahepatic HBV replicative intermediate, covalently closed circular DNA (cccDNA), tends to remain and is capable of reinitiating replication once NAs are ceased.[32] Measurement of HBV cccDNA has been reported to be useful for monitoring and predicting responses to antiviral treatments.[33] However, its measurement is difficult in the clinical setting as it requires a liver biopsy. Due to the mechanism of action of NAs mentioned above, serum HBV DNA does not reflect intrahepatic HBV cccDNA in patients undergoing NA treatment.[34] To address this, quantitative measurement of HBV antigens has been reported to be useful for predicting the effect of antiviral treatment in patients with chronic hepatitis B. Although HBsAg is usually used as a serum marker for the diagnosis of HBV infection, several groups have shown that HBsAg levels can also be reflective of the response to peg-interferon in chronic hepatitis B.[28,35,36] The HBcrAg assay measures serum levels of HB core and e antigens simultaneously using monoclonal antibodies that recognize the common epitopes of these two denatured antigens. Since the assay measures all antigens transcribed from the pre-core/core gene, it is regarded as core-related.[37] Serum HBcrAg has been reported to accurately reflect intracellular levels of HBV cccDNA even during NA treatment,[24,34,38] and was found to be useful for identifying patients who were likely to show relapse of hepatitis after the discontinuation of NAs.[39,40] It is possible that levels of HBsAg and HBcrAg have different roles in

monitoring antiviral effects because the transcription of these two antigens are regulated by alternative enhancer-promoter systems in the HBV genome.[3] Therefore, we analyzed both of these antigens to elucidate their ability to predict relapse of hepatitis after discontinuation of NAs.

Multivariate analysis demonstrated that levels of HBsAg and HBcrAg at the time of NA discontinuation were independent factors significantly associated with relapse of hepatitis. Thus, we believe these factors can also be applied for predicting relapse in patients whose HBV DNA is lower than 3.0 log copies/mL and whose HBeAg is negative at NA discontinuation. HBV DNA levels were further analyzed using a highly sensitive assay based on real-time polymerase chain reaction (PCR). However, even the level of a negative signal did not ensure successful discontinuation of NAs. The results obtained here indicate that the combined use of HBV-related antigens are useful makers for monitoring the effect of anti-viral treatment in ways different from HBV DNA. Finally, since prolonged NA administration was also a significant factor associated with safe discontinuation, physicians are advised to continue patient treatment for at least 16 months for the best possible outcome.

From our data, a tentative model for predicting relapse of hepatitis after discontinuation of NAs was constructed using levels of HBsAg and HBcrAg at discontinuation. A negative result for HBeAg and HBV DNA lower than 3.0 log copies/mL at the time of NA discontinuation are the essential conditions in this system. Levels of HBsAg and HBcrAg were each converted into scores from 0 to 2 partly because two cut-off values were needed for each antigen and partly because a scoring system may be more convenient for clinical use. The sum of the two scores, which ranged from 0 to 4, was used to prospect relapse. We found that group 1 patients who had a low score (0) could be recommended to discontinue NAs because nearly 90% of this group achieved successful discontinuation. Further analysis of factors associated with relapse are needed for group 2 patients who had middle range scores (1 or 2), since the odds of achieving successful discontinuation were approximately 50%. Continuation of NA treatment is recommended for group 3 patients having high scores (3 or 4) because nearly 90% of this group relapsed. However, this recommendation may be reconsidered in patients younger than 40 years; such cases tended to have a lower relapse rate in group 3. It is also noteworthy that relapse occurred mainly during the first and second years following NA discontinuation in all groups, similarly to a report by Liu *et al.*[14] Thus, clinicians should be vigilant in the early phase after discontinuation.

This study has several limitations. The patients who discontinued NAs were recruited retrospectively, and thus the decision to halt NA treatment was made by individual physicians without uniformly established criteria. Based on this, prospective studies are required to confirm our results. Furthermore, as over 90% of the patients we enrolled had genotype C and over 90% of cases were treated with LVD until discontinuation, the results obtained here can not be applied directly to other HBV genotypes or other types of NAs.

In conclusion, the present study showed that maximal levels of serum ALT and HBV DNA were useful for defining relapse patients after discontinuation of NAs. Along with serum HBV DNA of less than 3.0 log copies/mL and negative serum HBeAg, serum levels of HBsAg and HBcrAg at the time of NA discontinuation were able to predict relapse of hepatitis B and should therefore be considered when establishing uniform guidelines regarding the safe withdrawal of NA treatment. To this end, NA administration of more than 16 months is advisable to achieve successful discontinuation.

ACKNOWLEDGMENTS

THIS RESEARCH WAS supported in part by a research grant from the Ministry of Health, Labor and Welfare of Japan.

We thank Ms. Hiroe Banno for her secretarial assistance and thank Ms. Nozomi Kamijo and Ms. Etsuko Iigahama for their technical assistance. We also thank Mr Trevor Ralph for his English editorial assistance.

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Journal of Medical Virology 85:250–260 (2013)

Add-on Therapy of Pitavastatin and Eicosapentaenoic Acid Improves Outcome of Peginterferon Plus Ribavirin Treatment for Chronic Hepatitis C

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Despite the use of pegylated-interferon (peg-IFN) plus ribavirin combination therapy, many patients infected with hepatitis C virus (HCV)-1b remain HCV-positive. To determine whether addition of pitavastatin and eicosapentaenoic acid (EPA) is beneficial, the "add-on" therapy option (add-on group) was compared retrospectively with unmodified peg-IFN/ribavirin therapy (standard group). Association of host- or virus-related factors with sustained virological response was assessed. In HCV replicon cells, the effects of pitavastatin and/or EPA on HCV replication and expression of innate-immunity- and lipid-metabolism-associated genes were investigated. In patients infected with HCV-1b, sustained virological response rates were significantly higher in the add-on than standard group. In both groups, sustained virological response rates were significantly higher in patients with genotype TT of IL-28B (rs8099917) than in those with non-TT genotype. Among the patients with non-TT genotype, sustained virological response rates were markedly higher in the add-on than standard group. By multivariate analysis, genome variation of IL28B but not add-on therapy remained as a predictive factor of sustained virological response. In replicon cells, pitavastatin and EPA suppressed HCV replication. Activation of innate immunity was obvious in pitavastatin-treated cells and EPA suppressed the expression of sterol regulatory element binding protein-1c and low-density lipoprotein receptor. Addition of pitavastatin and EPA to peg-IFN/ribavirin treatment improved sustained virological response in patients infected with HCV-1b. Genotype variation of IL-28B is a strong predictive factor in add-on therapy. ***J. Med. Virol. 85:250–260, 2013.***

KEY WORDS: cholesterol; hepatitis C virus; IL28B; replicon system

Abbreviations: EPA, eicosapentaenoic acid; HCV, hepatitis C virus; HMGR, HMG-CoA reductase; IRF3, IFN regulatory factor 3; ISG15, IFN-stimulated gene 15; ITPA, inosine triphosphatase; LDLR, low-density lipoprotein receptor; MAVS, mitochondrial antiviral signaling; NPC1L1, Niemann-Pick C1 like 1; OR, odds ratio; PCR, polymerase chain reaction; peg-IFN, pegylated-interferon; PUFA, polyunsaturated fatty acid; RIG-I, retinoic acid inducible gene I; SNP, single nucleotide polymorphism; SREBP, sterol regulatory element binding protein; TRAF6, TNF receptor associated factor 6.

Grant sponsor: Ministry of Health, Labor, and Welfare of Japan (Research Program of Intractable Disease); Grant sponsor: National Hospital Organization of Japan (Grant-in-Aid for Clinical Research).

Disclosure Statement: No competing financial interests exist.

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Accepted 9 October 2012

DOI 10.1002/jmv.23464

Published online 14 November 2012 in Wiley Online Library (wileyonlinelibrary.com).

INTRODUCTION

Nearly, 170 million people are infected with hepatitis C virus (HCV) worldwide and natural history studies show that 5–20% of patients develop cirrhosis after approximately 20 years of infection [Alter, 2005]. Currently, pegylated-interferon (peg-IFN) plus ribavirin combination therapy has become the standard care for chronic hepatitis C because it achieves high rates of sustained virological response [Aghemo et al., 2009]. However, in patients infected with genotype 1b HCV (HCV-1b), at most, 50% of individuals achieve a sustained virological response following combination therapy, and HCV-1b in high viral loads (>5.0 log IU/ml) accounts for >70% of patients with HCV infection in Japan [Kumada et al., 2006]. The response to IFN-based treatment is influenced by virus-related factors including viral load and genotypes; host-related factors, such as sex, age, insulin resistance, staging of the disease and responses to previous antiviral therapies; as well as therapeutic factors, such as dose and duration of treatment [Shiffman, 2002; Backus et al., 2007; Kanwal et al., 2007; Bortoletto et al., 2010]. In addition, as a critical genetic factor for governing the outcomes of peg-IFN plus ribavirin combination therapy, genome variation of IL28B and inosine triphosphatase (ITPA) have been identified recently. At the spot of rs8099917 in the IL28B region, patients infected with HCV-1b with the major variation type (TT) show markedly higher sustained virological response rates than those with the minor variation type (TG + GG) [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Hayes et al., 2011]. Single nucleotide polymorphism (SNP) variation of the ITPA gene at rs1127354 is associated with anemia as an adverse effect during peg-IFN plus ribavirin combination therapy [Fellay et al., 2010; Azakami et al., 2011; Suzuki et al., 2011; Thompson et al., 2011]. In patients who have rs1127354 genotype CC (major type), ribavirin-induced anemia is more frequent and forces a reduction in dose of ribavirin, which worsens the therapeutic outcome. Alternatively, viral amino acid substitutions at core 70 and 91 are significant predictors of treatment outcome. In particular, a point mutation of core 70 from Arg to Gln is significantly associated with non-sustained virological response in patients infected with HCV-1b [Akuta et al., 2005, 2007; El-Shamy et al., 2012].

Investigation of patients treated by peg-IFN plus ribavirin combination therapy has indicated that serum cholesterol and statin use predict virological response to therapy [Harrison et al., 2010]. Recent studies have shown that virological response is improved by addition of fluvastatin or pitavastatin to peg-IFN and ribavirin treatment [Bader et al., 2008; Sezaki et al., 2009; Shimada et al., 2012]. Statins were associated with a reduced risk of hepatocellular carcinoma in a large cohort of patients with diabetes [El-Serag et al., 2009]. In other studies, it has been demonstrated that polyunsaturated fatty acids (PUFAs) inhibit HCV replication by a mechanism that is independent of their roles in regulating lipogenesis [Leu et al., 2004; Kapadia and Chisari, 2005; Huang et al., 2007]. Takaki et al. [2007] have reported that eicosapentaenoic acid (EPA), a type of n-3 PUFA, allows maintenance of the original ribavirin dose in chronic hepatitis C patients during peg-IFN plus ribavirin combination therapy. However, the effects of these lipid modulators on chronic hepatitis C patients with intractable IL-28B allele remain unknown.

As a result of this experimental and therapeutic evidence, a new antiviral strategy to improve treatment outcome for chronic hepatitis C was designed, that is, addition of pitavastatin and EPA to peg-IFN plus ribavirin combination therapy (add-on therapy). The validity of the add-on therapy was evaluated by comparing its effect on the final outcome (i.e., sustained virological response) with that of unmodified peg-IFN plus ribavirin combination therapy (standard therapy), and pretreatment predictors of virological response were investigated. Additionally, the antiviral effect of pitavastatin and/or EPA was estimated in HCV replicon cells.

MATERIALS AND METHODS

Study Patients

In Kyushu Medical Center, a standard protocol in Japan (subcutaneous peg-IFNα2a [180 μg] or peg-IFNα2b [median dose of 1.5 μg/kg, range 1.3–1.7] weekly, along with oral ribavirin daily for 48 weeks) was adopted for chronic hepatitis C patients from 2005 to 2008. The dose of ribavirin was adjusted according to body weight: 600 mg for patients weighing <60 kg, 800 mg for those weighing 60–80 kg, and 800 mg for those weighing >80 kg. From 2008, oral pitavastatin (2 mg/day) and ethyl icosapentate (1,800 mg/day) have been added to the standard protocol (add-on protocol). It has been shown that statins contribute to improving the virological response [Bader et al., 2008; Sezaki et al., 2009]. The add-on protocol was expected to improve treatment, and was applied to all patients after 2008 in Kyushu Medical Center, but a randomized study could not be designed. In these protocols, 48- and 24-week regimens were applied to patients infected with HCV-1b and HCV-2, respectively. Patients who experienced previous therapy using peg-IFN were excluded. Patients with cirrhosis were not included. Because of the possibility that vitamin E and bile acids including ursodeoxycholic acid promote HCV replication [Chang and George, 2007; Yano et al., 2007; Scholtes et al., 2008; Nakamura et al., 2010], treatment with these agents was withdrawn at least 1 month before the initiation of antiviral treatment. The study protocol was approved by the Ethics Committee of the National Hospital Organization, and written informed consent was obtained from all patients. Finally, 238 patients (genotype 1b/2 = 176/62) who were treated with the standard protocol (standard group) and 162 patients (genotype 1b/2 = 101/61) who were treated with the add-on protocol

TABLE I. Profile and Baseline Characteristics of Patients Infected With HCV-1b

Number of patients	Standard group	Add-on group	*P*
Gender: M/F	91/85	46/55	NS
Age (years)	59.5 ± 10.2	57.2 ± 12.5	NS
Past history of IFN therapy: naive/unmodified IFN/unmodified IFN + RBV	147/21/8	77/18/6	NS
HCV RNA (log IU/ml)	5.73 ± 0.16	6.08 ± 0.64	0.001
IL-28B (rs8099917): TT/TG + GG/ND	39/18/119	69/29/3	NS
ITPA (rs1127354): CC/CA + AA/ND	43/14/119	70/27/4	NS
Staging: F_{0-1}/F_{2-3}/ND	15/47/114	27/53/21	NS
ALT (IU/l)	74.5 ± 58.3	62.4 ± 45.2	NS
GGT (IU/l)	55.8 ± 46.8	51.9 ± 45.4	NS
WBC (/μl)	4,859 ± 1,239	4,870 ± 1,395	NS
Hemoglobin (g/dl)	13.9 ± 1.3	13.7 ± 1.5	NS
Platelet (/μl)	16.3 ± 5.7	19.1 ± 6.5	0.006
% of patients treated with enough total doses of Peg-IFN[a]	61.1	75.7	NS
% of patients treated with enough total doses of RBV[b]	76.4	77.1	NS

IFN, interferon; RBV, ribavirin; ITPA, inosine triphosphatase; ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase; WBC, white blood cell; Peg-IFN, pegylated-interferon; ND, not determined; NS, not significant.
[a]Enough total doses: >80% of planned doses.
[b]Enough total doses: >60% of planned doses.

(add-on group) were enrolled and retrospectively analyzed. The profile and baseline characteristics of patients infected with HCV-1b are shown in Table I. In all patients infected with HCV-1b or HCV-2, baseline HCV RNA levels in serum were ≥5.0 log IU/ml.

Laboratory Data

Hematological, biochemical and virological parameters were determined by the clinical laboratory at Kyushu Medical Center. Serum HCV RNA concentrations were determined by the COBAS TaqMan PCR HCV test (Roche Diagnostics, Tokyo, Japan). Sustained virological response was defined as undetectable HCV RNA at week 24 after completion of therapy. Genotyping for the IL28B (rs8099917) and ITPA (rs1127354) polymorphisms was performed by TaqMan® SNP Genotyping Assays (Applied Biosystems, Branchburg, NJ) that apply a polymerase chain reaction (PCR)-based restriction fragment length polymorphism assay. To determine amino acid polymorphism in HCV core protein, the PCR method with primers specific for polymorphism at core 70 was performed as described previously [Nakamoto et al., 2009].

Cell Lines and Treatment

The human-hepatoma-derived cell line, Huh7/Rep-Feo-1b, which stably expresses the HCV Rep-Feo replicon, was a kind gift from the Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University. The HCV subgenomic replicon plasmids, which contained NS3, NS4, NS5A, and NS5B, were derived from the HCV-N strain (genotype 1b), and the construct expressed a chimeric reporter protein of luciferase and neomycin phosphotransferase that allowed selection of cells and rapid measurement of the replication levels in stable replicon-expressing cells [Yokota et al., 2003; Tanabe et al., 2004; Toyoda et al., 2011]. Cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, and 0.1 mg/ml streptomycin in a humidified 37°C/5% CO_2 incubator. Pitavastatin (donated by Kowa Pharmaceutical Co, Tokyo, Japan) and EPA (Otsuka Pharmaceutical Co, Tokyo, Japan) were dissolved in 10% carboxyl methylcellulose and chloroform, respectively, and stored in stock solutions at a concentration of 10 and 20 M, respectively. According to previous reports and our pretests for inhibition rates of HCV replication and cytotoxicity [Ye et al., 2003; Leu et al., 2004; Kapadia and Chisari, 2005; Ikeda et al., 2006], Huh7/Rep-Feo-1b cells were treated with 20 μM EPA, 10 μM pitavastatin, or 20 μM EPA plus 10 μM pitavastatin for 48 hr. The concentrations of EPA and pitavastatin may have been reasonable because they were lower than the reported maximum blood concentration of EPA or pitavastatin in healthy adult men with usual daily doses. For control cells, the same volume of 10% carboxyl methylcellulose and chloroform used for treated cells was added to medium and incubated for 48 hr.

Cell Proliferation/Viability and Luciferase Assays

The proliferation and viability of cultured cells were checked by Cell Viability and Proliferation Assay Kit (Funakoshi, Tokyo, Japan). Luciferase activity assay was performed using the Bright-Glo Luciferase Assay System (Promega, Tokyo, Japan). According to the manufacturer's protocol, luciferase was extracted from control and treated cells, and luciferase activity was quantified by use of a luminometer.

Real-Time PCR

mRNA expression levels in Huh7/Rep-Feo-1b cells under EPA and/or pitavastatin treatment were

analyzed using real-time RT-PCR and compared with untreated Huh7/Rep-Feo-1b cells. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) and cDNA was synthesized from 1.0 μg RNA using GeneAmp™ RNA PCR (Applied Biosystems) with random hexamers. Real-time RT-PCR was performed using LightCycler-FastStart DNA Master SYBR Green 1 (Roche, Basel, Switzerland) according to the manufacturer's instructions. The reaction mixture (20 μl) contained LightCycler-FastStart DNA Master SYBR Green 1, 4 mM $MgCl_2$, 0.5 μM upstream and downstream PCR primers, and 2 μl first-strand cDNA as a template. To control for reaction variations, all PCR data were normalized against the expression of retinoblastoma binding protein 6 [Nakamuta et al., 2011]. The real-time RT-PCR primer sets in this study are listed in Table II.

Statistical Analysis

Statistical analysis was performed using JMP software (SAS Institute, Inc., Cary, NC). Differences between categorical variables were analyzed using Fisher's extract test or χ^2 test. Mann–Whitney U test was used for continuous variables. Multivariate analysis was used to identify factors independently associated with the achievement of sustained virological response. The odds ratio (OR) and 95% confidence intervals were also calculated. $P < 0.05$ was considered to be statistically significant.

RESULTS

Sustained Virological Response Rates in Patients Infected With HCV-1b and HCV-2

Peg-IFN and/or ribavirin were discontinued or their doses reduced, as required, upon reduction of hemoglobin levels, neutrophil counts or platelet counts, or the development of other adverse effects. Therefore, to evaluate therapeutic effects properly, sustained virological response rates were examined by intention to treat analysis. Within the enrolled patients, 62 and 61 patients infected with HCV-2 were included in the standard and add-on therapy groups, respectively. In these patients, no significant difference was found in sustained virological response rates between the standard and add-on therapy groups; 80.7% and 77.1%, respectively (Fig. 1A). Hence, all subsequent examinations were conducted on patients infected with HCV-1b.

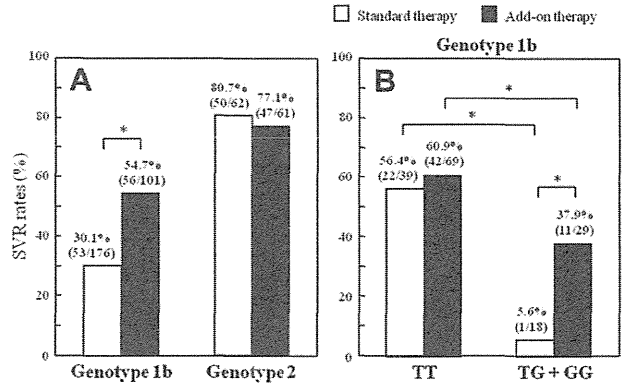

Fig. 1. Sustained virological response rates in chronic hepatitis C patients: comparison between standard and add-on therapy. **A**: Results for HCV genotype 1b and 2. **B**: Results for genome variation of IL28B (rs8099917); genotype TT and non-TT (TG + GG). Data for HCV-1b patients are shown. $^*P < 0.01$.

In patients infected with HCV-1b, sustained virological response rates were significantly higher in the add-on than in the standard therapy group (54.7% vs. 30.1%, $P < 0.0001$; Fig. 1A), although background HCV RNA levels were significantly higher in the add-on therapy group (Table I). Platelet counts were higher in the add-on therapy group but those in the standard therapy group were still sufficient for IFN-based therapy. Of note, no significant difference was found between the standard and add-on therapy groups for the rate of patients in whom sufficient total doses of peg-IFN (>80% of planned doses) and ribavirin (>60% of planned doses) were administered (Table I).

TABLE II. Sequences of Primers Used for Real-Time PCR

Genes	Forward (5′ → 3′)	Reverse (5′ → 3′)
RIG-I	GGCCCACTGCCCCAGGTCAT	TCCCCAACACCAACCGAGGC
MAVS	CCCTCTGGCATCTCTTCAATACC	TTCGTCCGCGAGATCAACTA
IRF3	CCAGCTTGGACAATCCCACTC	GAAGGCTGTCACCTCGAACTC
TRAF6	GAGGTCTCCACCCGCTTTGA	TTGAGCAAGTGAGGGCAAGCTA
IFNβ1	GCGACACTGTTCGTGTTGTCA	CCAAGCAAGTTGTAGCTCATGGA
ISG15	GGGCTGGGACCTGACGGTGA	GGACAGCCAGACGCTGCTGG
HMGR	GCCTGGCTCGAAACATCTGAA	CTGACCTGGACTGGAAACGGATA
SREBP-1	GCTGTCCACAAAAGCAAATCTCT	GTCAGTGTGTCCTCCACCTCAGT
LDLR	CAACGGCTCAGACGAGCAAG	AGTCACAGACGAACTGCCGAGA
RBBP6	GCGACCTGCAGATCACCAA	TGCCATCGCTGGTTCAGTTC

RIG-I, retinoic acid inducible gene I; MAVS, mitochondrial antiviral signaling; IRF, interferon regulatory factor; TRAF, TNF receptor associated factor; IFN, interferon; ISG, interferon-stimulated gene; HMGR, HMG-CoA reductase; SREBP, sterol regulatory element binding protein; LDLR, LDL receptor; RBBP, retinoblastoma binding protein.

Effect of IL28B and ITPA Genotypes on Viral Response

According to genetic variation of IL28B gene (rs8099917), sustained virological response rates in patients infected with HCV-1b were determined (Fig. 1B). In both the standard and add-on therapy groups, sustained virological response rates were significantly higher in patients with the major type genome variation (TT) than in those with the minor type (TG + GG). In the latter, sustained virological response rates were markedly higher in the add-on than in the standard therapy group (37.9% vs. 5.6%, $P = 0.007$). In patients with the major type genome variation, addition of pitavastatin and EPA induced higher sustained virological response rates although no significant difference was found between the two treatment groups. In comparison between the major (CC) and minor (non-CC) types of ITPA (rs1127354), sustained virological response rates were comparable between the standard and add-on therapy groups (Fig. 2A). However, in the add-on group, the percentage of patients infected with HCV-1b who completed therapy without dose reduction of ribavirin was significantly higher among those with the minor type of ITPA than the major type (45.8% vs. 21.5%, $P = 0.004$; Fig. 2B).

Viral Kinetics With Add-on Therapy

Viral kinetics in patients infected with HCV-1b were examined in the add-on therapy group according to genome variation of the IL28B (rs8099917), and compared between the sustained virological response and non-sustained virological response groups. In patients with major variation type (TT), viral decline was significantly greater at all times (days 3–84) in the sustained virological response than in the non-sustained virological response group (Fig. 3A). However, in patients with minor variation type (TG + GG), viral kinetics were similar within the first 2 weeks of treatment in the sustained virological response and non-sustained virological response groups (Fig. 3B). Accordingly, sustained virological response was affected by the depth of early phase viral decline in patients with major variation but not in patients with minor variation. Viral kinetics in patients with minor type variation (TG ± GG) of IL-28B were compared between the standard therapy and add-on therapy

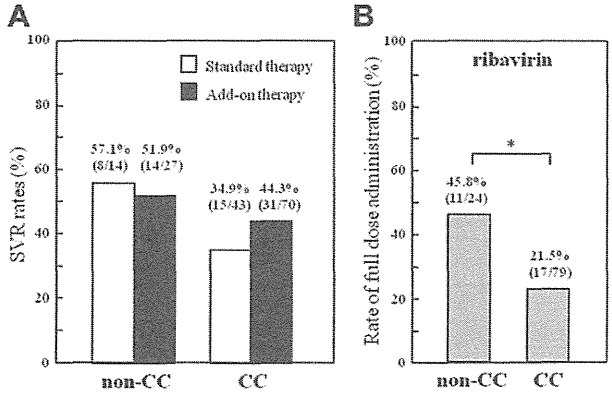

Fig. 2. Clinical data of patients infected with HCV-1b: comparison between genome variations of ITPA. A: Sustained virological response rates were compared between standard and add-on therapy. Results are presented for each genome variation of ITPA (rs1127354); genotype CC and non-CC. B: Numbers of patients in whom planned ribavirin doses were completed. Results in patients infected with HCV-1b treated with add-on therapy are shown in each genome variation of ITPA (rs1127354); genotype CC and non-CC. $^{*}P < 0.05$.

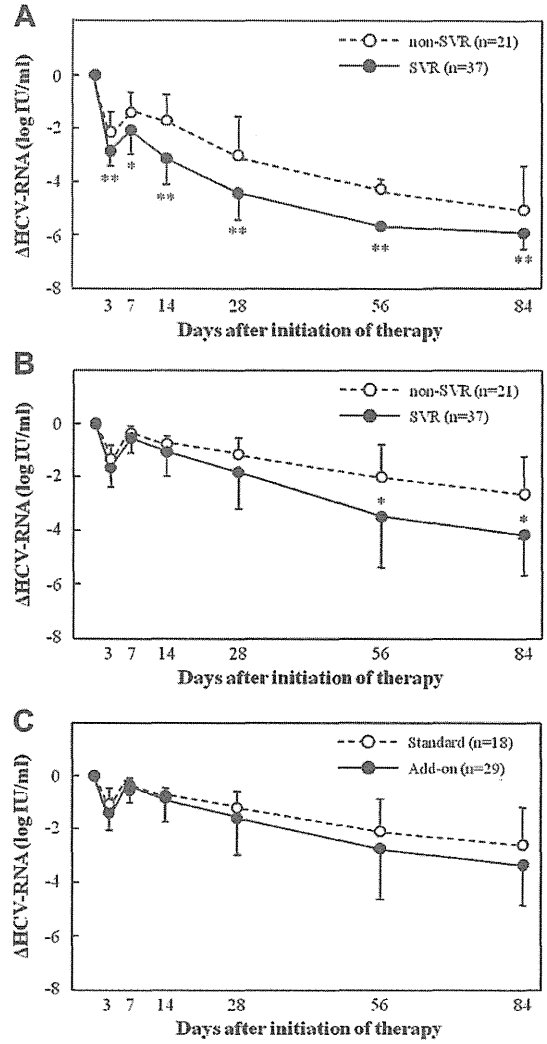

Fig. 3. Viral kinetics in patients infected with HCV-1b. A: Results in patients with major type variation (TT) of IL-28B: comparison between sustained virological response and non-sustained virological response groups. B: Results in patients with minor type variation (TG + GG) of IL-28B: comparison between sustained virological response and non-sustained virological response groups. C: Results in patients with minor type variation (TG + GG) of IL-28B: comparison between standard therapy and add-on therapy groups. $^{*}P < 0.05$, $^{**}P < 0.01$ (sustained virological response vs. non-sustained virological response).

groups (Fig. 3C). As a result, viral decline was somewhat greater after day 28 in the add-on therapy group but the difference was not significant.

Effect of Amino Acid Substitutions of HCV Core 70 on Viral Response

Add-on therapy was significantly more effective in patients with IL28B minor variation (TG + GG) compared with standard therapy, therefore, we investigated the association between HCV core 70 amino acid mutation and therapeutic outcome. In 27 patients infected with HCV-1b, who had minor variation of IL28B (TG + GG) and were treated with add-on therapy, core 70 amino acid mutation was determined. Sustained virological response was achieved in 10 patients and core 70 mutation (Gln) was found in 6 of the 10 patients (60%). Within the 17 non-sustained virological response patients, the mutation was identified in eight patients (47.1%). Accordingly, within these patients, the core 70 amino acid substitutions did not affect sustained virological response in the add-on therapy.

Predictive Factors Associated With Sustained Virological Response

Among the factors listed in Table III, predictive factors associated with sustained virological response were examined in patients infected with HCV-1b. Univariate analysis identified six parameters that correlated significantly with sustained virological response; age ($P = 0.0038$), fibrotic staging ($P = 0.0012$), γ-glutamyl transpeptidase ($P = 0.0009$), platelet count ($P = 0.0132$), genetic variation of IL28B ($P < 0.0001$) and add-on therapy ($P < 0.0001$; Table III). In multivariate analysis, significant contribution factors for sustained virological response were age (<60 years; OR 3.06, $P = 0.0221$), IL28B (genotype TT; OR 6.69, $P = 0.0019$) and staging (F_{0-1}; OR 5.71, $P = 0.0035$; Table IV). When IL28B was excluded from the factors in multivariate analysis, addition of pitavastatin and EPA (add-on therapy) was also selected as a significant contribution factor for sustained virological response (OR 2.13, $P = 0.0395$).

TABLE IV. Multivariate Analysis for Predictive Factors Associated With Sustained Virological Response

Factors	Category	95% confidence intervals	P
Age (years)	1. ≥60: 1.0		
	2. <60: 3.06	1.20–8.24	0.0221
IL-28B (rs8099917)	1. TG + GG: 1.0		
	2. TT: 6.69	2.17–24.66	0.0019
Staging	1. F_{2-3}: 1.0		
	2. F_{0-1}: 5.71	1.91–20.51	0.0035

Subgenomic HCV Replicon System

Suppression of HCV RNA replication by pitavastatin and/or EPA was examined in Huh7/Rep-Feo-1b cells by luciferase assay. The concentrations of pitavastatin and EPA for the following experiments were determined according to previous studies [Ye et al., 2003; Leu et al., 2004; Kapadia and Chisari, 2005; Ikeda et al., 2006] and our pilot study for cytotoxicity and luciferase assay (data not shown). Huh7/Rep-Feo-1b cells were incubated with or without 10 μM pitavastatin and/or 20 μM EPA for 48 hr. As a precondition, the proliferative activity and viability of pitavastatin- and/or EPA-treated cells were comparable with those of control cells (data not shown). As a result, luciferase activity was significantly suppressed in EPA- and/or pitavastatin-treated cells compared with the control cells (Fig. 4A). At these concentrations, the suppressive effect was more marked in pitavastatin-treated than EPA-treated cells.

TABLE III. Univariate Analysis Between Non-Sustained Virological Response and Sustained Virological Response Groups

Factors	Non-SVR	SVR	P
Gender (M/F)	82/86	55/54	NS
Age (years)	60.5 ± 10.6	55.7 ± 12.0	0.0038
Past history of IFN therapy: naive/unmodified IFN/unmodified IFN + RBV	136/24/8	88/15/6	NS
HCV RNA (log IU/ml)	6.03 ± 0.16	5.91 ± 0.55	NS
IL-28B (rs8099917) TT/TG + GG/ND	35/44/89	12/64/33	<0.0001
ITPA (rs1127354) CC/CA + AA/ND	59/20/89	54/21/34	NS
Staging (F_{0-1}/F_{2-3}/ND)	11/59/98	31/41/37	0.0012
Treatment add-on/standard	45/123	56/53	<0.0001
ALT (IU/l)	72.3 ± 57.7	63.9 ± 45.2	NS
GGT (IU/l)	65.3 ± 56.0	41.1 ± 27.1	0.0009
WBC (/μl)	4,935 ± 1,392	4,791 ± 1,254	NS
Hemoglobin (g/dl)	13.8 ± 1.4	13.8 ± 1.4	NS
Platelet (/μl)	16.7 ± 5.7	19.1 ± 6.6	0.0132
% of patients treated with enough total doses of Peg-IFN[a]	60.9	74.4	NS
% of patients treated with enough total doses of ribavirin[b]	71.9	80.8	NS

IFN, interferon; RBV, ribavirin; ITPA, inosine triphosphatase; ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase; WBC, white blood cell; Peg-IFN, pegylated-interferon; ND, not determined; NS, not significant.
[a]Enough total dose: >80% of planned doses.
[b]Enough total doses: >60% of planned doses.

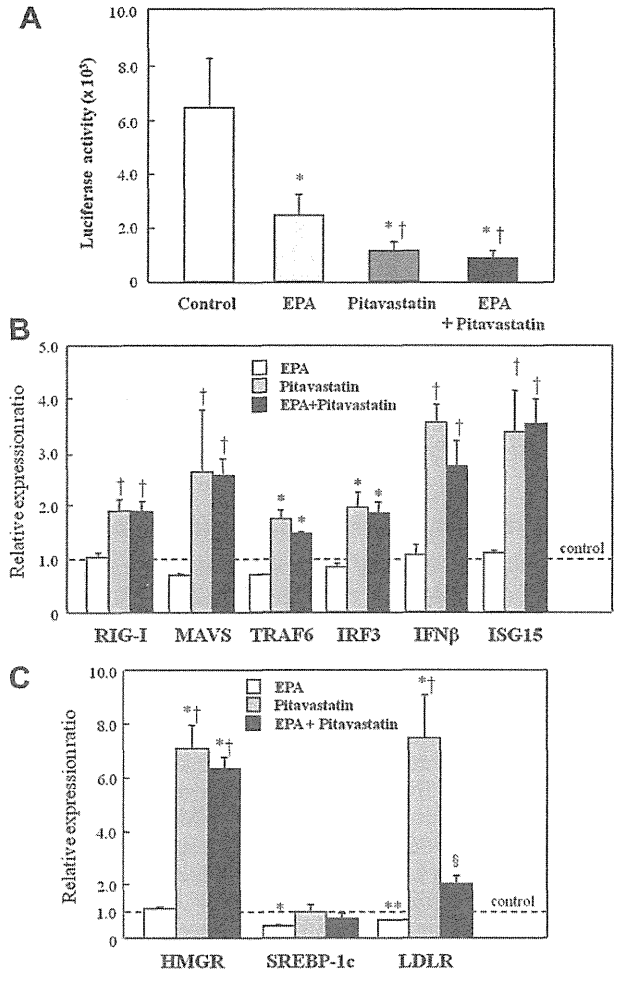

Fig. 4. Treatment of pitavastatin and/or EPA in HCV replicon cells. A: HCV replication was estimated by luciferase assay. Huh7/Rep-Feo-1b cells were treated with pitavastatin (10 μM) and/or EPA (20 μM) for 48 hr. $^{*}P < 0.01$ versus control, $^{\dagger}P < 0.01$ versus EPA. B: Expression levels of RIG-I, MAVS, TRAF6, IRF3, IFNβ, and ISG15 genes in Huh7/Rep-Feo-1b cells treated with pitavastatin (10 μM) and/or EPA (20 μM) for 48 hr. $^{*}P < 0.01$ versus control and EPA, $^{\dagger}P < 0.05$ versus control and EPA. C: Expression levels of HMGR, SREBP-1c and LDLR genes in Huh7/Rep-Feo-1b cells treated with pitavastatin (10 μM) and/or EPA (20 μM) for 48 hr. $^{*}P < 0.01$ versus control, $^{**}P < 0.05$ versus control, $^{\dagger}P < 0.01$ versus EPA, $^{\S}P < 0.01$ versus pitavastatin.

In Huh7/Rep-Feo-1b cells, the expression levels of innate-immunity-associated genes were examined after 48 hr treatment with 10 μM pitavastatin and/or 20 μM EPA. As shown in Figure 4B, retinoic acid inducible gene I (RIG-I), mitochondrial antiviral signaling (MAVS), TNF receptor associated factor 6 (TRAF6), IFN regulatory factor 3 (IRF3), IFNβ and IFN-stimulated gene 15 (ISG15) showed similar trend in expression. Accordingly, their expression was significantly increased by pitavastatin but not by EPA, and EPA did not show an additive effect with pitavastatin. With the same treatments, expression of lipid-metabolism-associated genes was analyzed (Fig. 4C). HMG-CoA reductase (HMGR) expression was significantly enhanced by pitavastatin but not by EPA. The sterol regulatory element binding protein 1c (SREBP-1c) expression was significantly suppressed by EPA but not by pitavastatin. Low-density lipoprotein receptor (LDLR) expression was significantly suppressed by EPA, whereas the expression was activated by pitavastatin, but the activation was lost in the presence of EPA.

DISCUSSION

For ethical reasons, standard therapy could not be selected after 2008; therefore, the present study was unable to eliminate some methodological issues that limit the interpretation and drawing of firm conclusions. For example, the percentage of patients receiving sufficient total dose of peg-IFN was lower in the historical standard group although the difference was not significant and, in order to prevent dose reduction, additional means might have been performed on the add-on group after 2008. However, in univariate and multivariate analyses, total dose of peg-IFN was not detected as a significant factor for sustained virological response. Nevertheless under these limitations, the presented clinical and in vitro studies indicate some sufficient trends in treatment response.

Previous studies on hepatic lipid metabolism have shown that, in the liver of patients with HCV infection, synthesis of cholesterol and fatty acids is still activated, regardless of overaccumulation of lipids [Kohjima et al., 2009; Nakamuta et al., 2009, 2011; Fujino et al., 2010]. This means that addition of pitavastatin and EPA to standard therapy is pathophysiologically reasonable for patients with chronic hepatitis C. Sustained virological response rates in patients infected with HCV-2 were sufficiently high and comparable between the standard and add-on therapy groups (Fig. 1A). Therefore, this study was focused on patients infected with HCV-1b with high virus load. This investigation of sustained virological response in patients treated with add-on or standard therapy had two clinically important findings.

First, add-on therapy led to significantly higher sustained virological response rates than did standard therapy (Fig. 1A). Although overall sustained virological response rate in this study was lower compared with the results from some other institutions, it may be because intention to treat analysis was used in this study and the ratio of IL28B minor (TG + GG) patients was higher in the standard and add-on groups. When sustained virological response rates were compared only in patients with IL28B major or in those with IL28B minor, the sustained virological response rates were not lower compared with those in other reports (data not shown). The suppressive effect against HCV replication by statins and EPA, and their synergistic action with IFN, has already been demonstrated in some HCV replicon systems [Ye

et al., 2003; Leu et al., 2004; Kapadia and Chisari, 2005; Huang et al., 2007; Ikeda and Kato, 2007]. In our investigation using the luciferase assay in Huh7/Rep-Feo-1b cells, a similar suppressive effect was seen with both pitavastatin and EPA treatments (Fig. 4A). It has been reported that the statins impede HCV replication through inhibition of host protein geranylgeranylation and FBL2 has been identified as a geranylgeranylated cellular protein required for HCV RNA replication [Wang et al., 2005; Nakamuta et al., 2011]. PUFAs, including EPA, inhibit HCV replication, although the precise mechanism is still unclear but may be independent of the route regulating lipogenesis [Leu et al., 2004; Kapadia and Chisari, 2005]. The synergistic and additive effect of EPA with pitavastatin was not significant in our luciferase assay; therefore, statins and EPA may act against cognate targets.

Second, the add-on therapy improved sustained virological response rates especially in patients with the minor type variation (TG + GG) of the IL28B gene (rs8099917), in whom sustained virological response is expected to be poor after standard therapy (Fig. 1B). Recent studies have revealed that SNPs within or adjacent to IL28B region provide a strong predictive value for the outcome of IFN-based therapy in patients infected with HCV-1b [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Hayes et al., 2011]. With add-on therapy, sustained virological response rates were significantly higher in patients with major type (TT) than minor type (TG + GG) variations, meaning that genome variation of IL28B (rs8099917) still governs the outcome even in add-on therapy. However, in the patients with minor type variation, the sustained virological response rate (37.9%) in the add-on group was markedly higher compared with that in the standard therapy group (Fig. 1B). This sustained virological response rate may be sufficiently high for clinical use of add-on therapy in patients infected with HCV-1b with minor type variation. From this point of view, the add-on therapy is a clinically valuable strategy for chronic hepatitis C. In the analysis of viral dynamics in patients with minor type variation of IL28B, no significant difference was found in viral decline within 84 days between the standard and add-on groups (Fig. 3C). Although there is still no evidence, in the add-on therapy, late phase viral decline (3 months after treatment initiation) may be more important for the achievement of sustained virological response in patients with minor type variation.

It has been emphasized that mutation of amino acids 70 and 91 in the core region of HCV-1b as a virus-related factor, as well as genome variation of IL28B gene as a host-related factor, greatly influences the outcome of IFN-based antiviral treatments. According to recent clinical studies in patients infected with HCV-1b, substitution of core 70 is assessed as a more influential factor affecting the outcome of peg-IFN plus ribavirin combination therapy, rather than that of core 91 [Akuta et al., 2007; Hayes et al., 2011; El-Shamy et al., 2012]. Even in the latest triple therapy with peg-IFN, ribavirin and a NS3/4A protease inhibitor, telaprevir, patients infected with HCV-1b with core 70 mutation were reported to be severely resistant to the therapy [Akuta et al., 2010]. In our assessment of patients with minor type IL28B variation at rs8099917 (TG + GG), mutation at core 70 was likely not to diminish the outcome of add-on therapy, although the number of patients examined was small (Fig. 4). Therefore, the lipid modulators, pitavastatin and EPA, may be expected to be more effective for patients infected with HCV-1b with core 70 mutation, compared with an NS3/4A protease inhibitor. However, for reliable assessment, further clinical data are needed from patients treated with add-on therapy.

As part of its pathogenic strategy, HCV interferes with the innate immune response of its host; mainly in the RIG-I/MAVS pathway [Breiman et al., 2005; Tasaka et al., 2007; Baril et al., 2009; Jouan et al., 2010; Lemon, 2010; Liu and Gale, 2010; Ekisioglu et al., 2011]. RIG-I undergoes a conformational change upon HCV RNA binding and interacts with MAVS, resulting in phosphorylation and nuclear translocation of IRF3, which leads to transcriptional activation and synthesis of IFNβ. IFNβ activates the Jak–STAT (Janus kinase–signal transducer and activator of transcription) signaling pathway and acts through the expression of ISGs. TRAF6 is recruited to the MAVS complex and is required for activation of nuclear factor-κB, which forms an enhanceosome on the IFNβ promoter in coordination with IRF3. In HCV-infected cells, NS3/4A protease cleaves MAVS, and the RIG-I/MAVS pathway is impeded. In the present study, the HCV replicon system was used to examine how pitavastatin and EPA influence the RIG-I/MAVS pathway, which plays an important role in the innate antiviral host response to HCV infection. The expression profile of innate-immunity-associated genes in pitavastatin- and/or EPA-treated Huh7/Rep-Feo-1b cells showed that only pitavastatin activated expression of the tested genes, RIG-I, MAVS, IRF3, TRAF6, IFNβ, and ISG15, similarly (Fig. 4B). EPA treatment did not increase expression levels of these genes. It is unclear whether the activation of these innate-immunity-associated factors directly contributes to elimination of HCV or whether inhibition of HCV replication by pitavastatin treatment directly leads to the activation of innate immunity through lowering NS3/4A protease expression.

Cholesterol, fatty acids, and lipid rafts have been demonstrated to be critical for efficient replication, infection and secretion of HCV [Simons and Ehehalt, 2002; Kushner et al., 2003]. For example, HCV replication was suppressed by inhibition of the liver X receptor α–SREBP-1c pathway [Kapadia and Chisari, 2005]. Therefore, negative modulation of lipid synthesis may be an antiviral step of statins and EPA. In pitavastatin treatment of HCV replicon cells, HMGR