Table 3 *Continued*

	Patient									
	1	2	3	4	5	6	7	8	9	10
Group B (500 mg q8h)										
Baseline characteristics										
Age/sex	64/M	54/F	36/F	60/F	52/M	46/F	56/F	65/M	56/F	54/M
Height (cm)	173.2	151.0	148.7	160.5	175.8	160.0	160.0	167.0	158.0	170.0
Weight (kg)	75.0	47.6	44.3	67.9	71.8	52.0	57.0	79.0	65.0	55.0
IL-28B SNP (rs8099917)	TT	TG	TG	TT	TG	TT	TG	TT	TT	TG
IL-28B SNP (rs12979860)	CC	CT	CT	CC	CT	CC	CT	CC	CC	CT
ITPA SNP (rs1127354)	CC	CC	CC	CC	CC	CC	CC	CC	CC	CA
Core a.a. 70 (W/M)	M	W	W	W	M	W	M	W	W	M
Core a.a. 91 (W/M)	W	W	W	W	M	W	M	W	M	M
ISDR substituted a.a. sites	6	0	1	0	0	0	0	0	0	0
History of IFN-based therapy†	Naïve	IFN	Naïve	Naïve	PR	Naïve	PR	IFN	IFN	PR
Baseline laboratory data										
HCV RNA (log$_{10}$ IU/mL)	5.50	7.15	6.15	6.80	6.80	7.00	6.10	7.20	6.85	6.75
Hb (g/dL)	16.1	11.7	12.1	13.6	14.5	12.3	16.8	14.3	13.7	14.8
Creatinine (g/dL)	0.78	0.50	0.45	0.56	0.87	0.58	0.80	0.89	0.75	0.70
Dose										
RBV, max/min (mg)	800/400	600/200	600/200	800/400	800/200	600/200	600/600	800/200	800/200	600/200
Duration of treatment (weeks)	12	12	11	12	12	3	12	12	5	12
Telaprevir, adherence (%)	98.0	99.2	91.0	99.2	101.6	25.5	98.8	99.2	43.1	98.4
PEG IFN, adherence (%)	98.3	66.7	87.5	100	91.7	25.0	100	100	41.7	100
RBV, adherence (%)	68.5	44.7	39.2	54.4	48.8	24.3	99.2	36.5	28.2	64.3
Pharmacokinetic parameter‡										
C_{trough} (µg/mL)	1.950	2.763	3.276	1.690	1.478	1.939	2.955	4.065	1.962	1.846
Outcome										
HCV RNA negativity (weeks)	2	6	2	2	4	-	2	2	2	8
Effect of therapy (SVR/BT/TR/NR)	SVR	TR	SVR	SVR	TR	NR	TR	SVR	SVR	TR

†Naïve, treatment naïve; IFN, IFN monotherapy; PR, PEG IFN/RBV.
‡Pharmacokinetic parameters of the patients who received triple therapy at weeks 2.
a.a., amino acid; ALT, alanine aminotransferase; C_{trough}, plasma trough concentrations; GGT, γ-glutamyltransferase; Hb, hemoglobin; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; ISDR, interferon sensitivity-determining region; M, mutant; PEG, pegylated; Plt, platelets; RBV, ribavirin; SNP, single nucleotide polymorphism; SVR, sustained virological response, BT, breakthrough, TR, transient response, NR, non-response; W, wild type; WBC, white blood cell.

Table 4 Adverse events developing in more than 20% of patients in total

MedDRA/J (ver. 12.0) PT	Group A (750 mg q8h) $n = 10$ n (%)	Group B (500 mg q8h) $n = 10$ n (%)	Total $n = 20$ n (%)
Platelet count decreased	10 (100.0)	10 (100.0)	20 (100.0)
Anemia	10 (100.0)	9 (90.0)	19 (95.0)
White blood cell count decreased	9 (90.0)	10 (100.0)	19 (95.0)
Rash	7 (70.0)	7 (70.0)	14 (70.0)
Pyrexia	6 (60.0)	8 (80.0)	14 (70.0)
Malaise	6 (60.0)	5 (50.0)	11 (55.0)
Blood triglycerides increased	6 (60.0)	5 (50.0)	11 (55.0)
Headache	3 (30.0)	7 (70.0)	10 (50.0)
Blood lactate dehydrogenase increased	3 (30.0)	7 (70.0)	10 (50.0)
Anorexia	3 (30.0)	6 (60.0)	9 (45.0)
Blood uric acid increased	4 (40.0)	4 (40.0)	8 (40.0)
Nausea	3 (30.0)	5 (50.0)	8 (40.0)
Pruritus	3 (30.0)	5 (50.0)	8 (40.0)
Protein total decreased	0 (0.0)	8 (80.0)	8 (40.0)
Hyperuricaemia	5 (50.0)	2 (20.0)	7 (35.0)
Blood creatinine increased	5 (50.0)	2 (20.0)	7 (35.0)
Nasopharyngitis	3 (30.0)	4 (40.0)	7 (35.0)
Neutrophil percentage decreased	3 (30.0)	4 (40.0)	7 (35.0)
Influenza-like illness	4 (40.0)	2 (20.0)	6 (30.0)
Abdominal discomfort	2 (20.0)	3 (30.0)	5 (25.0)
Vomiting	2 (20.0)	3 (30.0)	5 (25.0)
Dizziness	0 (0.0)	5 (50.0)	5 (25.0)
Dysgeusia	3 (30.0)	1 (10.0)	4 (20.0)
Stomatitis	3 (30.0)	1 (10.0)	4 (20.0)
Lymphocyte percentage increased	2 (20.0)	2 (20.0)	4 (20.0)
Diarrhea	1 (10.0)	3 (30.0)	4 (20.0)
Alopecia	1 (10.0)	3 (30.0)	4 (20.0)

contrary, there was observed a difference in serum creatinine concentrations between group A and group B; thus, the serum creatinine concentrations in group A were higher than those in group B at all of the time points examined with a statistical significance at weeks 4 and 8 ($P < 0.01$ and $P < 0.05$, respectively) as shown in Figure 2(b). The TVR Review Team confirms that higher exposure of TVR and RBV was significantly associated with increased risk of anemia and grade 2 or higher hemoglobin toxicity.[11] The behaviors of hemoglobin and creatinine in the triple therapy shown in Figure 2 are of interest from the viewpoints of development of anemia with TVR-based regimen and could be explained by the following possibilities: (i) the increase of plasma concentration of TVR may directly affect the renal function to cause the increase of creatinine especially in group A and the decrease of hemoglobin; (ii) TVR first caused the increase of systemic exposure to RBV which in turn additively or synergistically resulted in renal dys-

function. The decrease of renal function reportedly leads to the increase of RBV concentration in plasma, because RBV is mainly excreted via the renal route.[24,25] In this study, the AUC_{0-8h} on day 1 of patients who developed low hemoglobinemia (<8.5 g/dL) were significantly higher than those of the other patients. The pharmacokinetic parameters of TVR on day 14, at which plasma concentrations of TVR were in the steady state, did not affect low hemoglobinemia. The timing of reducing RBV dose may cause development of low hemoglobinemia, because the RBV dose reduction set in the protocol of this study was less strict than that in the previous reports.[7,8]

Because the present data show that the TVR exposure tended to be increased in a dose-dependent manner, there is a possibility that the triple therapy with TVR 500 mg q8h is advantageous in aged patients whose renal function, body water content or both are lower than those of younger patients. It should be noted,
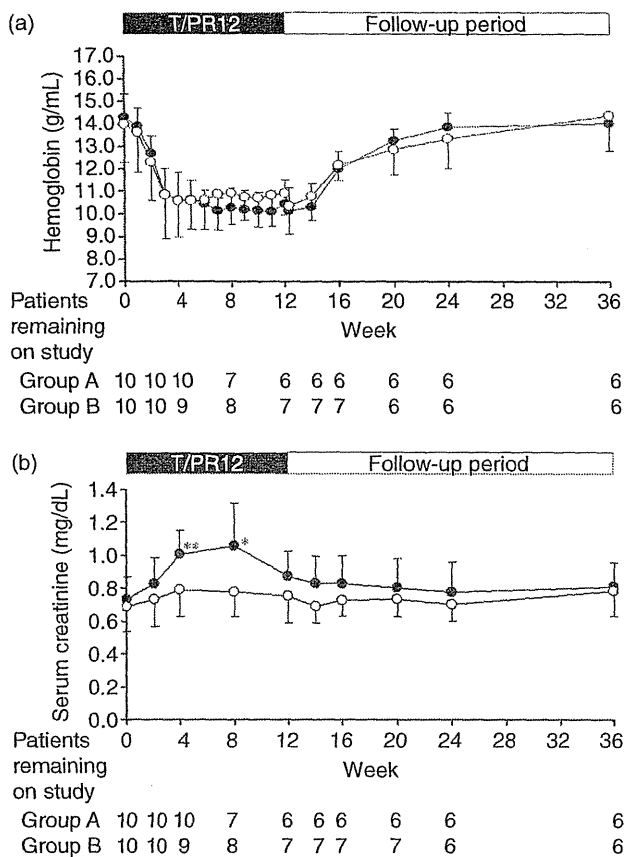
Figure 2 (a) Change from baseline of hemoglobin and (b) serum creatinine levels in Japanese patients with chronic hepatitis C during the telaprevir-based triple therapy. Each circle and bar represent mean values ± standard deviations, respectively. Number of patients at each time point is indicated below. Statistical tests were performed at each point. *P < 0.05 and **P < 0.01 difference. —●—, Group A (telaprevir 750 mg q8h); —○—, group B (telaprevir 500 mg q8h). T/PR12, triple therapy of telaprevir with peginterferon and ribavirin for 12 weeks.

however, that the small number of patients per arm in this study limits conclusions that can be drawn, and a future larger study is essential.

In conclusion, although the exposure to TVR tended to be lower in 500 mg q8h than that in 750 mg q8h in the TVR-based triple therapy, relatively high exposure of TVR was observed in Japanese CHC patients given TVR at the lower dose. The result suggests that the lower dose regimen may be one of the options for the treatment of Japanese patients. In addition, in the view of antiviral effects, TVR pharmacokinetics and safety profiles, the present findings indicate that development of adverse

events, specifically anemia and creatinine increase in the treatment with TVR-based regimen, could be avoided by dose adjustment of TVR as well as RBV.

ACKNOWLEDGMENT

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Long-Term Entecavir Treatment Reduces Hepatocellular Carcinoma Incidence in Patients With Hepatitis B Virus Infection

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Chronic hepatitis B virus (HBV) infection leads to cirrhosis and hepatocellular carcinoma (HCC). Antiviral agents are thought to reduce HCC development, but agents such as lamivudine (LAM) have a high rate of drug resistance. We compared the incidence of HCC in 472 entecavir (ETV)-treated patients and 1,143 nontreated HBV patients (control group). Propensity score matching eliminated the baseline differences, resulting in a sample size of 316 patients per cohort. The drug mutation resistance was 0.8% (4/472) in the ETV group. The cumulative HCC incidence rates at 5 years were 3.7% and 13.7% for the ETV and control groups, respectively ($P < 0.001$). Cox proportional hazard regression analysis, adjusted for a number of known HCC risk factors, showed that patients in the ETV group were less likely to develop HCC than those in the control group (hazard ratio: 0.37; 95% confidence interval: 0.15-0.91; $P = 0.030$). Both cohorts were applied in three previously reported risk scales and risk scores were generated based on age, gender, cirrhosis status, levels of alanine aminotransferase, hepatitis B e antigen, baseline HBV DNA, albumin, and bilirubin. The greatest HCC risk reduction occurred in high-risk patients who scored higher on respective risk scales. In sub analyses, we compared treatment effect between nucleos(t)ide analogs, which included matched LAM-treated patients without rescue therapy (n = 182). We found HCC suppression effect greater in ETV-treated ($P < 0.001$) than nonrescued LAM-treated ($P = 0.019$) cirrhosis patients when they were compared with the control group. *Conclusion:* Long-term ETV treatment may reduce the incidence of HCC in HBV-infected patients. The treatment effect was greater in patients at higher risk of HCC. (HEPATOLOGY 2013;58:98-107)

More than 2 billion people worldwide have been exposed to hepatitis B virus (HBV) and about 350 million people are chronically infected, the majority of whom are in Asia (75%). The prevalence of HBV in Japan is 0.8%, which is lower than other Asian countries such as Taiwan (>10%) and China.[1-3] As chronic HBV infection leads to cirrhosis and hepatocellular carcinoma (HCC), published studies have shown that up to 25% of chronically infected patients eventually die of liver cirrhosis or HCC.[4]

A large-scale longitudinal epidemiologic study has shown that a patient's baseline HBV DNA level is an independent predictor for the development of HCC.[5] Studies have begun to show that treatment to decrease HBV DNA reduces the risk of HCC development in HBV patients with cirrhosis or advanced fibrosis or in chronic HBV patients.[6,7]

Within the past 10 years, new antiviral therapies, including nucleos(t)ide analogs (NAs), have been approved and were successful in suppressing circulating serum viral loads. Studies that have examined the relationship between NA therapy and HCC almost exclusively used older drugs such as lamivudine and/or adefovir. Although results of long-term studies showed the importance of antiviral suppression, HCC risk among patients treated by newer NAs remains inconclusive. Entecavir (ETV) is a relatively new antiviral NA that has proved effective in suppressing HBV
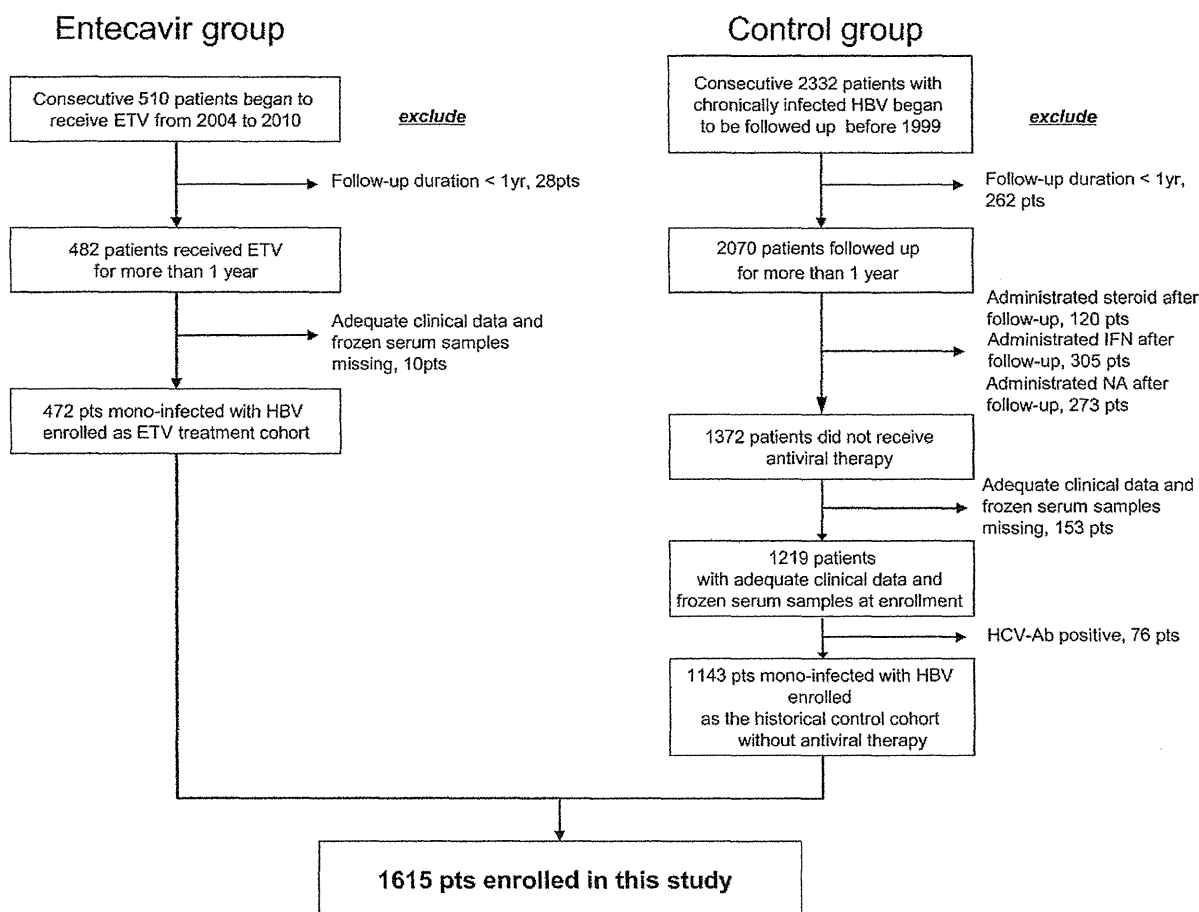

Fig. 1. Entecavir-treated and nontreated cohorts. ETV, entecavir; HBV, hepatitis B virus; IFN, interferon; NA, nucleos(t)ide; HCV-Ab, anti-hepatitis C virus antibody.

DNA replications with minimal drug resistance.[8,9] In this study we examined whether long-term ETV treatment would reduce HCC risk in HBV-infected patients when compared with NA-naïve patients.

Patients and Methods

Patients and Design. From 2004 to 2010, we consecutively recruited 510 patients treated with 0.5 mg ETV (ETV group); the ETV group was compared with a retrospective cohort of 2,332 NA-naïve, HBV-infected patients (control group).

These patients were chronically monoinfected with HBV and were confirmed as hepatitis B s antigen (HBsAg)-positive for at least 6 months. As a general rule,

ETV was initiated in a patient who had both abnormal alanine aminotransferase (ALT) levels (defined as ALT $\geq$45) and elevated HBV DNA levels of $\geq$4 log copies/mL. A patient with advanced fibrosis would be treated with ETV if the ALT level was normal; however, a patient without fibrosis or with a normal HBV DNA/ALT level would not be treated with ETV. Among the treated patients, 38 were excluded from the ETV group either because their follow-up period was less than 1 year (n = 28) or because the clinical data or serum samples were incomplete (n = 10). The remaining 472 ETV-treated patients were included in the analysis (Fig. 1). No patient in the ETV group received other NAs before ETV treatment.

The control group patients were recruited from 1973 to 1999. These patients were NA-naïve at baseline, as no NA therapy had yet been approved. Patients were excluded from the control group if (1) their follow-up duration was less than 1 year (n = 262); (2) corticosteroid withdrawal therapy (n = 120), IFN treatment (n = 305) or NA treatment (n = 273) was initiated during follow-up; (3) clinical data or serum samples were incomplete (n = 153); or (4) patients were found to be positive for anti-hepatitis C virus antibodies (HCV-Ab) (n = 76). The remaining 1,143 patients served as the control population (Fig. 1).

We also made subanalyses to examine the difference of HCC suppression effect between NAs. To make this comparison, we recruited a cohort of 949 consecutive patients from our hospital who were treated with lamivudine (LAM) (September 1995 to September 2007). LAM-treated patients who met the same inclusion criteria as the ETV group, who had no rescue therapy (LAM group, n = 492), were used in the comparison.

We received informed consent from each patient at their entry into the study. Informed consent for the clinical data collection and storage of serum samples were obtained from each patient in the historical control group. The study protocol was in accordance with the ethical guidelines of the Declaration of Helsinki and approved by the Toranomon Hospital Ethics Committee.

Clinical Data Collection and Follow-up. All ETV-treated and untreated patients were followed at 1- to 3-month intervals, during which biochemical and HBV virological markers, blood counts, tumor markers (e.g., alpha-fetoprotein and des-γ-carboxylprothrombin), and cirrhosis and HCC status were monitored. Viral response in the ETV group was defined as a reduction in HBV DNA levels to below 400 copies/mL. Cirrhosis was determined by laparoscopy, liver biopsy, imaging modalities, or portal hypertension. HCC was diagnosed predominantly via imaging, including dynamic computed tomography, magnetic resonance imaging, and/or digital subtraction angiography. When the hepatic nodule did not show typical imaging features, diagnosis was confirmed by fine-needle aspiration biopsy followed by histological examination. Patients were followed until any confirmed HCC diagnosis 1 year after the start of observation (primary outcome) or until the last visit before December 2011. All patients also underwent ultrasonography or helical dynamic computed tomography every 3 to 6 months (cirrhosis patients) or every 6 to 12 months (noncirrhosis patients).

HBV Infection Markers. HBV DNA levels were quantified using the COBAS Amplicor HBV Monitor Test (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.6 to 7.6 log copies/mL, or COBAS TaqMan HBV Test v2.0 (Roche Diagnostics) which has a dynamic range of over 2.1 to 9.0 log copies/mL. HBV DNA of the control group was measured from their stored frozen serum (−80°C) using COBAS TaqMan HBV v.2.0 once at the start of observation. Previous measurements were taken using the old DNA polymerase assay in the control group and thus were not used for comparisons. For the ETV group, drug-resistant mutations were determined from a nested polymerase chain reaction, using a primer specific at the polymerase region in patients who had an HBV DNA relapse of ≥1 log copies from nadir. Hepatitis B e antigen; (HBeAg) was determined by enzyme-linked immunosorbent assay with a commercial kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). 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 for each of the eight major genotypes (A to H).

HCC Incidence by Risk Scores. To examine HCC incidence by risk scores, we applied published HCC risk scales, which are based on the natural course of HCC among HBV-positive patients, to our cohorts. We first searched Medline/PubMed using "hepatitis B," "cancer," and "risk score" as keywords and found four publications in English that used risk-score estimations.[10-13] One article was rejected because we were unable to compute the risk scores with our variables, and therefore we used only the scales indicated by the remaining three publications to generate the risk scores.[13] The risk scales were based on parameters such as age, gender, cirrhosis, levels of ALT, HBeAg, baseline HBV DNA, albumin, and bilirubin. The original risk score formula and the risk score distributions for our two cohorts derived from these formulas are shown in Supporting Table 1. The risk score cutoff points were determined from the following original articles. In Yang et al.'s article,[10] the risk score was derived from 17-point categories. When we applied the scores to our control group, we found that the 12-point scale was at best in detecting a difference in HCC incidence. With that, we examined the HCC suppression treatment effect by dividing the patients into equal halves with 12 points as the cutoff. Yuen et al.[11] divided their cohort in half and found risk scores of 82 as the optimal cutoff point. We also applied the same cutoff point to our cohorts. Wong et al.[12] used their risk scores to categorize their cohort into low-risk, medium-risk, and high-risk groups with respective cutoff points at <4, 4-19, ≥20. We also applied the same cutoff points to our cohorts to examine the treatment effect. Cumulative

HCC incidence rates were compared by these risk scores between the ETV and control groups.

Statistical Analysis. Categorical data were compared using chi-square or Fisher's exact tests. Continuous variables with normal distributions were compared using Student's *t* test, and those without normal distributions were compared using the Mann-Whitney *U* test. Cumulative HCC incidence rates were analyzed using the Kaplan-Meier method; patients followed beyond 5 years were censored to better compare the two cohorts because the ETV group had a shorter follow-up period when compared with the historical control group. We compared the cumulative incidence of HCC using the log-rank test, and Cox proportional hazard regression analysis, which was used to assess the variables that were significantly associated with the development of HCC. Deaths before HCC development were censored. Significance was defined as $P < 0.05$ for all two-tailed tests.

We used the propensity score (PS) matching method to reduce significant differences in demographics between the ETV and control groups.[14,15] Using multiple logistic regression analysis, a PS was estimated for all patients treated with ETV.[14] Variables used in the model included age, sex, presence of cirrhosis, HBeAg, HBV DNA< aspartate aminotransferase (AST), ALT, γ-glutamyl transpeptidase; (γ-GTP), bilirubin, albumin, and platelet counts. We performed caliper matching on the PS (nearest available matching). Pairs (ETV and the control group) on the PS logit were matched to within a range of 0.2 standard deviation (SD).[16,17] The PS logit distributions for each cohort showing the overlaps and SD ranges are shown in Supporting Fig. 1. The balance of covariates was measured by their standardized differences. A difference >10% of the absolute value was considered significantly imbalanced.[17] The cohorts were divided into five PS quintiles (Supporting Table 2). We also made subanalyses to examine the difference of HCC suppression effect between NAs by comparing the HCC incidence between propensity score matched ETV- and lamivudine (LAM)-treated patients without a rescue therapy. The LAM-treated patients were derived from consecutive sampling at our institution and were PS matched with ETV group according to the same method described above. Interaction of the subgroups by pre-existing cirrhosis or risk scores and ETV treatment were evaluated. $P < 0.10$ was considered statistically significant. Data analysis was performed using IBM SPSS v. 19.0 software (Armonk, NY) and R software v. 2.13 (R Foundation for Statistical Computing, Vienna, Austria; www.r-project.org).

Results

Patient Characteristics. The patient characteristics at the baseline, before PS matching are shown in Table 1. The ETV group was followed for an average of 3.2 years (1,561 person-years), whereas the control group was followed for an average of 9.5 years (12,381 person-years). Before matching, patients in the ETV group and the control group differed significantly in age, gender, genotype, baseline HBV DNA level, and other clinical data. In the ETV group, 421 patients (89%) had HBV DNA (<400 copies/mL) at year 1. Not all patients in the control group were tested for HBV DNA level during follow-up. The drug mutation resistance was 0.8% (4/472). The four patients who had drug mutation did not develop HCC. During follow-up, 12 patients (2.54%) in the ETV group and 144 patients (12.60%) in the control group developed HCC. The incidence rates of HCC for the ETV and the control groups were 76/10,000 patient-years and 116/10,000 patient-years, respectively. During this period, 21 patients in the control group developed liver cirrhosis while no patient developed liver cirrhosis in the ETV group. During the same observation period, there were four deaths in the ETV group and 10 deaths in the control group. We took competing risk into account[18,19] and compared incidence of non-HCC deaths between the cohorts and the results were not different. However, because there were only four patients in the non-HCC deaths in the ETV group (two patients in the PS matched cohort) and 10 patients in the control group (six patients in the PS matched cohort), we considered that it was not meaningful to apply competing risk analysis in our cohorts.

Factors Associated with HCC and Effect of ETV Treatment on HCC Development. To allow a common ground for comparison between the two cohorts, we used PS matching with selected key characteristics and compared the two groups within the same time period of 5 years. The PS matching process resulted in a matched sample size that consisted of 316 patients in each group (Table 1). The PS matching reduced the significant variability of the two cohorts. While five (42%) of the 12 covariates varied by >10% before matching, all covariates differed by <10% of the absolute value after matching (Supporting Fig. 2). In the PS score matched cohort, 10 out of the 231 noncirrhosis patients progressed to liver cirrhosis within 5 years of observation. The cumulative incidence rates of HCC in the matched ETV groups were 0.7% at year 2, 1.2% at year 3, 2.5% at year 4, and 3.7% at year 5. The cumulative incidence rates of HCC in the

Table 1. Patient Characteristics and Demographics

Characteristics	Entire Cohort				Propensity Score Matched Cohort		
	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/mm³) (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 year‡	–	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. Pro-

gression 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 non-rescued 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
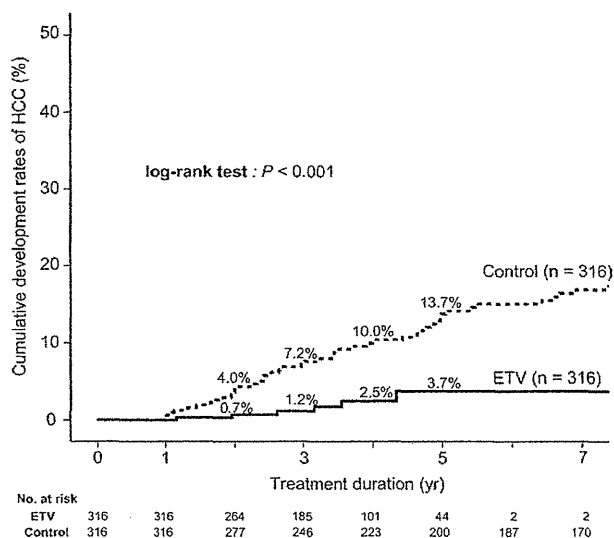
Fig. 2. Comparison of HCC cumulative incidence rates between the entecavir-treated group and the nontreated control group after propensity score matching. The log-rank test revealed a statistically significant difference between the ETV and the control group in the incidence of HCC at 5 years time (log-rank 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 ≥ 12, $P = 0.006$; risk score ≥ 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 (≥5.0 log copies/mL)	4.66 (1.44-15.1)	0.010		
ALT (≥45 IU/L)	2.29 (1.10-4.77)	0.027		
GGTP (≥50 IU/L)	3.79 (2.02-7.09)	<0.001		
Total bilirubin (≥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 (<1.5×10^5/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.
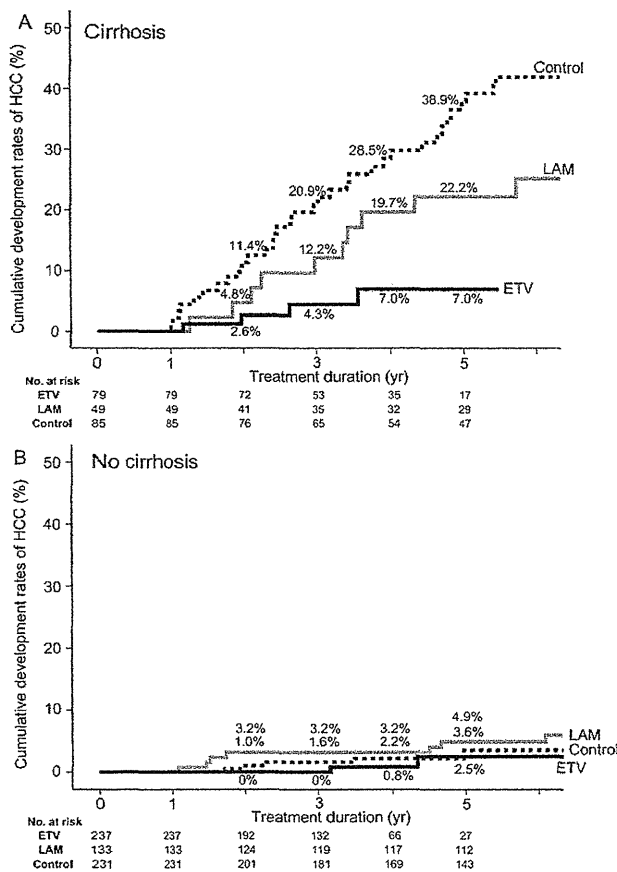
Fig. 3. Comparison of HCC cumulative incidence rates between the entecavir (ETV)-treated group, lamivudine (LAM)-treated, and the non-treated control group after PS matching stratified by cirrhosis. The log-rank test revealed a statistically significant difference in the incidence of HCC at 5 years time 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 non-responding 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
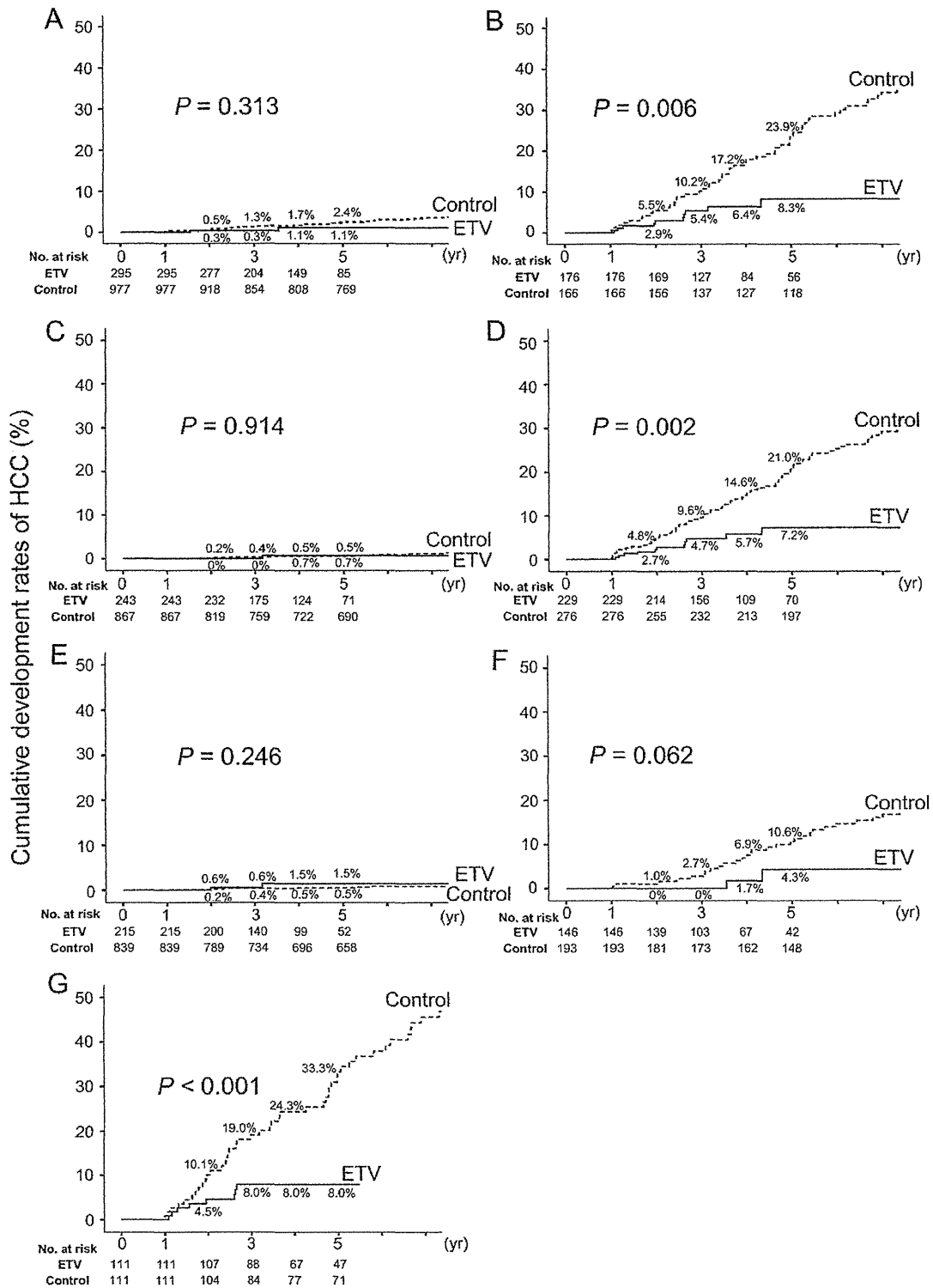
Fig. 4. Cumulative incidence of HCC by risk score scales: comparison between entecavir-treated and nontreated control patients: Risk score cutoff points were based on those presented in articles by the following: A,B (Yang et al.[10]): low-risk score cutoff point < 12; high-risk score cutoff point ≥ 12. C,D (Yuen et al.[11]): low-risk score cutoff point < 82; high-risk score cutoff point ≥ 82. E-G (Wong et al.[12]): low-risk score cutoff point < 4; medium-risk cutoff point 4-19; high-risk score cutoff point ≥ 20. A statistically significant difference in HCC incidence was seen between the ETV group and the control group in the higher-risk groups when observed the incidence of HCC over time (log-rank test $P = 0.006$ for risk score ≥ 12; $P = 0.002$ for risk score ≥ 82; $P = 0.062$ for patients with medium risk; $P < 0.001$ for patients at high risk for HCC).

easy-to-use nomograms based on clinical characteristics to predict the risk of HCC in patients with HBV. These scales have been validated, and can accurately estimate the risk of HCC up to 10 years. The cutoff scores used in these studies were based on their sensitivity to detect HCC derived and validated with non-treated HBV cohorts. The importance of our study using these risk scales in our cohorts was to see the change in risk with the initiation of therapy. We found that the ETV treatment effect to reduce the risk of HCC was more prominent among cirrhosis and high-risk patients despite the lack of interactions between ETV treatment and preexisting cirrhosis or risk factors. The lower treatment effect among lower-risk patients was somewhat not surprising. HCC development among low-risk patients is generally rare, and therefore, the treatment effect may not have occurred in large enough numbers during the treatment period allotted in our study to be able to detect a difference. In addition, HCC development differs greatly by cirrhotic status and risk factors in the control group. The treatment effect of ETV to reduce HCC is probably more likely reflected among cirrhosis or high-risk patients. A study with a longer observation period and higher patient numbers might be necessary to examine this ETV treatment effect among low-risk patients. The development of a scoring system to predict treatment effect of HBV patients with different risk levels will be useful in determining the most appropriate timing of treatment initiation in clinical settings.

Study Limitations. There were several limitations to our study. First, because our patients were recruited from one hospital, they might not have been representative of the general Japanese HBV population. Second, our control group included historically observed patients who entered the cohort long before the ETV group, resulting in treatment differences during the time gap. However, we used PS matching and a similar follow-up period between the two cohorts to minimize this bias. Third, our study was an observational study with patients having large demographic differences. Although we used a PS to match ETV-treated and control groups, our sample size did not take into account other unobserved confounding factors such as HCC family history, stage of cirrhosis, and comorbidities when determining associating factors for carcinogenesis in HBV. Finally, the observation period of the ETV group was relatively short, and patients in the ETV-treated cohort at 5 years consisted of only less than ~25% of the initial recruited patients. Because of this limitation, we censored patients who were followed for more than 5 years. The observed treatment effect would require confirmation over a longer period and a more complete follow-up.

Conducting a long-term study to examine the effect of antiviral therapy with HCC as the endpoint would be time-consuming and challenging. Such a study would require a large sample size and would, therefore, be costly. In addition, the increases in choices of therapy over time would make it difficult to conduct a long-term study using a single therapy. Owing to ethical issues, it would be difficult to recruit or follow a naïve, untreated cohort over an extended period of time. Because of these challenges, most studies have examined the relationship between antiviral treatment and the risks of HCC involved older drugs, lacked a control group, or were of relatively short duration. Consequently, the association between antiviral treatment and carcinogenesis is inferential and requires additional confirmatory studies.

In conclusion, in our study we observed the effect of HCC risk among HBV-infected patients treated by ETV by comparing them with a group of NA-naïve patients. We followed these Japanese patients for a relatively long period of time and compared them with a large pool of untreated control patients. In this long-term study among Japanese patients, ETV significantly reduced the incidence of HCC among chronic HBV-infected patients, and was more prominent among patients at higher risk for HCC.

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

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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.*
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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

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) | | | | Efficacy |
								Baseline	12 weeks	24 weeks	48 weeks	
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			Viral loads	At point of re-elevation of viral loads			Viral loads	Efficacy
		Direct	Deep	Coverage		Direct	Deep	Coverage		
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)