Table 1. Patient Characteristics and Demographics

	Entire Cohort				Propensity Score Matched Cohort		
Characteristics	All Patients (n = 1,615)	Entecavir (n = 472)	Control (n = 1,143)	P	Entecavir (n = 316)	Control (n = 316)	P
Age (y)†	42 (13.5)	47 (12.4)	39 (13.1)	<0.001	46 (12.1)	46 (13.5)	0.907
Gender (male:female)	1,035:580	315:157	720: 423	0.171	210:106	210:106	1.000
Alcohol consumption (>200kg)	355 (22)	97 (20.5)	288 (25.1)	0.013	62 (20)	105 (33)	<0.001
Cigarette smoking	443 (27)	157 (33.2)	286 (25.0)	0.005	110 (35)	110 (35)	1.000
Preexisting cirrhosis	311 (19)	116 (25)	195 (17)	0.001	79 (25)	85 (29)	0.324
HBV genotype	–	–	–	<0.001	–	–	0.843
A	53 (3.3)	12 (2.5)	41 (3.6)	–	8 (2.5)	9 (2.8)	–
B	254 (15.7)	66 (14.0)	188 (16.4)	–	49 (15.5)	50 (15.8)	–
C	1,135 (70.3)	344 (72.9)	791 (69.2)	–	225 (71.2)	226 (71.5)	–
D	1 (0.06)	0	1 (0.09)	–	0	0	–
F	1 (0.06)	0	1 (0.09)	–	0	0	–
H	2 (0.1)	2 (0.4)	0	–	0	0	–
Unclassified / missing	169 (10.4)	48 (10.2)	121 (10.5)	–	34 (10.7)	31 (9.8)	–
Baseline HBeAg positive	617 (38)	219 (46)	398 (35)	<0.001	135 (43)	133 (42)	0.936
Baseline HBV DNA (log copies/mL)	6.0 (4.3-7.7)	6.7 (5.3-8.0)	5.8 (4.0-7.5)	<0.001	6.3 (5.2-7.9)	6.6 (4.5-7.8)	0.795
Baseline AST level (IU/L)	35 (22-63)	53 (35-95)	28 (20-50)	<0.001	45 (32-70)	49 (27-98)	0.956
Baseline AST level (x ULN)	1.1 (0.7-1.9)	1.6 (1.1-2.9)	0.8 (0.6-1.5)	<0.001	1.4 (1.0-2.1)	1.5 (0.8-3.0)	0.989
Baseline ALT level (IU/L)	42 (22-88)	70 (42-163)	33 (20-68)	<0.001	61 (39-109)	60 (28-144)	0.110
Baseline ALT level (x ULN)	1.1 (0.7-2.4)	1.9 (1.2-4.3)	0.9 (0.6-1.8)	<0.001	1.7 (1.0-3.3)	1.6 (0.8-3.7)	0.086
Baseline GGTP level (IU/L)	28 (16-59)	39 (24-72)	24 (14-52)	<0.001	34 (23-64)	34 (18-68)	0.088
Baseline total bilirubin level (mg/dL)	0.7 (0.5-0.9)	0.7 (0.5-1.0)	0.6 (0.5-0.9)	<0.001	0.7 (0.5-1.0)	0.7 (0.5-0.9)	0.210
Baseline serum albumin level (g/L)	4.2 (3.9-4.5)	3.9 (3.6-4.1)	4.4 (4.1-4.6)	<0.001	3.9 (3.7-4.2)	4.0 (3.8-4.3)	0.084
†Platelet count (10^5/mm^3) (SD)	19.1 (6.3)	16.9 (5.6)	20.0 (6.4)	<0.001	17.5 (5.2)	17.2 (6.0)	0.349
Follow-up duration (yrs)	5.4 (3.1-13.2)	3.2 (2.1-4.3)	9.5 (4.4-16.1)	<0.001	3.3 (2.3-4.3)	7.6 (3.4-13.7)	<0.001
Person-years of follow-up	13,986	1561	12381	–	1064	2978	–
No. of HCC cases	156	12	144	–	6	72	–
Incidence rates per 1000 person-years	11.15	7.69	11.63	–	5.63	24.1	–
Progression of cirrhosis within 5 year	21 (1.3)	0	21 (1.8)	0.001	0	10 (3.2)	0.001
HBV DNA <400 copies/mL at 1 yearr	–	421 (89)	NA	–	288 (90)	NA	–
Emergence of drug-resistant mutants during ETV treatment	–	4 (0.8)	NA	–	2 (0.6)	NA	–

HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; AST, aspartate aminotransferase; GGTP, gamma glutamyltransferase (ULN=33 IU/L); ALT, alanine aminotransferase (ULN=42 IU/L for men and 27 IU/L for women); HCC, hepatocellular carcinoma; ETV, entecavir.
*P <0.05.
**P <0.001, comparison of entecavir-treated group and control group.
†Data displayed as mean ± standard deviation. ‡All other values are expressed as median (25th to 75th percentile) or number (percentage of total, %).

matched control group were 4.0% at year 2, 7.2% at year 3, 10.0% at year 4, and 13.7% at year 5. Log-rank test revealed a statistically significant difference between the incidence of HCC in the ETV group and the control group over time ($P < 0.001$) (Fig. 2). We then used Cox proportional regression analysis to estimate the effects of ETV treatment on HCC risk. Factors that were associated with HCC at year 5 in the propensity score matched cohort were age, gender, alcohol consumption (>200 kg), the presence of cirrhosis, HBeAg positivity, baseline viral load, ALT, γ-GTP, total bilirubin, serum albumin, and platelet counts (Table 2). For ETV treatment effect, we estimated the hazard ratio of HCC development, adjusting for multiple baseline variables (age, gender, alcohol consumption, smoking, preexisting cirrhosis, HBeAg, HBV DNA, ALT, albumin, γ-GTP, total bilirubin, and platelet count) in the propensity matched cohort. Progression of cirrhosis within 5 years was used as a time-dependent covariate in the proportional hazard regression but it did not show a statistically significant hazard to HCC development.

Subanalyses Showing HCC Suppression Effect Between ETV and LAM. PS matching of the LAM-treated patients without rescue therapy (n = 492) with ETV-treated patients resulted in a matched cohort of 182 patients (Supporting Table 3). The rate of nonrescued LAM-treated group having undetectable HBV DNA at 1 year after treatment was lower when compared with the ETV-treated group. The LAM-treated group also had a higher drug-resistant mutation rate. Comparisons of HCC incidence among the ETV-treated group, nonrescued LAM-treated group, and control showed that the HCC suppression effect was greater in ETV-treated ($P < 0.001$) than nonrescued LAM-treated ($P = 0.019$) when compared with the

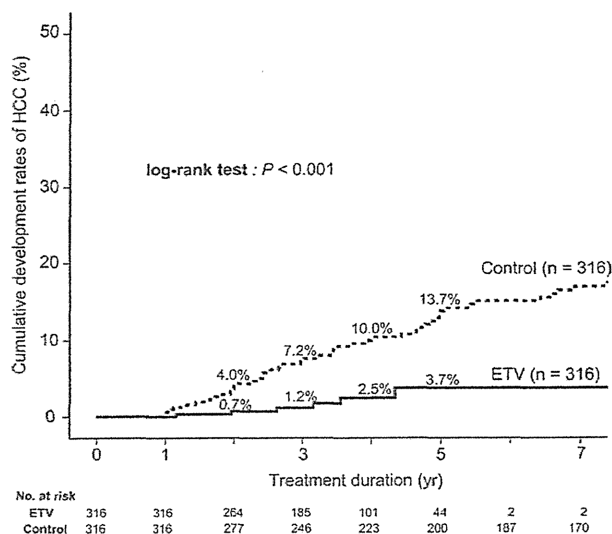

Fig. 2. Comparison of HCC cumulative incidence rates between the entecavir-treated group and the nontreated control group after propensity score matching. The log-rank test revealed a statistically significant difference between the ETV and the control group in the incidence of HCC at 5 years time (log-rank test: $P < 0.001$).

control group (Fig. 3). The difference of effect between ETV and LAM was also significant ($P = 0.043$). The treatment effect was seen in cirrhosis patients but not in noncirrhosis patients. The result showed ETV's superiority to LAM in suppressing HCC.

Effect of ETV on the Reduction of HCC Development by Preexisting Cirrhosis and Risk Scores. To further examine the ETV treatment effect, we compared the ETV and the control groups by preexisting cirrhosis and published risk scores. Viral response rates (HBV DNA < 400 copies/mL) of 1-year post-ETV treatment was 87% in the noncirrhosis patients and 91% in the cirrhosis patients (LC). ALT normalization was 94% and 90% in the chronic hepatitis and cirrhosis patients, respectively. The treatment effect was not inferior by cirrhosis status. Among those who developed HCC, 97 out of 144 patients in the control group and 9 out of 12 patients in the ETV group had cirrhosis. Interactions between preexisting cirrhosis and ETV treatment were not observed ($P = 0.177$).

Cumulative HCC incidence rates by risk scores are compared between the two cohorts in Fig. 4A-G. Figure 4A,B shows the risk scores developed by Yang et al.[10] Figure 4C,D shows the risk scores developed by Yuen et al.[11] Figure 4E-G shows the risk scores developed by Wong et al.[12] All three risk score scales showed that ETV significantly reduced HCC incidence in patients with a higher risk (risk score $\geq$12, $P = 0.006$; risk score $\geq$82, $P = 0.002$; medium risk, $P = 0.062$; high risk, $P < 0.001$). Interactions between risk scores and ETV treatment were not observed (Yang et al.: $P = 0.713$, Yuen et al.: $P = 0.267$, Wong et al.: $P = 0.265$).

Discussion

Our study suggests that long-term ETV therapy would significantly suppress the development of HCC in HBV-infected patients when compared with HBV-infected patients in the control group. The treatment effect was more prominent among patients at high risk of HCC than those at low risk.

Table 2. Factors Associated with HCC Development as Determined by Cox Proportional Hazard Regression Analysis at 5-Year (Propensity Score Matched Cohort)

Variable	Univariate HR (95% CI)	P	Multivariate Adjusted HR (95% CI)	P
Age (per year)	1.05 (1.02-1.07)	<0.001	1.06 (1.03-1.09)	<0.001
Gender (M)	2.81 (1.25-6.32)	0.012		
Alcohol consumption (>200kg)	2.71 (1.49-4.92)	0.001	2.21 (1.18-4.16)	0.013
Cigarette smoking	1.53 (0.84-2.80)	0.164		
Preexisting cirrhosis	12.0 (5.57-25.9)	<0.001	4.28 (1.88-9.73)	0.001
HBV genotype (C)	2.73 (0.98-7.65)	0.056		
HBeAg (positive)	2.64 (1.41-4.94)	0.002	2.26 (1.18-4.34)	0.014
HBV DNA ($\geq$5.0 log copies/mL)	4.66 (1.44-15.1)	0.010		
ALT ($\geq$45 IU/L)	2.29 (1.10-4.77)	0.027		
GGTP ($\geq$50 IU/L)	3.79 (2.02-7.09)	<0.001		
Total bilirubin ($\geq$1.5 mg/dL)	5.51 (2.87-10.6)	<0.001		
Serum albumin (<3.8 g/L)	4.44 (2.42-8.14)	<0.001		
Platelet count ($<1.5\times10^5$/mm^3)	14.8 (5.84-37.7)	<0.001	5.64 (2.13-15.0)	0.001
*Progression of cirrhosis within 5 years	1.80 (0.25-13.2)	0.562		
ETV treatment	0.23 (0.09-0.55)	0.001	0.37 (0.15-0.91)	0.030

Asterisks (*) indicate time-dependent covariates.

†Adjusted for age, gender, alcohol, cigarette, cirrhosis, genotype, HBeAg, HBV DNA, ALT, albumin, GGTP, total bilirubin, and platelet counts

Abbreviations: ETV, entecavir; HR, hazard ratio; CI, confidence interval; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; GGTP, gamma glutamyltransferase.

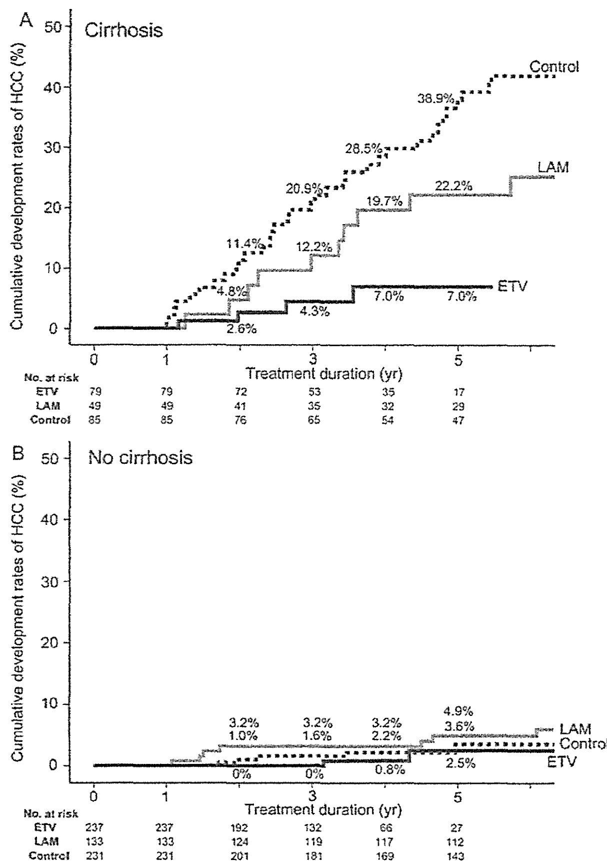

Fig. 3. Comparison of HCC cumulative incidence rates between the entecavir (ETV)-treated group, lamivudine (LAM)-treated, and the non-treated control group after PS matching stratified by cirrhosis. The log-rank test revealed a statistically significant difference in the incidence of HCC at 5 years time in cirrhosis patients: ETV versus control group ($P < 0.001$); LAM versus control ($P = 0.019$); ETV versus LAM ($P = 0.043$). The differences were not seen in the noncirrhosis patients: ETV versus control ($P = 0.440$); LAM versus control ($P = 0.879$); ETV versus LAM ($P = 0.126$).

HBV has been previously shown to influence HCC development. Ikeda et al.[20] reported that the cumulative HCC incidence rates among Japanese HBV patients were 2.1% at 5 years, 4.9% at 10 years, and 18.8% at 15 years among NA-naïve patients. Other studies, both from Japan and other countries, have reported a 5-year cumulative HCC incidence rate of 3.3% among chronic HBV, and 21.2% to 59% among cirrhosis patients.[21,22] The incidence of HCC varies significantly by country and ethnic group,[4] which seems to be attributable to diverse exposure to HCC risk factors.

Carcinogenicity related to HBV infection is somewhat complex and multifactorial when compared with carcinogenicity related to HCV infection. Known HCC risk factors among HBV-infected patients include older age, male gender, cirrhotic status, diabetes mellitus, family history, alcohol consumption, AST, HBsAg, HBeAg, and genotype C.[20,23,25] Chen et al.[5] found a dose-response relationship between pretreatment serum HBV DNA levels and the development of HCC. Baseline ALT is another risk factor for HCC, as elevated ALT levels indicate an active immune response against HBV, resulting in repetitive hepatocyte injury.[5] Our study corroborates these findings on these factors influence on HCC development.

The potential ability of ETV to reduce the risk of HCC is an additional example of a long-term NA treatment effect. Some studies have shown that ETV has low incidence of HCC but these studies did not have a control arm.[9] A meta-analysis and a systematic review showed that NAs can reduce liver complications, including HCC.[26,27] Other studies have begun to show that control of sustained viral loads through drugs such as NAs is important in preventing long-term complications. Chen et al.[28] showed that greater decreases in serum HBV DNA levels ($<10^4$ copies/mL) during follow-up were associated with a lower risk of HCC.

Our comparison among the PS-matched ETV-treated group, nonrescued LAM-treated patients, and the control showed that ETV is superior to LAM in HCC suppression. Kurokawa et al.[29] showed that treatment with lamivudine for an average of 5 years reduced the incidence of HCC in HBV-infected cirrhosis patients, who showed sustained viral response at a median HBV DNA of <4.0 log copies/mL. Unfortunately, only 48% of the patients in this study achieved sustained viral response, while 51% developed lamivudine-resistant tyrosine-methionine-aspartate-aspartate mutation (YMDD mutation) during follow-up.[29] Patients with drug resistance were reported to have a 2.6 times greater chance of developing long-term complications.[26] A systematic review of 21 studies showed that HCC occurred more (2.3% versus 7.5%, $P < 0.001$) in nonresponding patients or in patients with viral breakthrough compared with those who experienced remission.[28] On-treatment drug resistance could subject patients to a variable viral status. Suppression of HCC by NAs requires NAs that do not lead to drug resistance. Compared with other NAs, ETV shows minimal drug resistance. Our results showed that ~90% of the ETV-treated patients had sustained viral suppression at year 1, and that drug resistance was minimal (0.8%) during the median follow-up period of 3.2 years.

We found that the effect of ETV treatment in reducing the risk of HCC was more prominent among high-risk patients. This phenomenon was observed by examining the combination of parameters associated with the recently developed risk scores (Fig. 4). The published risk scores were developed mainly to create

8. Yokosuka O, Takaguchi K., Fujioka S, Shindo M, Chayama K, Kobashi H, et al. Long-term use of entecavir in nucleoside-naïve Japanese patients with chronic hepatitis B infection. J Hepatol 2010;52:791-799.
9. Chang TT, Lai CL, Yoon SK, Lee SS, Coelho HSM, Carrilho FJ, et al. Entecavir treatment for up to 5 years in patients with hepatitis B e antigen-positive chronic hepatitis B. HEPATOLOGY 2010;51:422-430.
10. Yang HI, Yuen MF, Chan HLY, Han KH, Chen PJ, Kim DY, et al. Risk estimation for hepatocellular carcinoma in chronic hepatitis B (REACH-B): development and validation of a predictive score. Lancet Oncol 2011;12:568-574.
11. Yuen MF, Tanaka Y, Fong DYT, Fung J, Wong DKH, Yuen JCH, et al. Independent risk factors and predictive score for the development of hepatocellular carcinoma in chronic hepatitis B. J Hepatol 2009;50:80-88.
12. Wong VWS, Chan SL, Mo F, Chan TC, Loong HH, Wong GL, et al. Clinical scoring system to predict hepatocellular carcinoma in chronic hepatitis B carriers. J Clin Oncol 2010;28:1660-1665.
13. Yang HI, Sherman M, Su J, Chen PJ, Liaw YF, Iloeje UH, et al. Nomograms for risk of hepatocellular carcinoma in patients with chronic hepatitis B virus infection. J Clin Oncol 2010;28:2437-2444.
14. Rosenbaum PR, Rubin DB. Reducing bias in observational studies using subclassification on the propensity score. J Am Stat Assoc 1984; 79:516-524.
15. Braitman LE, Rosenbaum PR. Rare outcomes, common treatments: analytic strategies using propensity scores. Ann Intern Med 2002;137:693-695.
16. Rosenbaum PR, Rubin DB. Constructing a control group using multivariate matched sampling methods that incorporate the propensity score. J Am Stat Assoc 1985;39:33-38.
17. D'Agostino RB Jr. Propensity score methods for bias reduction in the comparison of a treatment to a non-randomized control group. Stat Med 1998;17:2265-2281.
18. Gray RJ. A class of k-sample tests for comparing the cumulative incidence of a competing risk. Ann Stat 1988;16:1141-1154.
19. Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. J Am Stat Assoc 1999;94:496-509.
20. Ikeda K, Saitoh S, Suzuki Y, Kobayashi M, Tsubota A, Koida I, et al. Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. J Hepatol 1998;28:930-938.
21. Kato Y, Nakata K, Omagari K, Furukawa R, Kusumoto Y, Mori I, et al. Risk of hepatocellular carcinoma in patients with cirrhosis in Japan. Cancer 1994;74:2234-2238.
22. Lo KJ, Tong MJ, Chien MC, Tsai YT, Liaw YF, Yang KC, et al. The natural course of hepatitis B surface antigen-positive chronic active hepatitis in Taiwan. J Infect Dis 1982;146:205-210.
23. Yang HI, Lu SN, Liaw YF, You SL, Sun CA, Wang LY, et al. Hepatitis B e antigen and the risk of hepatocellular carcinoma. N Engl J Med 2022;347:168-174.
24. Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology 2000;118:554-559.
25. Chen CJ, Yang HI, Iloeje UH. Hepatitis B virus DNA levels and outcomes in chronic hepatitis B. HEPATOLOGY 2009;49:S72-S84.
26. Zhang Q-Q, An X, Liu YH, Li SY, Zhong Q, Wang J, et al. Long-term nucleos(t)ide analogues therapy for adults with chronic hepatitis B reduces the risk of long-term complications: a meta-analysis. Virol J 2011;8:72.
27. Papatheodoridis GV, Lampertico P, Manolakopoulos S, Lok A. Incidence of hepatocellular carcinoma in chronic hepatitis B patients receiving nucleos(t)ide therapy: a systematic review. J Hepatol 2010;53: 348-356.
28. Chen CF, Lee WC, Yang HI, Chang HC, Jen CL, Iloeje UH, et al. Changes in serum levels of HBV DNA and alanine aminotransferase determine risk for hepatocellular carcinoma. Gastoenterology 2011;141: 1240-1248.
29. Kurokawa M, Hiramatsu N, Oze T, Yakushijin T, Miyazaki M, Hosui A, et al. Long-term effect of lamivudine treatment on the incidence of hepatocellular carcinoma in patients with hepatitis B virus infection. J Gastroenterol 2012;47:577-585.

Journal of Medical Virology 85:1028–1036 (2013)

Emergence of Telaprevir-Resistant Variants Detected by Ultra-Deep Sequencing After Triple Therapy in Patients Infected With HCV Genotype 1

Norio Akuta,[1*] Fumitaka Suzuki,[1] Yuya Seko,[1] Yusuke Kawamura,[1] Hitomi Sezaki,[1] Yoshiyuki Suzuki,[1] Tetsuya Hosaka,[1] Masahiro Kobayashi,[1] Tasuku Hara,[1] Mariko Kobayashi,[2] Satoshi Saitoh,[1] Yasuji Arase,[1] Kenji Ikeda,[1] and Hiromitsu Kumada[1]

[1]*Department of Hepatology, Toranomon Hospital, and Okinaka Memorial Institute for Medical Research, Tokyo, Japan*
[2]*Liver Research Laboratory, Toranomon Hospital, Tokyo, Japan*

Using ultra-deep sequencing technology, the present was designed to investigate whether the emergence of telaprevir-resistant variants (amino acid substitutions of aa36, aa54, aa155, aa156, and aa170 positions in HCV NS3 region) after commencement of triple therapy of telaprevir/peginterferon (PEG-IFN)/ribavirin could be predicted at baseline in previous non-responders to dual therapy. Fourteen patients infected with HCV genotype 1 who did not respond to previous PEG-IFN/ribavirin, received a 24-week regimen of triple therapy, and were evaluated for appearance of telaprevir-resistant variants (amino acid substitutions of more than 0.2% among the total coverage) by ultra-deep sequencing. The sustained virological response rate was 28.6% (4 of 14 patients), which was significantly higher in patients with Arg70 (substitution at core aa70) and partial response (type of previous response to PEG-IFN/ribavirin) than in other patients. Telaprevir-resistant variants at baseline were detected in 7.1% (1 of 14 patients) by direct sequencing and in 21.4% (3 of 14 patients) by ultra-deep sequencing. The appearance of telaprevir-resistant variants was examined by ultra-deep sequencing in 10 who did not show sustained virological responders. De novo variants emerged at re-elevation of viral load, regardless of variant frequencies at baseline (one patient with very high frequency variants [T54S: 99.9%], two patients with very low frequency variants [V36A: 0.2%; and V170A: 0.4%], and seven patients of undetectable variants). It is concluded that it is difficult to predict at baseline the emergence of telaprevir-resistant variants after commencement of triple therapy in prior non-responders of HCV genotype 1, even with the use of ultra-deep sequencing. ***J. Med. Virol. 85:1028–1036, 2013.***

© 2013 Wiley Periodicals, Inc.

KEY WORDS: HCV; ultra-deep sequence; telaprevir; resistant variants; non-response

INTRODUCTION

New strategies have been introduced for the treatment of chronic hepatitis C virus (HCV) infection based on inhibition of protease in the NS3/NS4 of the HCV polyprotein. Of these, telaprevir (VX-950) was selected as a candidate agent for treatment of chronic HCV infection [Lin et al., 2006]. Three studies (PROVE1, PROVE2, and Japanese study) showed that a 24-week regimen of triple therapy (telaprevir, peginterferon [PEG-IFN], and ribavirin) for 12 weeks followed by dual therapy (PEG-IFN and ribavirin) for 12 weeks (also called the T12PR24 regimen) achieved sustained virological response (lasting more than 24 weeks after withdrawal of treatment) rates of 61%, 69%, and 73%, respectively, in patients infected with HCV genotype 1 (HCV-1) [Hézode et al., 2009; McHutchison et al., 2009; Kumada et al., 2012]. However, a recent study (PROVE3) showed lower sustained virological response rates for the T12PR24 regimen (39%) in non-responders to previous PEG-IFN/ribavirin therapy infected with HCV-1, who did not achieve HCV-RNA negativity during or at the

Grant sponsor: Ministry of Health, Labor and Welfare, Government of Japan; Grant sponsor: Ministry of Education Culture Sports Science and Technology, Government of Japan.

*Correspondence to: Norio Akuta, MD, Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-0001, Japan. E-mail: akuta-gi@umin.ac.jp

Accepted 30 January 2013
DOI 10.1002/jmv.23579
Published online in Wiley Online Library (wileyonlinelibrary.com).

© 2013 WILEY PERIODICALS, INC.

end of the initial combination therapy [McHutchison et al., 2010]. Furthermore, telaprevir-based regimen is reported to induce resistant variants [Lin et al., 2005; Kieffer et al., 2007]. Thus, there is a need to determine the predictive factors for non-response to triple therapy before administration of such treatment in order to avoid the appearance of telaprevir-resistant variants.

Though Sanger sequencing has been used to determine viral sequences, ultra-deep sequencing technology is much faster and can perform large-scale sequencing. Recent reports have indicated that ultra-deep sequencing technology provides a better understanding of the dynamics of variants in HCV quasispecies [Bull et al., 2011; Hiraga et al., 2011; Nasu et al., 2011; Ninomiya et al., 2012]. However, it is not clear at this stage whether this can be useful to predict treatment response and treatment resistant variants, including telaprevir-resistant variants by triple therapy.

The aim of this study using ultra-deep sequencing technology was to investigate whether the presence of low frequency resistant variants at baseline could predict the emergence of telaprevir-resistant variants after the start of telaprevir/PEG-IFN/ribavirin triple therapy, in adult Japanese patients infected with HCV-1 who did not respond to previous PEG-IFN/ribavirin dual therapy.

PATIENTS AND METHODS

Study Patients

Between May 2008 and September 2009, 61 patients infected with HCV were recruited in this study at the Department of Hepatology, Toranomon Hospital, which is located in Metropolitan Tokyo. The study protocol was in compliance with the Good Clinical Practice Guidelines and the 1975 Declaration of Helsinki, and was approved by the institutional review board. Each patient gave an informed consent before participation in this trial. Patients were assigned to a 24-week regimen of triple therapy (telaprevir [MP-424], PEG-IFN and ribavirin) for 12 weeks followed by dual therapy of PEG-IFN and ribavirin for 12 weeks (the T12PR24 regimen).

Fourteen of the 61 patients met the following inclusion and exclusion criteria: (1) diagnosis of chronic hepatitis C. (2) HCV-1b confirmed by sequence analysis. (3) HCV RNA levels of $\geq$5.0 log IU/ml determined by the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). (4) Japanese (Mongoloid) ethnicity. (5) Age at study entry of 20–65 years. (6) Body weight $\geq$35 and $\leq$120 kg at the time of registration. (7) Absence of decompensated cirrhosis of the liver. (8) No detectable hepatitis B surface antigen in serum. (9) No history of hepatocellular carcinoma. (10) No previous treatment for malignancy. (11) No history of autoimmune hepatitis, alcohol liver disease, hemochromatosis, or chronic liver disease other than chronic hepatitis C. (12) No history of depression, schizophrenia or suicide attempts, hemoglobinopathies, angina pectoris, cardiac insufficiency, myocardial infarction or severe arrhythmia, uncontrollable hypertension, chronic renal dysfunction or creatinine clearance of $\leq$50 ml/min at baseline, diabetes requiring treatment or fasting glucose level of $\geq$110 mg/dl, autoimmune disease, cerebrovascular disorders, thyroid dysfunction uncontrollable by medical treatment, chronic pulmonary disease, allergy to medication or anaphylaxis at baseline. (13) Hemoglobin level of $\geq$12 g/dl, neutrophil count $\geq$1,500/mm^3, and platelet count of $\geq$100,000/mm^3 at baseline. Pregnant or breast-feeding women or those willing to become pregnant during the study and men with a pregnant partner were excluded from the study. (14) Prior non-responders, who did not achieve HCV-RNA negativity during or at the end of 24- to 48-week PEG-IFN plus ribavirin combination therapy.

Non-response to previous therapy was defined as null response (a reduction of less than 2 $\log_{10}$ in HCV RNA during treatment) or partial response (a reduction of 2 $\log_{10}$ or more in HCV RNA during treatment). Table I summarizes the profiles and laboratory data of the 14 patients at commencement of treatment with the T12PR24 regimen. The study patients included seven males and seven females, aged 40–65 years (median, 56 years).

All 14 patients were followed-up for at least 24 weeks after the completion of treatment. The efficacy of treatment was evaluated by HCV-RNA negativity at 24 weeks after the completion of therapy (sustained virological response), based on the COBAS TaqMan HCV test (Roche Diagnostics). Furthermore, failure to achieve sustained virological response was classified as non-response (HCV-RNA detected during or at the end of treatment), viral breakthrough (re-elevation of viral loads before the end of treatment, even when HCV-RNA was temporarily negative during treatment), and relapse (re-elevation of viral loads after the end of treatment, even when HCV-RNA was negative at the end of treatment).

Telaprevir (MP-424; Mitsubishi Tanabe Pharma, Osaka, Japan) was administered at 750 mg three times a day at an 8-hr (q8) interval after the meal. PEG-IFNα-2b (PEG-Intron; Schering Plough, Kenklworth, NJ) was injected subcutaneously at a median dose of 1.5 μg/kg (range: 1.3–1.7 μg/kg) once a week. Ribavirin (Rebetol; Schering Plough) was administered at 200–600 mg twice a day after breakfast and dinner (daily dose: 600–1,000 mg). PEG-IFN and ribavirin were discontinued or their doses reduced, as required, upon reduction of hemoglobin level, leukocyte count, neutrophil count or platelet count, or the development of adverse events. Thus, the dose of PEG-IFN was reduced by 50% when the leukocyte count decreased below 1,500/mm^3, neutrophil count below 750/mm^3 or platelet count below 80,000/mm^3; PEG-IFN was discontinued when these counts decreased below 1,000/mm^3, 500/mm^3, or 50,000/mm^3, respectively. When hemoglobin decreased to $<$10 g/dl, the daily dose of ribavirin was reduced from 600 to 400 mg, from 800 to 600 mg,

TABLE I. Profile and Laboratory Data at Commencement of Telaprevir, Peginterferon and Ribavirin Triple Therapy of 14 Japanese Patients Infected With HCV Genotype 1b, Who Did Not Respond to Previous Peginterferon Plus Ribavirin Combination Therapy

Demographic data	
Number of patients	14
HCV genotype 1b	14
Japanese (Mongoloid) ethnicity	14
Sex (male/female)	7/7
Age (years)*	56 (40–65)
History of blood transfusion	3 (21.4%)
Family history of liver disease	2 (14.3%)
Body mass index (kg/m^2)*	23.0 (18.1–26.5)
Laboratory data*	
Level of viremia (log IU/ml)	6.7 (5.8–7.4)
Serum aspartate aminotransferase (IU/L)	35 (20–108)
Serum alanine aminotransferase (IU/L)	45 (17–135)
Serum albumin (g/dl)	3.9 (3.4–4.5)
Gamma-glutamyl transpeptidase (IU/L)	50 (20–154)
Leukocyte count (/mm^3)	4,500 (3,300–6,500)
Hemoglobin (g/dl)	14.5 (12.6–16.6)
Platelet count ($\times 10^4$/mm^3)	16.2 (10.4–23.9)
Alpha fetoprotein (μg/L)	7 (2–38)
Total cholesterol (mg/dl)	180 (132–228)
Fasting plasma glucose (mg/dl)	89 (81–102)
Treatment*	
PEG-IFNα-2b dose (μg/kg)	1.5 (1.3–1.7)
Ribavirin dose (mg/kg)	11.7 (8.1–14.5)
Amino acid substitutions in the HCV genotype 1b	
Core aa 70 (arginine/glutamine (histidine))	6/8
Core aa 91 (leucine/methionine)	6/8
ISDR of NS5A (wild-type/non wild-type)	13/1
IL28B genotype	
rs8099917 genotype (TT/TG/GG)	1/11/2
Type of previous response to peginterferon/ribavirin	
Partial response/null response	8/6

ND, not determined.
Data are number and percentages of patients.
*Median (range) values.

and from 1,000 to 600 mg, depending on the initial dose. Ribavirin was withdrawn when hemoglobin decreased to <8.5 g/dl. However, the dose of telaprevir (MP-424) remained the same, and its administration was stopped only when the discontinuation was considered appropriate for the development of adverse events. In those patients in whom telaprevir was discontinued, treatment with PEG-IFNα-2b and ribavirin was also terminated.

Measurement of HCV RNA

The antiviral effects of the triple therapy on HCV were assessed by measuring plasma HCV RNA levels. In this study, HCV RNA levels during treatment were evaluated at least once every month before, during, and after therapy. HCV RNA concentrations were determined using the COBAS TaqMan HCV test (Roche Diagnostics). The linear dynamic range of the assay was 1.2–7.8 log IU/ml, and the undetectable samples were defined as negative.

Assessment of Telaprevir-Resistant Variants

The genome sequence of the *N*-terminal 609 nucleotides (203 amino acids) in the NS3 region of HCV isolates from the patients was examined before, during, and after triple therapy. HCV RNA was extracted from 100 μl of serum and the nucleotide sequences were determined by direct sequencing and deep sequencing. The primers used to amplify the NS3 region were NS3-F1 (5′-ACA CCG CGG CGT GTG GGG ACA T-3′; nucleotides 3295–3316) and NS3-AS2 (5′-GCT CTT GCC GCT GCC AGT GGG A-3′; nucleotides 4040–4019) as the first (outer) primer pair and NS3-F3 (5′-CAG GGG TGG CGG CTC CTT-3′; nucleotides 3390–3407) and NS3-AS2 as the second (inner) primer pair [Akuta et al., 2012a,b; Suzuki et al., 2012]. Thirty-five cycles of first and second amplifications were performed as follows: denaturation for 30 sec at 95°C, annealing of primers for 1 min at 63°C, extension for 1 min at 72°C, and final extension was performed at 72°C for 7 min. The PCR-amplified DNA was purified after agarose gel electrophoresis, and then used for direct sequencing and deep sequencing.

All patients were tested at baseline. The analysis was also repeated at the time of re-elevation of viral loads in those patients who did not achieve sustained virological response. Telaprevir-resistant variants included V36A/C/M/L/G, T54A/S, R155K/T/I/M/G/L/S/Q, A156V/T/S/I/G, V170A [Barbotte et al., 2010; Romano et al., 2010].

Direct sequencing was analyzed by standard Sanger sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator v1.1 Cycle Sequencing kit (Life Technologies, Carlsbad, CA) [Akuta et al., 2012a,b; Suzuki et al., 2012]. On the other hand, ultra-deep sequencing was performed using the Ion Personal Genome Machine (PGM) Sequencer (Life Technologies). An Ion Torrent adapter-ligated library was prepared using an Ion Xpress Plus Fragment Library Kit (Life Technologies). Briefly, 100 ng of fragmented genomic DNA was ligated to the Ion Torrent adapters P1 and A. The adapter-ligated products were nick-translated and PCR-amplified for a total of eight cycles. Subsequently, the library was purified using AMPure beads (Beckman Coulter, Brea, CA) and the concentration determined using the StepOne Plus RealTime PCR (Life Technologies) and Ion Library Quantitation Kit, according to the instructions provided by the manufacturer. Emulsion PCR was performed using Ion OneTouch (Life Technologies) in conjunction with Ion OneTouch 200 Template Kit v2 (Life Technologies). Enrichment for templated Ion spheres particles (ISPs) was performed using Ion OneTouch Enrichment System (Life Technologies), according to the instructions provided by the manufacturer. Templated ISPs was loaded onto an Ion 314 chip, and subsequently sequenced using 130 sequencing cycles according to the Ion PGM 200 Sequencing Kit user guide. Total output read length per run is over 10 Mbase (0.5M-tag, 200 base read) [Elliott et al., 2012]. The results were analyzed with the CLC Genomics Workbench software (CLCbio, Aarhus, Denmark) [Vogel et al., 2012].

A control experiment was included to validate the error rates in ultra-deep sequencing of the viral genome. In this study, amplification products of the second-round PCR were ligated with plasmid and transformed in *Escherichia coli* in a cloning kit (TA Cloning; Invitrogen, Carlsbad, CA). A plasmid-derived NS3 sequence was determined as the template, by the control experiment. The numbers of coverage evaluated per position for aa36, aa54, aa155, aa156, and aa170 in NS3 region, were 359379, 473716, 106435, 105979, and 49058, respectively. Thus, using the control experiment based on plasmid encoding HCV NS3 sequence, amino acid mutations were defined as amino acid substitutions at frequency of more than 0.2% among the total coverage. This frequency ruled out putative errors caused by deep sequence platform used in this study (Table II).

Detection of Amino Acid Substitutions in Core, and NS5A Regions of HCV-1b

With the use of HCV-J (accession no. D90208) as a reference [Kato et al., 1990], the sequence of 1–191 aa in the core protein of HCV-1b was determined and then compared with the consensus sequence constructed in a previous study to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and aa 91 of leucine (Leu91) or methionine (Met91) [Akuta et al., 2005, 2007, 2012a,b]. The sequence of 2,209–2,248 aa in the NS5A of HCV-1b (ISDR) reported by Enomoto et al. [1996] was determined, and the numbers of aa substitutions in ISDR were defined as wild-type (0, 1) or non wild-type (≥ 2) in comparison with HCV-J. In the present study, aa substitutions of the core region, and NS5A-ISDR of HCV-1b were analyzed by direct sequencing.

Determination of *IL28B* Genotype

IL28B (rs8099917 and rs12979860) were genotyped by the Invader assay, TaqMan assay, or direct sequencing, as described previously [Ohnishi et al., 2001; Suzuki et al., 2003].

Statistical Analysis

Non-parametric tests (chi-squared test and Fisher's exact probability test) were used to determine those factors that significantly contributed to sustained virological response and end-of-treatment response. All P-values less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were determined. For statistical analysis, each variable was transformed into categorical data consisting of two simple ordinal numbers. The potential pretreatment factors associated with sustained virological response included the following variables: sex, age, body mass index, HCV RNA level, type of previous response to PEG-IFN/ribavirin, *IL28B* genotype, and amino acid substitution in the core region/NS5A-ISDR.

TABLE II. Error Rates of Ultra-Deep Sequencing for the Plasmid Encoding HCV NS3 Sequence, Determined by the Control Experiment

Position	Coverage	Frequencies (%)	Error rates (%)[a]
aa36	359,415	V (99.9%), A/F/I (0.1%)	0.1
aa54	473,716	T (99.9%), A/I (0.1%)	0.1
aa155	106,435	R (99.9%), Q/W (0.1%)	0.1
aa156	105,979	A (99.9%), T/V (0.1%)	0.1
aa170	49,058	V (99.9%), A/I (0.1%)	0.1

[a]Amino acid mutations were defined as amino acid substitutions at a frequency of more than 0.2% among the total coverage. This frequency ruled out putative errors caused by deep sequence platform used in this study.

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, Inc., Chicago, IL).

RESULTS

Virological Response to Therapy

Table III summarizes the profiles and laboratory data of the 14 patients at commencement of triple therapy, virological course, and efficacy of treatment. The sustained virological response rate was 28.6% (four patients [Cases 1–4]). Of the 10 patients (Cases 5–14) who did not show sustained virological response, the relapse, breakthrough and non-response rates were 50.0% (five patients [Cases 5–9]), 40.0% (4 [Cases 10–13]), and 10.0% (one [Cases 14]), respectively. Two patients (Cases 10, 13) stopped telaprevir before the completion of 12-week treatment (PEG-IFN and ribavirin continued), and one patient (Case 9) stopped the triple therapy at 9 weeks before the completion of the 24-week regimen, due to a fall in Hb concentration.

Thirteen of 14 patients showed *IL28B* rs8099917 non-TT and rs12979860 non-CC, whereas the other one patient (Case 4) had rs8099917 TT and rs12979860 CC. Thus, in non-responders to previous treatment, *IL28B* genotype did not play a role in sustained virological response. The sustained virological response rate was significantly higher in patients with Arg70 (66.7% [four of six patients]) than in those with Gln70(His70) (0% [zero of eight]; $P = 0.015$). Furthermore, the rate tended to be higher in patients with partial response to previous treatment (50.0% [four of eight patients]) than those with null response (0% [zero of six]; $P = 0.085$). Especially, the sustained virological response rate was significantly higher in patients with Arg70 plus partial response (80.0% [four of five patients]) than in other patients (0% [zero of nine]; $P = 0.005$). Thus, all four patients (100%) who achieved sustained virological response had Arg70 and showed partial response (Table III).

Detection of Telaprevir-Resistant Variants by Direct and Ultra-Deep Sequencing

Baseline telaprevir-resistant variants were detected by direct sequencing in 7.1% (one patient [Case 12 with T54S]), and by ultra-deep sequencing in 21.4% (three patients [Case 9 with V170A: 0.4% of 29,881 coverage], [Case 11 with V36A: 0.2% of 27,915 coverage], and [Case 12 with T54S: 99.9% of 33,830 coverage]; Table IV).

Of 10 patients who did not show sustained virological response to triple therapy, telaprevir-resistant variants were detected by direct sequencing during and after treatment in 80.0% (eight patients [Cases 7–14]), and not detected in 20.0% (two patients [Cases 5, 6]). However, telaprevir-resistant variants were detected by ultra-deep sequencing during and after treatment in all 10 patients (Cases 5–14; Table IV).

Evolution of Telaprevir-Resistant Variants Over Time Detected by Ultra-Deep Sequencing

In 3 (Cases 9, 11, 12) of 10 patients who did not show sustained virological response to triple therapy, telaprevir-resistant variants were detected by ultra-deep sequencing at baseline. In Case 9 (relapse), very low frequency variants of V170A (0.4% of 29,881 coverage) at baseline were replaced after treatment by de novo very high frequency variants of A156T (99.6% of 14,757 coverage). In Case 11 (breakthrough), very low frequency variants of V36A (0.2% of 27,915 coverage) at baseline persisted during treatment as very low frequency variants of V36A (0.2% of 5,835 coverage), but de novo high frequency

TABLE III. Profile at Commencement of Triple Therapy, Virological Course, and Efficacy of Treatment

Case	Sex	Age (yrs)	BMI (kg/m^2)	*IL28B*	Core aa70	NS5A ISDR	Previous response	RNA (log IU/ml) Baseline	12 weeks	24 weeks	48 weeks	Efficacy
1	M	50	22.6	TG/CT	Arg70	Wild	Partial	6.6	Negative	Negative	Negative	SVR
2	F	52	25.3	TG/CT	Arg70	Wild	Partial	7.3	Negative	Negative	Negative	SVR
3	M	63	25.5	TG/CT	Arg70	Wild	Partial	7.0	Negative	Negative	Negative	SVR
4	M	50	18.1	TT/CC	Arg70	Wild	Partial	6.6	Negative	Negative	Negative	SVR
5	F	61	26.5	GG/TT	Arg70	Wild	Null	7.1	Negative	Negative	6.9	Relapse
6	M	56	23.6	TG/CT	Gln70	Wild	Partial	6.6	Negative	Negative	6.0	Relapse
7	M	48	24.9	TG/CT	Gln70	Wild	Partial	6.7	Negative	Negative	6.1	Relapse
8	M	40	23.3	TG/CT	Gln70	Wild	Partial	6.4	Negative	Negative	6.8	Relapse
9[a]	F	65	22.7	GG/TT	Gln70	Wild	Null	5.8	Negative[b]	—	6.6[c]	Relapse
10	F	59	22.7	TG/CT	Arg70	Wild	Partial	6.3	3.9	4.3	6.0	Breakthrough
11	M	47	23.7	TG/CT	Gln70	Wild	Null	7.2	Negative	3.8	7.6	Breakthrough
12	F	60	20.9	TG/CT	Gln70	Non-Wild	Null	6.4	Negative	2.1	6.0	Breakthrough
13	F	63	20.4	TG/CT	Gln70	Wild	Null	6.8	2.7	5.9	7.2	Breakthrough
14	F	55	21.0	TG/CT	Gln70	Wild	Null	7.4	4.2	6.9	7.6	Non-response

IL28B, rs8099917/rs12979860 genotypes; SVR, sustained virological response.
[a]Case 9 stopped the triple therapy at 9 weeks due to a fall in Hb concentration.
[b]At 9 weeks.
[c]HCV RNA at 24 weeks after stopping treatment.

TABLE IV. Detection of Telaprevir-Resistant Variants by Direct and Ultra-Deep Sequencing, at Two Time Points (Baseline and Re-Elevation of Viral Load)

Case	Position	At point of baseline Direct	At point of baseline Deep	At point of baseline Coverage	Viral loads	At point of re-elevation of viral loads Direct	At point of re-elevation of viral loads Deep	At point of re-elevation of viral loads Coverage	Viral loads	Efficacy
1	aa36	—	—	31,204	6.6	ND	ND	ND	ND	SVR
	aa54	—	—	33,284		ND	ND	ND		
	aa155	—	—	19,468		ND	ND	ND		
	aa156	—	—	22,657		ND	ND	ND		
	aa170	—	—	20,762		ND	ND	ND		
2	aa36	—	—	44,203	7.4	ND	ND	ND	ND	SVR
	aa54	—	—	66,117		ND	ND	ND		
	aa155	—	—	48,863		ND	ND	ND		
	aa156	—	—	55,519		ND	ND	ND		
	aa170	—	—	60,022		ND	ND	ND		
3	aa36	—	—	43,620	7.0	ND	ND	ND	ND	SVR
	aa54	—	—	58,753		ND	ND	ND		
	aa155	—	—	33,249		ND	ND	ND		
	aa156	—	—	36,227		ND	ND	ND		
	aa170	—	—	33,005		ND	ND	ND		
4	aa36	—	—	60,773	6.6	ND	ND	ND	ND	SVR
	aa54	—	—	68,541		ND	ND	ND		
	aa155	—	—	46,512		ND	ND	ND		
	aa156	—	—	48,389		ND	ND	ND		
	aa170	—	—	62,197		ND	ND	ND		
5	aa36	—	—	47,769	7.1	—	—	34,279	5.8	Relapse
	aa54	—	—	66,508		—	—	31,842		
	aa155	—	—	23,751		—	Q (0.2%)	11,572		
	aa156	—	—	25,317		—	T (0.2%)	16,040		
	aa170	—	—	30,807		—	—	10,637		
6	aa36	—	—	70,158	6.6	—	—	49,523	6.0	Relapse
	aa54	—	—	78,419		—	—	73,216		
	aa155	—	—	15,606		—	—	35,998		
	aa156	—	—	15,175		—	T (1.4%)	56,171		
	aa170	—	—	15,218		—	—	52,691		
7	aa36	—	—	40,035	6.7	A	A (81.6%)	16,952	4.5	Relapse
	aa54	—	—	52,685		—	A (0.2%)	30,353		
	aa155	—	—	19,171		—	Q (1.8%)	17,847		
	aa156	—	—	21,407		—	T (0.7%)	19,988		
	aa170	—	—	26,457		—	—	17,339		
8	aa36	—	—	63,035	6.4	—	—	29,802	5.4	Relapse
	aa54	—	—	54,526		A	A (98.2%) · S (0.9%)	23,781		
	aa155	—	—	60,030		—	K (0.3%)	26,846		
	aa156	—	—	59,571		—	—	30,403		
	aa170	—	—	44,816		—	—	24,467		
9	aa36	—	—	32,598	5.8	—	—	8,968	5.4	Relapse
	aa54	—	—	29,903		—	—	14,013		
	aa155	—	—	22,440		—	—	14,499		
	aa156	—	—	26,318		T	T (99.6%)	14,757		
	aa170	—	A (0.4%)	29,881		—	—	17,139		
10	aa36	—	—	299,668	6.3	M	M (94.6%)	51,403	3.9	Breakthrough
	aa54	—	—	370,253		—	S (0.2%)	42,176		
	aa155	—	—	123,791		—	—	9,581		
	aa156	—	—	126,538		—	S (0.7%)	13,028		
	aa170	—	—	116,279		—	—	5,033		
11	aa36	—	A (0.2%)	27,915	7.2	—	A (0.2%)	5,853	3.8	Breakthrough
	aa54	—	—	31,822		A	A (27.7%)	5,032		
	aa155	—	—	8,016		—	Q (0.4%)	9,873		
	aa156	—	—	8,134		—	T (0.3%)	17,178		
	aa170	—	—	19,360		—	—	27,246		
12	aa36	—	—	30,743	6.4	—	C (5.4%) · A (0.2%)	16,095	5.8	Breakthrough
	aa54	S	S (99.9%)	33,830		S	S (99.7%)	26,348		
	aa155	—	—	14,931		K	K (96.1%) · Q (0.4%)	20,630		
	aa156	—	—	15,930		—	—	22,087		
	aa170	—	—	38,447		—	—	24,204		
13	aa36	—	—	75,460	6.8	—	C (0.4%)	36,193	2.7	Breakthrough
	aa54	—	—	80,066		—	S (0.9%) · A (0.2%)	41,127		
	aa155	—	—	18,658		—	—	29,921		
	aa156	—	—	17,175		S	S (99.2%)	29,582		

(Continued)

TABLE IV. (*Continued*)

Case	Position	At point of baseline Direct	Deep	Coverage	Viral loads	At point of re-elevation of viral loads Direct	Deep	Coverage	Viral loads	Efficacy
	aa170	—	—	82,725		—	—	48,491		
14	aa36	—	—	131,802	7.4	—	—	9,803	3.0	Non-response
	aa54	—	—	163,800		S	S (79.4%)	17,273		
	aa155	—	—	65,310		—	—	19,885		
	aa156	—	—	64,594		S	S (99.8%)	20,207		
	aa170	—	—	121,327		—	—	31,243		

Substituted amino acids are shown by standard single-letter codes, and frequencies among the total coverage by ultra-deep sequencing are also presented. Dashes indicate amino acids, except for telaprevir-resistant variants included V36A/C/M/L/G, T54A/S, R155K/T/I/M/G/L/S/Q, A156V/T/S/I/G, V170A. Viral loads (log IU/ml) at two points of baseline or re-elevation are shown.
ND, not done; Direct, direct sequencing; deep, ultra-deep sequencing.

variants of T54A (27.7% of 5,032 coverage) emerged during treatment. In Case 12 (breakthrough), very high frequency variants of T54S (99.9% of 33,830 coverage) at baseline persisted during treatment as very high frequency variants of T54S (99.7% of 26,348 coverage), and de novo very high frequency variants of R155K (96.1% of 20,630 coverage) also emerged during treatment (Table IV).

In 7 (Cases 5, 6, 7, 8, 10, 13, 14) of 10 patients, who did not show sustained virological response to triple therapy, telaprevir-resistant variants were not detected at baseline by ultra-deep sequencing, but de novo resistant variants were detected according to treatment (three patients with V36A/C/M [Cases 7, 10, 13], five with T54A/S [Cases 7, 8, 10, 13, 14], three with R155K/Q [Cases 5, 7, 8], and six with A156S/T [Cases 5, 6, 7, 10, 13, 14]; Table IV).

Thus, the present study using ultra-deep sequencing indicates the emergence of de novo telaprevir-resistant variants regardless of variants frequencies at baseline, and that the emergence of variants after the start of treatment could not be predicted at baseline.

DISCUSSION

Employing ultra-deep sequencing, the present study indicated that de novo telaprevir-resistant variants emerged regardless of variants frequencies at baseline, and that the emergence of variants after the start of triple therapy could not be predicted at baseline. Hence, non-responders to previous therapy (those who failed to achieve sustained virological response to triple therapy) need to be identified to avoid the emergence of telaprevir-resistant variants. Host genetic factors (e.g., *IL28B* genotype), and viral factors (e.g., amino acid substitutions in the core/NS5A region) have often been used as pretreatment predictors of poor virological response to PEG-IFN/ribavirin dual therapy [Akuta et al., 2005; Donlin et al., 2007; El-Shamy et al., 2008; Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Maekawa et al., 2012], and telaprevir/PEG-IFN/ribavirin triple therapy [Akuta et al., 2010; Chayama et al., 2011]. However, it is not clear at this stage whether these factors can be used to predict the virological response to triple therapy in non-responders to previous therapy. The present study identified that the T12PR24 regimen could achieve a higher sustained virological response rate in non-responders to previous therapy with the combination of Arg70 and partial response, and suggests that this group of non-responders should especially be selected for triple therapy to overcome problem of telaprevir-resistant variants.

Three previous studies of patients naïve to direct-acting antiviral agents detected telaprevir-resistant variant rates of 13.4% [Shindo et al., 2011], 4.9% [Suzuki et al., 2012], and 9.2% [Vicenti et al., 2012] at baseline using direct sequencing. In the present study, the telaprevir-resistant variant rate detected at baseline by direct sequencing was 7.1%, which is similar to that reported in the above three studies. A recent study using ultra-deep sequencing of patients naïve to direct-acting antiviral agents, showed that rates of telaprevir-resistant variants at baseline were 44.4, 74.1, 18.5, and 25.9% per position for aa36, aa54, aa155, and aa156, respectively [Nasu et al., 2011]. However, in the present study, the rates of resistant variants at baseline using the same technology were 7.1%, 7.1%, 0%, 0%, and 7.1% per position for aa36, aa54, aa155, aa156, and aa170, respectively (Table IV). The following differences could probably explain the differences in the rates between the two studies: (1) Ultra-deep sequencing was performed using the Ion PGM Sequencer (Life Technologies) in the present study, compared with the Illumina Genome Analyzer II (Illumina, San Diego, CA) in the previous study and (2) The previous study was performed in treatment-naïve patients, while the present study was conducted in non-responders to previous PEG-IFN/ribavirin therapy. Further studies of larger number of patients matched for deep sequence platform and clinical background, including past history of treatment, are required to investigate the true frequency of telaprevir-resistant variants.

Interestingly, ultra-deep sequencing identified telaprevir-resistant variants at baseline in two patients (Case 9 with V170A [0.4%], and Case 11 with V36A [0.2%])at very low frequency, but the frequency of resistant variants did not increase over time. This

finding may be due to one or more reasons. One reason is probably related to the high susceptibility of telaprevir-resistant variants to IFN [Hiraga et al., 2011]. Furthermore, this finding probably suggests that a small number of mutant type viral RNA may be incomplete or defective, since a large proportion of viral genomes are thought to be defective due to the high replication and mutation rates of the virus [Bartenschlager and Lohmann, 2000]. Further studies should be performed in order to interpret the significance of the presence of low frequency variants detected by ultra-deep sequencing.

A recent study using the human hepatocyte chimeric mouse model and deep sequencing reported that the rapid emergence of de novo telaprevir-resistant HCV quasispecies was induced by mutation of the wild type strain of HCV in vivo [Hiraga et al., 2011]. In the present study, ultra-deep sequencing did not detect any telaprevir-resistant variants at baseline in seven patients, although de novo resistant variants emerged in all seven patients over time. The present clinical results provide evidence in support of de novo emergence of telaprevir resistance induced by viral mutation.

Recent studies described the use of deep sequencing in detecting resistant variants induced by NS3 protease inhibitors, except for telaprevir. One study applied deep sequencing analysis of HCV-1 in patients treated with protease inhibitor GS-9256/GS-9451 [Svarovskaia et al., 2012]. The results suggested limited viral load suppression with protease inhibitor monotherapy, which failed to suppress preexisting resistant variants, highlighting the need for combination therapy. Another report described the efficacy of re-treatment with protease inhibitor TMC435 as combination therapy in six patients infected with HCV-1, after a full-course of TMC435 monotherapy. Direct sequencing before re-treatment did not detect TMC435-resistant variants, but deep sequencing indicated persistence of low-level of resistant variants in two patients, which could have affected their response to re-treatment [Lenz et al., 2012].

One limitation of the present study is that the existence of very low frequency telaprevir-resistant variants was not investigated long after the cessation of therapy, by ultra-deep sequencing. Further large-scale studies using ultra-deep sequencing should be performed to investigate the effects of telaprevir-resistant variants on the response to treatment using new drugs, including direct-acting antiviral agents.

In conclusion, the present study could not predict the emergence of telaprevir-resistant variants after the start of triple therapy in non-responders to previous therapy, even by using ultra-deep sequencing at baseline. Further large-scale prospective studies are needed to investigate the clinical utility of the ultra-deep sequencing technology in detecting low frequency telaprevir-resistant variants, and to help in the design of more effective therapeutic regimens.

REFERENCES

Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, Matsuda M, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2005. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 48:372–380.

Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2007. Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepatocarcinogenesis. Hepatology 46:1357–1364.

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, Chayama K, Nakamura Y, Kumada H. 2010. Amino acid substitution in hepatitis C virus core region and genetic variation near the interleukin 28B gene predict viral response to telaprevir with peginterferon and ribavirin. Hepatology 52:421–429.

Akuta N, Suzuki F, Seko Y, Kawamura Y, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2012a. Determinants of response to triple therapy of telaprevir, peginterferon, and ribavirin in previous non-responders infected with HCV genotype 1. J Med Virol 84:1097–1105.

Akuta N, Suzuki F, Seko Y, Kawamura Y, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Hara T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2012b. Complicated relationships of amino acid substitution in HCV core region and *IL28B* genotype influencing hepatocarcinogenesis. Hepatology 56:2134–2141.

Barbotte L, Ahmed-Belkacem A, Chevaliez S, Soulier A, Hézode C, Wajcman H, Bartels DJ, Zhou Y, Ardzinski A, Mani N, Rao BG, George S, Kwong A, Pawlotsky JM. 2010. Characterization of V36C, a novel amino acid substitution conferring hepatitis C virus (HCV) resistance to telaprevir, a potent peptidomimetic inhibitor of HCV protease. Antimicrob Agents Chemother 54: 2681–2683.

Bartenschlager R, Lohmann V. 2000. Replication of hepatitis C virus. J Gen Virol 81:1631–1648.

Bull RA, Luciani F, McElroy K, Gaudieri S, Pham ST, Chopra A, Cameron B, Maher L, Dore GJ, White PA, Lloyd AR. 2011. Sequential bottlenecks drive viral evolution in early acute hepatitis C virus infection. PLoS Pathog 7:e1002243.

Chayama K, Hayes CN, Abe H, Miki D, Ochi H, Karino Y, Toyota J, Nakamura Y, Kamatani N, Sezaki H, Kobayashi M, Akuta N, Suzuki F, Kumada H. 2011. *IL28B* but not *ITPA* polymorphism is predictive of response to pegylated interferon, ribavirin, and telaprevir triple therapy in patients with genotype 1 hepatitis C. J Infect Dis 204:84–93.

Donlin MJ, Cannon NA, Yao E, Li J, Wahed A, Taylor MW, Belle SH, Di Bisceglie AM, Aurora R, Tavis JE. 2007. Pretreatment sequence diversity differences in the full-length hepatitis C virus open reading frame correlate with early response to therapy. J Virol 81:8211–8224.

Elliott AM, Radecki J, Moghis B, Li X, Kammesheidt A. 2012. Rapid detection of the ACMG/ACOG-recommended 23 CFTR disease-causing mutations using ion torrent semiconductor sequencing. J Biomol Tech 23:24–30.

El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. 2008. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology 48:38–47.

Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. 1996. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 334:77–81.

Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB. 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461:399–401.

Hézode C, Forestier N, Dusheiko G, Ferenci P, Pol S, Goeser T, Bronowicki JP, Bourlière M, Gharakhanian S, Bengtsson L, McNair L, George S, Kieffer T, Kwong A, Kauffman RS, Alam J,

Pawlotsky JM, Zeuzem S, PROVE2 Study Team. 2009. Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. N Engl J Med 360:1839–1850.

Hiraga N, Imamura M, Abe H, Hayes CN, Kono T, Onishi M, Tsuge M, Takahashi S, Ochi H, Iwao E, Kamiya N, Yamada I, Tateno C, Yoshizato K, Matsui H, Kanai A, Inaba T, Tanaka S, Chayama K. 2011. Rapid emergence of telaprevir resistant hepatitis C virus strain from wildtype clone in vivo. Hepatology 54:781–788.

Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimotohno K. 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA 87:9524–9528.

Kieffer TL, Sarrazin C, Miller JS, Welker MW, Forestier N, Reesink HW, Kwong AD, Zeuzem S. 2007. Telaprevir and pegylated interferon-alpha-2a inhibit wild-type and resistant genotype 1 hepatitis C virus replication in patients. Hepatology 46:631–639.

Kumada H, Toyota J, Okanoue T, Chayama K, Tsubouchi H, Hayashi N. 2012. Telaprevir with peginterferon and ribavirin for treatment-naive patients chronically infected with HCV of genotype 1 in Japan. J Hepatol 56:78–84.

Lenz O, de Bruijne J, Vijgen L, Verbinnen T, Weegink C, Van Marck H, Vandenbroucke I, Peeters M, Simmen K, Fanning G, Verloes R, Picchio G, Reesink H. 2012. Efficacy of re-treatment with TMC435 as combination therapy in hepatitis C virus-infected patients following TMC435 monotherapy. Gastroenterology 143:1176–1178.

Lin C, Gates CA, Rao BG, Brennan DL, Fulghum JR, Luong YP, Frantz JD, Lin K, Ma S, Wei YY, Perni RB, Kwong AD. 2005. In vitro studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061. J Biol Chem 280:36784–36791.

Lin C, Kwong AD, Perni RB. 2006. Discovery and development of VX-950, a novel, covalent, and reversible inhibitor of hepatitis C virus NS3. 4A serine protease. Infect Disord Drug Targets 6:3–16.

Maekawa S, Sakamoto M, Miura M, Kadokura M, Sueki R, Komase K, Shindo H, Komatsu N, Shindo K, Kanayama A, Ohmori T, Amemiya F, Takano S, Yamaguchi T, Nakayama Y, Kitamura T, Inoue T, Okada S, Enomoto N. 2012. Comprehensive analysis for viral elements and interleukin-28B polymorphisms in response to pegylated interferon plus ribavirin therapy in hepatitis C virus 1B infection. Hepatology 56:1611–1621.

McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R, McNair L, Alam J, Muir AJ, PROVE1 Study Team. 2009. Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. N Engl J Med 360:1827–1838.

McHutchison JG, Manns MP, Muir AJ, Terrault NA, Jacobson IM, Afdhal NH, Heathcote EJ, Zeuzem S, Reesink HW, Garg J, Bsharat M, George S, Kauffman RS, Adda N, Di Bisceglie AM, PROVE3 Study Team. 2010. Telaprevir for previously treated chronic HCV infection. N Engl J Med 362:1292–1303.

Nasu A, Marusawa H, Ueda Y, Nishijima N, Takahashi K, Osaki Y, Yamashita Y, Inokuma T, Tamada T, Fujiwara T, Sato F, Shimizu K, Chiba T. 2011. Genetic heterogeneity of hepatitis C virus in association with antiviral therapy determined by ultra-deep sequencing. PLoS ONE 6:e24907.

Ninomiya M, Ueno Y, Funayama R, Nagashima T, Nishida Y, Kondo Y, Inoue J, Kakazu E, Kimura O, Nakayama K, Shimosegawa T. 2012. Use of illumina deep sequencing technology to differentiate hepatitis C virus variants. J Clin Microbiol 50:857–866.

Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y. 2001. A high-throughput SNP typing system for genome-wide association studies. J Hum Genet 46:471–477.

Romano KP, Ali A, Royer WE, Schiffer CA. 2010. Drug resistance against HCV NS3/4A inhibitors is defined by the balance of substrate recognition versus inhibitor binding. Proc Natl Acad Sci USA 107:20986–20991.

Shindo H, Maekawa S, Komase K, Sueki R, Miura M, Kadokura M, Shindo K, Amemiya F, Kitamura T, Nakayama Y, Inoue T, Sakamoto M, Okada SI, Asahina Y, Izumi N, Honda M, Kaneko S, Enomoto N. Characterization of naturally occurring protease inhibitor-resistance mutations in genotype 1b hepatitis C virus patients. Hepatol Int 2011. [Epub ahead of print].

Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Müller T, Bahlo M, Stewart GJ, Booth DR, George J. 2009. *IL28B* is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41:1100–1104.

Suzuki A, Yamada R, Chang X, Tokuhiro S, Sawada T, Suzuki M, Nagasaki M, Nakayama-Hamada M, Kawaida R, Ono M, Ohtsuki M, Furukawa H, Yoshino S, Yukioka M, Tohma S, Matsubara T, Wakitani S, Teshima R, Nishioka Y, Sekine A, Iida A, Takahashi A, Tsunoda T, Nakamura Y, Yamamoto K. 2003. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginie deminase 4, are associated with rheumatoid arthritis. Nat Genet 34:395–402.

Suzuki F, Sezaki H, Akuta N, Suzuki Y, Seko Y, Kawamura Y, Hosaka T, Kobayashi M, Saito S, Arase Y, Ikeda K, Kobayashi M, Mineta R, Watahiki S, Miyakawa Y, Kumada H. 2012. Prevalence of hepatitis C virus variants resistant to NS3 protease inhibitors or the NS5A inhibitor (BMS-790052) in hepatitis patients with genotype 1b. J Clin Virol 54:352–354.

Svarovskaia ES, Martin R, McHutchison JG, Miller MD, Mo H. 2012. Abundant drug-resistant NS3 mutants detected by deep sequencing in hepatitis C virus-infected patients undergoing NS3 protease inhibitor monotherapy. J Clin Microbiol 50:3267–3274.

Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41:1105–1109.

Vicenti I, Rosi A, Saladini F, Meini G, Pippi F, Rossetti B, Sidella L, Di Giambenedetto S, Almi P, De Luca A, Caudai C, Zazzi M. 2012. Naturally occurring hepatitis C virus (HCV) NS3/4A protease inhibitor resistance-related mutations in HCV genotype 1-infected subjects in Italy. J Antimicrob Chemother 67:984–987.

Vogel U, Szczepanowski R, Claus H, Jünemann S, Prior K, Harmsen D. 2012. Ion torrent personal genome machine sequencing for genomic typing of Neisseria meningitidis for rapid determination of multiple layers of typing information. J Clin Microbiol 50:1889–1894.

J Gastroenterol (2013) 48:930–941
DOI 10.1007/s00535-012-0688-7

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Clearance of hepatitis B surface antigen during long-term nucleot(s)ide analog treatment in chronic hepatitis B: results from a nine-year longitudinal study

Tetsuya Hosaka · Fumitaka Suzuki · Masahiro Kobayashi · Yuya Seko · Yusuke Kawamura · Hitomi Sezaki · Norio Akuta · Yoshiyuki Suzuki · Satoshi Saitoh · Yasuji Arase · Kenji Ikeda · Mariko Kobayashi · Hiromitsu Kumada

Received: 27 March 2012 / Accepted: 12 September 2012 / Published online: 12 October 2012
© Springer Japan 2012

Abstract
Background Clearance of hepatitis B surface antigen (HBsAg) is considered the ultimate goal in chronic hepatitis B treatment. One treatment option is long-term nucleot(s)ide analog (NA) therapy. We followed a group of long-term NA therapy patients to evaluate the efficacy of this treatment in promoting clearance and longitudinal declines of HBsAg.
Method The study included 791 NA therapy patients who received lamivudine as their first drug. At the baseline, 442 patients were hepatitis B e antigen (HBeAg)+ and 349 were HBeAg−. All analyses were performed after separating the HBeAg+ and HBeAg− cohorts. Cox proportional hazards models were used to determine which factors were associated with HBsAg clearance.
Results HBsAg clearance was observed in 18 (4.1 %) of the HBeAg+ patients and 20 (5.7 %) of the HBeAg− patients at baseline, giving seroclearance rates of 6.4 and 6.9 %, respectively, over the nine-year study period. HBsAg clearance was influenced by several independent factors that varied according to HBeAg cohort. For HBeAg+ patients, these included previous interferon therapy, infection with hepatitis B virus (HBV) genotype A, a ≥0.5 log IU/mL decline in HBsAg level within six months, and clearance of HBeAg at six months. For HBeAg− patients, these included infection with HBV genotype A, decline in HBsAg at six months, and a baseline HBsAg level of <730 IU/mL.
Conclusion This study suggests that both direct antiviral potential and host immune response are needed to achieve HBsAg clearance by NA therapy. Viral genotype strongly influenced HBsAg clearance during NA therapy.

Keywords Hepatitis B surface antigen · Nucleot(s)ide analog · Lamivudine · Interferon

T. Hosaka (✉) · F. Suzuki · M. Kobayashi · Y. Seko · Y. Kawamura · H. Sezaki · N. Akuta · Y. Suzuki · S. Saitoh · Y. Arase · K. Ikeda · H. Kumada
Department of Hepatology, Toranomon Hospital, 2-2 Toranomon, Minato-ku, Tokyo, Japan
e-mail: hosa-p@toranomon.gr.jp

M. Kobayashi
Research Institute for Hepatology, Toranomon Hospital, 1-3-1, Kajigaya, Takatsu-ku, Kawasaki, Japan

Introduction

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 analogs (NAs) have been used as a mainstay therapeutic strategy against chronic hepatitis B. Five such antiviral agents—lamivudine (LAM), entecavir (ETV), telbivudine, adefovir dipivoxil (ADV), and tenofovir disoproxil fumarate—which inhibit viral replication [e.g., hepatitis B virus DNA (HBV DNA) priming, reverse transcription of negative-stranded HBV DNA, and synthesis of positive-stranded HBV DNA] have been approved; these NAs vary in both the strength and the rapidity with which they suppress HBV DNA [3–10]. Sustained viral suppression by NA therapy can improve liver fibrosis and clinical outcomes of patients [11, 12]. LAM was the first NA to be approved to treat chronic hepatitis B in Japan, followed by ADV and ETV.

Responses to antiviral treatments can be evaluated by monitoring serum HBV DNA levels, hepatitis B e antigen (HBeAg) and antibody levels, and hepatitis B surface

Antiviral therapy and drug resistance

All 791 patients received 100 mg LAM daily as an initial therapy, but a LAM-resistant rtM204I/V mutation developed in 439 (55 %) of these patients. Over time, 334 (42 %) individuals experienced an increase in HBV DNA (≥1 log copies/mL) [e.g., virological breakthrough (VBT)] and, as a result, 299 (98.5 %) individuals were also provided with ADV treatment (10 mg) added onto LAM as a rescue therapy. The remaining patients continued to receive LAM monotherapy and were lost to follow-up before the administration of ADV because of the lack of approval for ADV administration in Japan at the time. The resistant mutation for rtM204I/V was detected in 312 of 334 patients who experienced VBT using a commercial kit (as described below). Patients who had achieved an optimal or suboptimal virological response or who wished to participate in the clinical trial of ETV for LAM-refractory patients (ClinicalTrials.gov: NCT 1037166)—152 and 17 patients, respectively—switched from LAM to ETV (0.5 mg/day). Additionally, patients in whom subsequent ADV- or ETV-resistant mutants emerged received an optimal rescue therapy with other NAs (ETV + ADV combination for ADV resistance, and LAM + ADV combination for ETV resistance).

NA treatment was continued as a rule; median NA treatment duration was 75 months (25th–75th percentile, 55–102) in the HBeAg+ cohort and 92 months (67–119) in the HBeAg− cohort. Ultimately, 55 (7 %) of the 791 patients discontinued treatment; 16 of these individuals terminated treatment after achieving HBsAg seroclearance. Follow-ups were conducted for all patients, regardless of length of treatment, for as long as possible.

Clinical data collection and follow-ups

Data on patient characteristics, biochemistry, hematology, virology, histology, and previous treatments were collected and registered in our institute's database at the time of patient enrollment. Prior to beginning LAM, all patients were surveyed about the presence of a family history of HBV infection. Data on treatment dose and duration of previous IFN therapy were collected from our hospital's IFN therapy database or requested from other hospitals as necessary. Complete details on the previous treatment were lacking for 29 (9.7 %) of 297 patients who received IFN therapy before starting LAM.

At least every 1–3 months, liver function and virological markers of HBV infection were measured in all patients. All serum HBsAg titers were measured from frozen serum samples collected at six months, one year, three years, five years, and once annually for 6–10 years, and then stored at −80 °C. The day of HBsAg clearance was defined by the measurement in consecutive available serum samples before it was undetected in subsequent samples. A genotypic analysis of drug resistance was performed in cases of insufficient virological response or VBT, defined as an increase in serum HBV DNA levels ≥1 log above the nadir measured after the initial virological response. Cirrhosis was diagnosed by laparoscopy, liver biopsy, or clinical data such as imaging modalities and portal hypertension. The primary outcome for this study was HBsAg clearance. The endpoint of the follow-up was HBsAg clearance or last visit before January 2011.

Markers of HBV infection

Serum HBsAg titers were measured using ARCHITECT HBsAg QT assay kits (Abbott Laboratories, Tokyo, Japan), which have a lower limit of detection of 0.05 IU/mL and an upper limit of detection of 250 IU/mL. To expand the upper range from 250 to 125,000 IU/mL, serum samples, going off the scale, were diluted stepwise to 1:20 and 1:500 with ARCHITECT diluents as the product document described. HBeAg was determined by enzyme-linked immunosorbent assay with a commercial kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). HBV DNA was quantified using the Amplicor monitor assay (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.6–7.6 log copies/mL, or COBAS TaqMan HBV v.2.0 (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.1–9.0 log copies/mL. A commercial kit (HBV Genotype EIA; Institute of Immunology) was used to serologically determine HBV genotypes using the combination of epitopes expressed on the pre-S2 region product, which is specific to each of the seven major genotypes (A–G). YMDD mutants (rt M204I/V) were determined by polymerase chain reaction-based enzyme-linked mini-sequence assay with a commercial kit (Genome Science Laboratories, Tokyo, Japan).

Statistical analyses

Categorical data were compared between groups using chi-square or Fisher's exact tests. Continuous variables with a nonparametric distribution were analyzed with Mann–Whitney U tests, while those with a parametric distribution were analyzed with Student's t tests. When appropriate, Kruskal–Wallis tests were used to conduct pairwise comparisons of specific variables. Cox regression analyses were used to assess which variables were significantly associated with HBsAg clearance. Cut-off values were provided using the area under the receiver operating characteristic curve (ROC) only after rejecting the null hypothesis for the ROC curve. All baseline factors that were found to be significantly associated with HBsAg clearance by univariate analysis

antigen (HBsAg) and antibody levels. Serum HBsAg levels appear to reflect the amount of intrahepatic covalently closed circular DNA (cccDNA), which acts as a template for the transcription of viral genes [13–15]. Previous studies have shown that both interferon (IFN) and NA therapy result in a reduction of intrahepatic cccDNA [16, 17], suggesting that these treatments may be helpful in achieving the ultimate therapeutic goal of antiviral therapy for chronic hepatitis B (i.e., total clearance of HBsAg).

Very low rates of HBsAg clearance have been reported in the past [18–22]. Recent work has shown that over a one-year period, pegylated (PEG)-IFN therapy is more successful than ETV at reducing serum HBsAg [23]; furthermore, PEG-IFN therapy has also been reported to promote the complete clearance of HBsAg [24–27]. Several studies have detailed similar successes achieved by NA therapy but over relatively short (<5 years) treatment durations [18–20, 22, 28, 29]. The kinetics of HBsAg during long-term (>5 years) treatment remain unknown. NA therapy leads to time-dependent decreases in intrahepatic cccDNA and serum HBsAg levels if sustained viral suppression is longer term, and may therefore increase the rates of HBsAg clearance.

In order to evaluate this possibility empirically, we conducted a ten-year-long study in which we followed patients who received NA therapy initiated by the administration of LAM. We evaluated the resulting clearance and longitudinal declines of HBsAg using highly sensitive assays. Our aim was to determine whether long-term NA therapy can lead to HBsAg clearance, as suggested; if so, we also wished to elucidate the factors associated with its success.

Methods

Study population

Over a period of 12 years (September 1995 to September 2007), 949 consecutive patients who were chronically monoinfected with HBV (confirmed HBsAg positivity for at least six months), were treated with LAM monotherapy at the Department of Hepatology, Toranomon Hospital, Metropolitan Tokyo. The indication for antiviral therapy was abnormal ALT levels accompanying the increase in HBV DNA (over 4 log copies/mL) as a rule. However, in cases where ALT levels were normal, patients with advanced fibrosis were administered LAM. We did not treat patients without fibrosis who had low HBV DNA and normal ALT levels as a rule. We selected 791 patients for the final study after we had excluded all those who had been treated with LAM for <6 months, were co-infected with hepatitis C virus, had not provided sufficient serum samples, and/or had insufficient clinical records (Fig. 1). No patient was co-infected with human immunodeficiency virus in this cohort. Seven hundred ninety-one patients were enrolled in this cohort study. Of these 791 patients, 442 were HBeAg+ and 349 were HBeAg− at baseline. All analyses were performed after separating the HBeAg+ and HBeAg− cohorts. Written informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved a priori by the institution's human research committee. This study has been registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN CTR) as the number UMIN000007993.

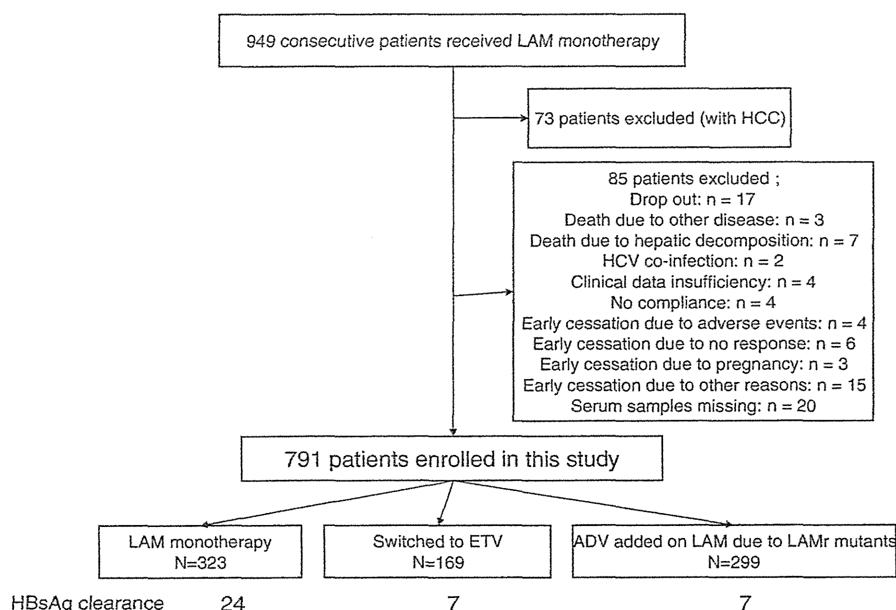

Fig. 1 Schematic of study protocol. *LAM* lamivudine, *HCC* hepatocellular carcinoma, *HCV* hepatitis C virus, *ETV* entecavir, *ADV* adefovir dipivoxil, *HBsAg* hepatitis B surface antigen

were entered into a multivariate analysis. Independent baseline factors associated with clearance of HBsAg were calculated using a stepwise Cox regression analysis. We then performed a time-dependent Cox regression to analyze independent factors associated with HBsAg while adjusting for on-treatment factors and independent baseline factors. Three covariates of the on-treatment response factors—emergence of rtM204I/V mutants, VBT, and biochemical breakthrough—were set as the time-dependent covariates. Cumulative HBsAg clearance rates were analyzed using the Kaplan–Meier method; differences in the resulting curves were tested using log-rank tests. We performed Cox regression analysis, Kaplan–Meier curve analysis, and HBsAg kinetics analysis for no more than nine years, as the number of patients with a long-term follow-up of over ten years was too small to permit analysis [30]. Bonferroni adjustments were used to correct for the number of different ways a single predictor variable can be split. Significance was defined as $P < 0.05$ for all two-tailed tests. Data analysis was performed with IBM SPSS version 19.0 software (IBM Corp., Armonk, NY, USA).

Results

Patient characteristics

Thirty-eight (4.8 %) of 791 patients successfully cleared HBsAg. Of these, 24 had received LAM, 7 had switched to ETV treatment, and 7 had been treated with both LAM and ADV (Fig. 1). Of the 38 patients who achieved HBsAg clearance, 18 were HBeAg+, whereas 20 were HBeAg− at baseline. Table 1 provides a comparison of the baseline and on-treatment characteristics between patients who were and were not able to successfully clear HBsAg (all patients, HBeAg+ and − cohorts, respectively). In the HBeAg+ cohort, baseline characteristics that were significantly associated with HBsAg clearance included previous IFN therapy, HBV genotype, HBV DNA, and AST and ALT levels; in the HBeAg− cohort, significant characteristics included HBV genotype and HBsAg levels. Significant on-treatment characteristics in the HBeAg+ cohort included decline in HBsAg, clearance of HBeAg, and decline in HBV DNA to <2.6 log copies/mL at six months;

Table 1 Baseline, demographic, and on-treatment characteristics of patients with and without HBsAg seroclearance

Characteristics	All patients ($n = 791$)	HBeAg+ at baseline ($n = 442$)			HBeAg− at baseline ($n = 349$)		
		Persistently HBsAg+ ($n = 424$)	HBsAg seroclearance ($n = 18$)	*P*	Persistently HBsAg+ ($n = 329$)	HBsAg seroclearance ($n = 20$)	*P*
Baseline							
Age[a] (years) (SD)	43 (11.1)	41 (11.2)	44 (10.5)	0.177	47 (10.3)	46 (10.3)	0.899
Gender (male:female)	627:164	329:95	16:2	0.385	265:64	16:4	1.000
Race				0.446			
Japanese	768 (97)	411 (97)	17 (94)		320 (97)	20 (100)	1.000
Non-Japanese (%) (Asian:Caucasian)	23 (3) (21:2)	13 (3) (20:2)	1 (3) (1:0)		9 (3) (20:2)	0 (3) (1:0)	
Family history of HBV infection	539 (68)	311 (73)	10 (56)	0.107	208 (63)	10 (50)	0.238
Previous IFN therapy	297 (38)	167 (39)	15 (83)	**<0.001**	106 (32)	9 (45)	0.326
IFN duration (weeks)	27 (20–58)	26 (18–53)	52 (21–79)	0.214	32 (22–89)	23 (14–72)	0.457
Duration from the end of IFN to start of lamivudine (weeks)	50 (3–189)	26 (7–124)	37 (2–89)	0.505	119 (3–316)	102 (18–289)	0.746
Previous NA therapy	34 (4)	21 (5)	2 (11)	0.239	10 (3)	1 (5)	0.483
Presence of cirrhosis	169 (21)	76 (18)	2 (11)	0.752	87 (26)	4 (20)	0.610
HBV genotype				**<0.001**			**<0.001**
A	28 (3.5)	14 (3.3)	6 (33)		6 (1.8)	2 (10)	
B	67 (8.5)	16 (3.8)	0 (0)		48 (14.6)	3 (15)	
C	664 (83.9)	374 (88.2)	12 (67)		265 (80.5)	13 (65)	
D	3 (0.4)	2 (0.4)	0 (0)		0 (0)	1 (5)	
F	2 (0.3)	2 (0.4)	0 (0)		0 (0)	0 (0)	
Unclassified/missing	27 (3.4)	16 (3.8)	0 (0)		10 (3.0)	1 (5)	

Table 1 continued

Characteristics	All patients (n = 791)	HBeAg+ at baseline (n = 442)			HBeAg− at baseline (n = 349)		
		Persistently HBsAg+ (n = 424)	HBsAg seroclearance (n = 18)	P	Persistently HBsAg+ (n = 329)	HBsAg seroclearance (n = 20)	P
Baseline HBV DNA (log copies/mL)	7.0 (5.8–8.0)	7.6 (6.7–8.2)	8.0 (7.5–8.4)	**0.027**	6.3 (5.2–7.2)	6.1 (5.0–7.0)	0.652
Baseline HBsAg level (IU/mL)	2530 (907–6590)	3910 (1690–12300)	5280 (943–67600)	0.331	1590 (599–3050)	529 (58–1610)	**0.004**
Baseline AST level (IU/L)	74 (48–135)	81 (52–165)	201 (78–666)	**0.011**	66 (42–113)	57 (39–96)	0.694
Baseline AST level (×ULN)	2.2 (1.5–4.1)	2.5 (1.6–5.0)	6.1 (2.3–20.2)	**0.011**	2.0 (1.3–3.4)	1.7 (1.2–2.9)	0.736
Baseline ALT level (IU/L)	115 (63–252)	130 (72–290)	326 (104–775)	**0.021**	101 (56–194)	101 (55–215)	0.904
Baseline ALT level (×ULN)	3.0 (1.7–6.4)	3.5 (1.9–7.8)	7.8 (2.5–20.3)	**0.040**	2.6 (1.4–5.2)	2.6 (1.4–5.2)	0.955
Baseline total bilirubin level (mg/dL)	0.8 (0.6–1.1)	0.8 (0.5–1.1)	0.9 (0.6–1.9)	0.117	0.7 (0.6–1.0)	0.8 (0.6–0.9)	0.556
Platelet count[a] (10^5/mm^3) (SD)	16.1 (5.7)	16.5 (6.1)	14.7 (3.5)	0.221	15.6 (5.1)	17.7 (6.9)	0.216
On-treatment response							
Decline of HBsAg level (≥0.5 log IU/mL within six months)	97 (1)	67 (16)	13 (72)	**<0.001**	11 (3)	6 (30)	**<0.001**
HBeAg positive → clearance within six months	109 (14)	94 (22)	10 (56)	**0.005**	NA	NA	
Undetectable HBV DNA (<400 copies/mL) at six months	532 (67)	221 (52)	15 (83)	**0.014**	277 (84)	19 (95)	0.330
Emergence of rtM204I/V mutants	439 (55)	251 (59)	9 (50)	0.469	170 (52)	9 (45)	0.646
Viral breakthrough due to mutants	334 (42)	216 (51)	5 (28)	0.055	108 (33)	5 (25)	0.473
Biochemical breakthrough due to mutants	318 (40)	200 (47)	5 (28)	0.146	108 (33)	5 (25)	0.473

Except where marked with a superscript letter a, values are expressed as the median and 25th–75th percentiles (parenthetically), or number and percentage (parenthetically). ULN; AST = 33 IU/L, ALT = 42 IU/L (male), and 27 IU/L (female). *Asterisks* indicate data displayed as mean values and standard deviations. *Bold text* indicates statistically significant P values

the only significant characteristic in the HBeAg− cohort was a decline in HBsAg within six months. ROC curve analysis confirmed a cut-off value of 0.5 log IU/mL for a decline in HBsAg level within six months in the HBeAg+ and − cohorts [area under the curve = 0.810 (95 % CI 0.673–0.947) (HBeAg+ cohort) and 0.760 (95 % CI 0.611–0.909) (HBeAg− cohort)].

LAM-resistant rtM204I/V mutants were detected in 439 (55.5 %) of 791 patients. Of these, 334 (42.2 % of all patients) also developed VBT accompanied by an increase in HBV DNA (≥1 log copies/mL). The rate of VBT was marginally significantly lower in the HBsAg clearance group in the HBeAg+ cohort (Table 1).

Factors associated with HBsAg clearance

The overall cumulative rates of HBsAg clearance were 0.2 % at one year, 1.2 % at three years, 2.6 % at five years, 4.2 % at seven years, and 6.4 % at nine years in the HBeAg+ cohort; and 0.6 % at one year, 0.9 % at three - years, 2.2 % at five years, 5.2 % at seven years, and 6.9 % at nine years in the HBeAg− cohort. Univariate Cox

Table 2 Baseline and on-treatment response factors associated with HBsAg clearance, as determined by time-dependent univariate and multivariate analyses at year 9 (HBeAg+ cohort)

Variable	Univariate		Multivariate	
	HBsAg clearance rate ratio (95 % CI)	P	HBsAg clearance rate ratio (95 % CI)	P
Baseline factors				
Age (≥50 years)	1.36 (0.48–3.86)	0.564		
Gender (F)	0.51 (0.12–2.23)	0.371		
Family history of HBV infection	0.42 (0.16–1.09)	0.074		
Previous IFN therapy	**5.60 (1.61–19.5)**	**0.007**	**6.15 (1.69–22.4)**	**0.006**
Previous NA therapy	2.42 (0.55–10.6)	0.242		
Presence of cirrhosis	0.85 (0.52–1.40)	0.527		
HBV genotype (A)	**3.64 (2.21–5.99)**	**<0.001**	**3.18 (1.80–5.62)**	**<0.001**
HBV DNA (≥6.0 log copies/mL)	2.56 (0.34–19.3)	0.362		
HBsAg (<730 IU/mL)	1.57 (0.51–4.81)	0.432		
AST (≥4.5 × ULN)	**4.53 (1.68–12.2)**	**0.003**		
ALT (≥7.2 × ULN)	**3.56 (1.35–9.36)**	**0.010**		
Total bilirubin (≥1.5 mg/dL)	2.63 (0.92–7.46)	0.070		
Platelet count (<1.2 × $10^5/mm^3$)	0.58 (0.13–2.59)	0.476		
On-treatment response factors				
Decline of HBsAg level (≥0.5 log IU/mL within six months)	**15.8 (5.14–48.5)**	**<0.001**	**18.6 (5.78–60.0)**	**<0.001**
HBeAg positive → clearance within six months	**4.33 (1.65–11.4)**	**0.003**	**2.95 (1.04–8.39)**	**0.042**
Undetectable HBV DNA (<400 copies/mL) at six months	**3.95 (1.14–13.7)**	**0.031**		
Emergence of rtM204I/V mutants[a]	0.88 (0.32–2.44)	0.802		
Viral breakthrough due to mutants[a]	**0.32 (0.10–1.00)**	**0.050**		
Breakthrough hepatitis due to mutants[a]	0.41 (0.13–1.31)	0.134		

[a] Time-dependent covariates. *Bold text* indicates statically significant P valuesVariables analyzed in multivariate analysis: previous IFN therapy, HBV genotype, ALT, decline of HBsAg levels, HBeAg clearance within six months, undetectable HBV DNA at six months, and viral breakthrough due to mutants (time-dependent covariate)

regression analysis identified four baseline characteristics and four on-treatment responses that were associated with HBsAg clearance in the HBeAg+ cohort (Table 2), and two baseline characteristics and two on-treatment responses in the HBeAg− cohort (Table 3). ROC curve analysis provided the optimal cut-off values and indices for the prediction of HBsAg clearance. ROC curve analysis confirmed cut-off indices of 4.5 × ULN for AST and 7.2 × ULN for ALT for HBsAg clearance in the HBeAg+ cohort [area under the curve = 0.677 (95 % CI 0.524–0.830) (AST) and 0.643 (95 % CI 0.503–0.783) (ALT)]. Meanwhile, ROC curve analysis confirmed a cut-off value of 730 IU/mL (2.86 log IU/mL) for HBsAg for HBsAg clearance in the HBeAg− cohort [area under the curve = 0.696 (95 % CI 0.556–0.836)]. Time-dependent multivariate Cox regression analysis identified two significant baseline characteristics and two on-treatment responses related to HBsAg clearance: previous IFN therapy, infection with HBV genotype A, a decline in HBsAg level of ≥0.5 log IU/mL within six months, and HBeAg clearance within six months in the HBeAg+ cohort (Table 2). In the HBeAg− cohort, two baseline characteristics and one on-treatment response were identified in multivariate analysis: infection with HBV genotype A, HBsAg level of <730 IU/mL (2.86 log IU/mL), and a decline in HBsAg level of ≥0.5 log IU/mL within six months (Table 3).

Association between HBV genotype and HBsAg clearance

We performed a detailed analysis of the association between HBV genotype and HBsAg clearance in patients treated with NAs. Median baseline HBsAg levels were 4.7 log IU/mL (25th–75th percentile, 4.4–5.1) among patients with genotype A, 3.8 (3.5–4.2) among patients with genotype B, and 3.5 (3.2–4.0) among patients with genotype C in the HBeAg+ cohort (Fig. 2a); and 3.7 (2.5–4.1) in patients with genotype A, 2.9 (2.6–3.5) in patients with genotype B, and 3.2 (2.8–3.5) in patients with genotype C in the HBeAg− cohort (Fig. 2b). HBeAg+ patients with genotype A had higher baseline HBsAg levels than those with genotypes B or C ($P < 0.001$) (Fig. 2a). There were no significant differences in baseline HBsAg levels between the genotypes in the HBeAg− cohort.

Table 3 Baseline and on-treatment response factors associated with HBsAg clearance, as determined by time-dependent univariate and multivariate analyses at year 9 (HBeAg− cohort)

Variable	Univariate		Multivariate	
	HBsAg clearance rate ratio (95 % CI)	*P*	HBsAg clearance rate ratio (95 % CI)	*P*
Baseline factors				
Age (≥50 years)	1.39 (0.54–3.60)	0.498		
Gender (F)	0.98 (0.28–3.40)	0.971		
Family history of HBV infection	0.49 (0.19–1.27)	0.140		
Previous IFN therapy	0.88 (0.32–2.38)	0.797		
Previous NA therapy	2.41 (0.32–18.2)	0.394		
Presence of cirrhosis	0.71 (0.43–1.16)	0.173		
HBV genotype (A)	**2.79 (1.33–5.85)**	**0.007**	**2.73 (1.29–5.81)**	**0.009**
HBV DNA (≥6.0 log copies/mL)	1.16 (0.43–3.14)	0.772		
HBsAg (<730 IU/mL)	**3.91 (1.59–9.52)**	**0.003**	**4.90 (1.85–10.6)**	**0.001**
AST (≥4.5 × ULN)	1.76 (0.57–5.40)	0.324		
ALT (≥7.2 × ULN)	1.89 (0.62–5.81)	0.265		
Total bilirubin (≥1.5 mg/dL)	1.18 (0.27–5.20)	0.825		
Platelet count (<1.2 × 10^5/mm^3)	0.77 (0.17–3.55)	0.733		
On-treatment response factors				
Decline of HBsAg level (≥0.5 log IU/mL within six months)	**11.5 (4.24–31.0)**	**<0.001**	**16.9 (5.89–48.4)**	**<0.001**
Undetectable HBV DNA (<400 copies/mL) at six months	2.78 (0.37–20.8)	0.322		
Emergence of rtM204I/V mutants[a]	0.64 (0.23–1.79)	0.392		
Viral breakthrough due to mutants[a]	0.72 (0.23–2.29)	0.581		
Breakthrough hepatitis due to mutants[a]	0.65 (0.21–2.06)	0.465		

[a] Time-dependent covariates. *Bold text* indicates statically significant *P* values

Variables analyzed in multivariate analysis: HBV genotype, baseline HBsAg, decline of HBsAg levels

HBsAg kinetics over time in the HBeAg+ and − cohorts are shown in Fig. 2c, d, respectively. Among patients with genotype A in the HBeAg+ cohort, the median HBsAg change from baseline was −0.44 log IU/mL at six months, −0.56 at one year, −0.58 at three years, −1.08 at five years, and −1.33 at six years. Among patients with genotype B in the HBeAg+ cohort, median changes were −0.30 log IU/mL at six months, −0.30 at one year, −0.43 at three years, and −0.46 at five years. Kinetics were not calculated for some groups (genotype A at seven years, genotype B at six years) because the number of patients was too small. Finally, among patients with genotype C in the HBeAg+ cohort, median changes were 0.00 log IU/mL at six months, 0.03 at one year, −0.08 at three years, −0.29 at five years, −0.53 at six - years, −0.62 at seven years, −0.70 at eight years, and −0.82 at nine years. Genotype had a significant effect on the slopes between data collection points at six months and six years. In the HBeAg+ cohort, declines were faster in patients with genotype A than in those with genotypes B or C. HBeAg− patients with genotype A displayed a median HBsAg change from baseline of 0.05 log IU/mL at six months, 0.05 at one year, −0.11 at three years, −0.21 at five years, and −0.26 at six years. Among patients with genotype B in the HBeAg− cohort, median changes were −0.03 log IU/mL at six months, −0.06 at one year, −0.25 at three years, −0.31 at five years, −0.51 at six years, −0.62 at seven years, −0.66 at eight years, and −0.61 at nine years. Among patients with genotype C in the HBeAg− cohort, median changes were 0.03 log IU/mL at six months, 0.04 at one year, −0.08 at three years, −0.19 at five years, −0.32 at six years, −0.39 at seven years, −0.46 at eight years, and −0.62 at nine years. The decline was slightly faster in patients with genotype B than in those with genotypes A and C in the HBeAg− cohort.

We investigated whether HBsAg clearance were influenced by genotype or baseline HBeAg. Cumulative HBsAg clearance rates in the HBeAg+ cohort were as follows: 15 % at year 3, and 35 % at year 5 in patients with genotype A; 0 % over all years in patients with genotype B; and 0.6 % at year 3, 1.2 % at year 5, and 5.4 % at year 9 in patients with genotype C (Fig. 2e). In the HBeAg− cohort, clearance rates were 12 % at year 3, and 25 % at year 5 in patients with genotype A; 0 % at year 3, 0 % at year 5, and 11.5 % at year 9 in patients with genotype B; and 0.4 % at year 3, 1.6 % at year 5, and 5.1 % at year 9 in