combination therapy were examined as correlates of HCC development. The Kaplan–Meier method was used to assess the cumulative incidence of HCC, and the groups were compared using the log-rank test. The Cox proportional hazards model was used to identify the independent factors associated with developing HCC. A value of $P < 0.05$ (two-tailed) was considered to indicate significance. Statistical software IBM SPSS for Windows, version 19.0.0 (IBM, Armonk, NY, USA), was used for this analysis.

RESULTS

Baseline characteristics of patients with chronic HCV infection and NALT

Baseline characteristics of the 809 HCV-infected patients with NALT are shown in Table 1. The mean age was 56.7 ± 11.0 years, and the ratio of men was 67%. A total of 550 patients (69%) were infected with HCV genotype 1. Liver biopsies were performed for 587 cases, and the ratio of patients with progression of liver fibrosis (METAVIR fibrosis score 3 or 4) was 7.8%. The rate of patients with SVR was 48.7% (263 of 550) in genotype 1 and 74.5% (184 of 247) in genotype 2. The mean observation period was 36.2 ± 16.5 months.

Baseline characteristics of patients who developed HCC

Eleven patients developed HCC during the observation period. The baseline clinical features before Peg-IFN plus ribavirin combination therapy and the features of the HCC that developed are shown in Table 2. HCC development was observed often in aged patients and male patients. All who developed HCC were infected with HCV genotype 1 and had a high viral load.

Risk factors associated with incidence of HCC among patients with chronic HCV infection and NALT

Patients with chronic HCV infection and NALT showed HCC incidences of 1.1% and 3.3% at 3 years and 5 years, respectively, in this study. Significant association with HCC development on univariate analysis was found for age, sex, platelet count and virological response to combination therapy, but not for the amount of HCV RNA (Table 3). Significant factors included older age, male gender and virological response, which were independent factors of HCC incidence in the Cox proportional hazards model (Table 3). The adjusted cumulative incidence of HCC at 3 years by the Cox proportional hazards model was 0.19% among patients <60 years of age, 0.59% among patients 60–64 years of age and 1.12% among patients ≥65 years of age. The cumulative incidence of HCC at 3 years was 0.96% in men and 0.25% in women. As shown in Fig. 1, patients with SVR or relapse had a significantly lower cumulative incidence of HCC than that of patients with NR (cumulative incidence of HCC at 3 years: SVR, 0.20%; relapse, 0.30%; NR, 1.89%).

Baseline characteristics and risk factors associated with HCC incidence among patients with chronic HCV infection and PNALT

In this study, patients who met the definition of PNALT40 were 431 of the 809 patients with NALT during the mean observation period of 35.5 ± 15.9 months. Baseline characteristics of the 431 HCV-infected patients with PNALT40 are shown in Table 1. Among the PNALT40 patients, three developed HCC during the observation period (Table 2, cases 4, 9 and 10). Significant association with HCC development by log-rank test was found for older age, male gender, lower platelet count and NR (Table 4). No significant risk factors were found in multivariate analyses of these four variables.

Baseline characteristics of the 176 HCV-infected patients with PNALT30 are shown in Table 1. There were more female PNALT30 patients, more HCV genotype 2 patients, lower BMI, lower activity and lower fibrosis compared with PNALT30–40 patients. Among them, one patient developed HCC during the mean observation period of 33.5 ± 15.9 months (Table 2, case 4). There were no significant risk factors associated with HCC development among these patients (Table 4).

DISCUSSION

Continuous infection with HCV is a leading cause of liver fibrosis which may progress to cirrhosis and HCC [10,11]. HCV carriers with NALT levels are generally considered to have a low risk of carcinogenesis because of minimal liver inflammation and slow progression of liver fibrosis [12,13]. Thus, IFN therapy has not been used for NALT patients. Against this background, there has been no report of antiviral therapy for HCV carriers with NALT reducing HCC incidence. However, a simulation using the Markov model has suggested that Peg-IFN plus ribavirin combination therapy could decrease HCV-related morbidity and mortality in patients with NALT [14]. This needed to be evaluated with patients using Peg-IFN plus ribavirin combination therapy. In the present study, we examined the efficacy of antiviral therapy in patients with chronic HCV infection and NALT from the viewpoint of reducing the risk of HCC. This is the first report indicating that virological responders for antiviral therapy had a significantly lower cumulative incidence rate of HCC than those with NR in patients with chronic HCV infection and NALT.

In the present study, we examined whether successful virological response to Peg-IFN plus ribavirin combination therapy was associated with a low HCC incidence in NALT patients. Considering that reducing the risk of HCC

Table 1 Baseline characteristics of NALT patients and PNALT patients

	NALT (n = 809)	PNALT40 (n = 431)	PNALT40		P value *
			PNALT30-40 (n = 255)	PNALT30 (n = 176)	
Age (years)	56.7 ± 11.0	57.3 ± 10.7	57.1 ± 11.1	57.4 ± 10.1	0.912
Sex: male/female (%)	269 (33)/540 (67)	119 (28)/312 (72)	87 (34)/168 (66)	32 (18)/144 (82)	<0.001
BMI (kg/m^2)	22.7 ± 3.2	22.5 ± 3.3	22.7 ± 3.2	22.1 ± 3.3	0.020
HCV genotype: 1/2	550 (69)/247 (31)	279 (66)/143 (34)	186 (74)/67 (26)	93 (55)/76 (45)	<0.001
HCV RNA (log IU/mL)	6.5 ± 0.7	6.6 ± 0.7	6.6 ± 0.6	6.5 ± 0.7	0.132
Past IFN therapy†: naïve/experienced (%)	580 (74)/207 (26)	320 (76)/100 (24)	184 (74)/63 (26)	136 (79)/37 (21)	0.195
Liver histology‡					
Activity: A0/A1/A2/A3	58/394/131/3	46/222/60/1	17/120/42/1	26/102/18/0	0.032
Fibrosis: F0/F1/F2/F3/F4	61/359/121/36/10	42/204/66/12/5	17/112/38/11/5	25/92/28/1/0	0.006
White blood cell (per mm^3)	5228 ± 1591	5173 ± 1628	5233 ± 1660	5086 ± 1581	0.326
Neutrophil (per mm^3)	2847 ± 1190	2850 ± 1213	2812 ± 1209	2902 ± 1220	0.370
Haemoglobin (g/dL)	13.5 ± 1.4	13.4 ± 1.2	13.5 ± 1.3	13.2 ± 1.1	0.023
Platelet (×10^4/mm^3)	18.6 ± 5.8	18.8 ± 5.6	18.7 ± 5.9	19.0 ± 5.2	0.299
ALT (IU/mL)	28.2 ± 7.6	26.2 ± 7.3	29.9 ± 6.6	21.2 ± 4.9	<0.001
AFP (ng/L)	6.2 ± 23.9	5.0 ± 9.4	5.0 ± 9.5	5.0 ± 9.3	0.409
Observation period (month)	36.2 ± 16.5	35.5 ± 15.9	36.9 ± 16.2	33.5 ± 15.4	0.038
Viral response: SVR/relapse/NR (%)	454 (56)/191 (24)/164 (20)	225 (52)/113 (26)/83 (19)	129 (51)/71 (28)/55 (22)	106 (60)/42 (24)/28 (16)	0.126

NALT, defined as an ALT value of ≤40 IU/mL at the start of therapy; PNALT40, defined as an ALT value of ≤40 IU/mL on two to three occasions separated by at least a month over a period of 6 months; PNALT30, PNALT with an ALT value of ≤30 IU/mL on 2 to 3 occasions separated by at least a month over a period of 6 months; PNALT30–40, ALT values of 31–40 IU/mL on at least one occasion among PNALT40 patients; BMI, body mass index; HCV, hepatitis C virus; IFN, interferon; AST, aspartate aminotransferase; ALT, alanine aminotransferase; SVR, sustained virological response; NR, nonresponse. †Interferon therapy history was not known for 22 patients. ‡222 patients, no fibrosis data; 223 patients, no activity data. *P value was calculated between PNALT30–40 patients and PNALT30 patients.

Table 2 Baseline characteristics of NALT patients with HCC development

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10	Case 11
Age (years)	64	67	70	72	59	55	73	65	61	68	64
Sex: male/female	female	male	male	male	male	male	female	female	male	male	female
BMI (kg/m^2)	17.4	24.5	23.3	22.9	22.7	22.7	23.4	19.4	20.0	22.3	21.6
HCV genotype: 1/2	1	1	1	1	1	1	1	1	1	1	1
HCV RNA (log IU/mL)	6.9	7.0	7.3	7.3	7.3	6.8	6.1	6.6	6.3	7.1	7.3
Past IFN therapy: naïve/NR/relapse	relapse	naïve	naïve	NR	NR	naïve	naïve	naïve	NR	relapse	NR
Liver biopsy*											
Activity: A0/A1/A2/A3	1	2	1	1	2	–	–	1	2	0	–
Fibrosis: F0/F1/F2/F3/F4	1	3	1	2	2	–	–	1	1	2	–
Virological response: SVR/relapse/NR	SVR	SVR	SVR	relapse	NR	NR	NR	NR	NR	NR	NR
Platelet ($\times 10^4$ per mm^3)	15.1	24.3	21.4	9.9	12.0	22.3	8.7	12.7	18.8	11.4	10.1
ALT (IU/mL)	34	34	31	21	28	27	35	33	36	11	40
AFP (ng/L)	2.0	16.0	7.0	4.0	–	6.3	–	6.0	3.3	3.0	2.0
Timing of HCC development (month)	22.9	32.8	21.4	56	31.7	36.1	47.2	29.5	25.8	12.9	39.6
HCC size† (mm)	10	12	25	9	16	17	10	10	30	30	20
(number)	(1)	(1)	(≥5)	(1)	(2)	(1)	(1)	(1)	(1)	(1)	(1)

BMI, body mass index; HCV, hepatitis C virus; IFN, interferon; SVR, sustained virological response; NR, nonresponse; ALT, alanine aminotransferase; AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma. *Liver biopsy was not performed for cases 6, 7 and 11. †HCC size was defined as size of the largest HCC if patients had many HCCs.

Table 3 *Factors associated with HCC incidence among patients with NALT*

Factor	Category	Univariate analysis			Multivariate analysis		
		Hazard ratio	95% CI	*P* value	Hazard ratio	95% CI	*P* value
Age (years)	<60 *vs* 60–65	3.00	0.50–17.96	0.229	3.15	0.52–19.12	0.212
	<60 *vs* 65≤	7.51	1.51–37.35	0.014	6.03	1.17–31.22	0.032
Sex	female *vs* male	3.55	1.04–12.14	0.039	3.91	1.13–13.49	0.031
BMI (kg/m^2)	<25 *vs* 25≤	0.04	0.00–20.33	0.302			
HCV genotype	genotype 2 *vs* genotype 1	30.68	0.07–13048.42	0.268			
HCV RNA (log IU/mL)	<6.5 *vs* 6.5≤	2.49	0.54–11.55	0.242			
Activity	A0–A1 *vs* A2–A4	1.79	0.43–7.50	0.442			
Fibrosis	F0–F1 *vs* F2–F4	2.44	0.61–9.75	0.208			
Platelet (×10^4 per μL)	12≤ *vs* <12	4.16	1.22–14.25	0.038	2.86	0.81–10.04	0.101
ALT (IU/mL)	<30 *vs* 30≤	2.10	0.61–7.19	0.226			
AFP (ng/mL)	<5 *vs* 5	2.67	0.75–9.45	0.129			
Virological response	NR *vs* SVR	0.13	0.03–0.49	0.003	0.16	0.04–0.63	0.009
	NR *vs* relapse	0.11	0.01–0.87	0.037	0.11	0.01–0.87	0.037

HCC, hepatocellular carcinoma; NR, nonresponse.

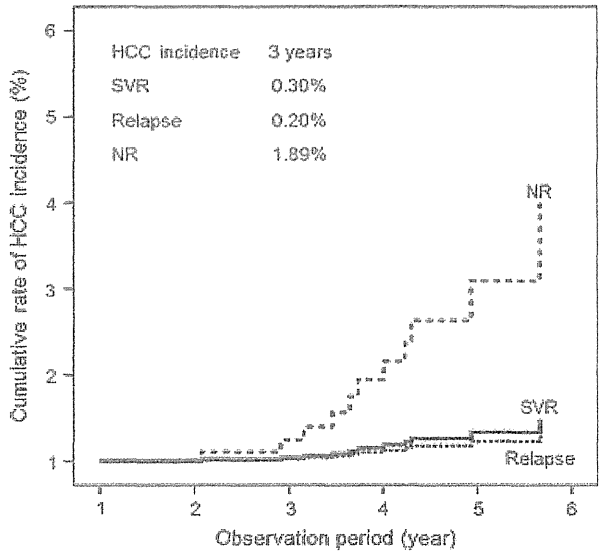

Fig. 1 Cumulative rate of HCC incidence by Cox proportional hazards model according to antiviral effect.

incidence is the goal of antiviral therapy, this result indicates that patients with HCV infection and NALT can also be candidates for antiviral therapy. In particular, male patients ≥65 years of age should be treated earlier even if the ALT level is within the normal range because the factors of age ≥65 years and male gender were identified as independent risk factors for HCC incidence. Several studies have reported that patients ≥65 years of age or male patients generally had a higher risk of HCC incidence among CHC patients with EALT [15–17]. Our results suggest that older male patients with HCV infection should be treated even if they are NALT patients.

All patients who developed HCC had HCV genotype 1 and a high viral load. The first reason is that the patient group with genotype 1 and high viral load is refractory to antiviral therapy. Non-SVR patients are liable to develop HCC. However, three of the 11 patients in this group had SVR. As some researchers have hypothesized, HCV genotype 1 may pose a higher carcinogenic hazard than other genotypes [18,19].

In Japanese treatment guidelines for patients with chronic HCV infection and NALT levels, HCV carriers with NALT (≤40 IU/mL) at the initial visit are classified into four groups according to their ALT levels (≤30 IU/mL or ≥31 IU/mL) and platelet counts (≥15 × 10^4 per mm^3 or <15 × 10^4 per mm^3). In the univariate analysis according to those categories, low platelet counts (<15 × 10^4 per mm^3) were shown to be the only risk factor for HCC incidence, irrespective of the ALT levels [20]. In the present study, risk factors for HCC incidence were examined among NALT patients by multivariate analysis. As a result, no significant relationship was found between platelet counts or progression of liver fibrosis and HCC incidence. In fact, older patients who developed HCC in this study had relatively high platelet counts and minimal liver fibrosis. Previous studies have shown that older patients with HCV infection even without severe liver fibrosis are at risk of developing HCC [21]. Although the reason for this is not known, mechanisms such as those associated with telomeres may be in part responsible [22,23]. Older patients require extra attention with respect to HCC surveillance, regardless of the progression of liver fibrosis.

Table 4 Factors associated with HCC incidence among patients with PNALT by log-rank test

Factor	Category	PNALT40 (n = 431) P value	PNALT30-40 (n = 255) P value	PNALT30 (n = 176) P value
Age (years)	<60/60–64	0.171	0.192	–*
	60–64/65≤	0.561	0.945	0.480
	<60/65≤	0.028	0.165	0.157
Sex	male/female	0.008	0.040	0.248
BMI (kg/m^2)	<25/25≤	0.459	0.529	0.887
HCV genotype	genotype 1/genotype 2	0.343	0.430	–*
HCV RNA (log IU/mL)	<6.5/6.5≤	0.891	0.599	0.456
Activity	A 0–1/A 2–3	0.473	0.397	0.580
Fibrosis	F 0–1/F 2–4	0.104	0.542	0.186
Platelet ($\times 10^4$ per mm^3)	<12/12≤	0.007	0.100	0.114
AFP (ng/mL)	<5/5≤	0.272	0.363	0.602
Virological response	SVR/relapse	0.150	–*	0.273
	relapse/NR	0.314	0.094	0.439
	SVR/NR	0.011	0.018	–*

PNALT40, defined as an ALT value of ≤40 IU/mL on two to three occasions separated by at least a month over a period of 6 months; PNALT30, PNALT with an ALT value of ≤30 IU/mL on two to three occasions separated by at least a month over a period of 6 months; PNALT30–40, ALT values of 31–40 IU/mL on at least one occasion among PNALT40 patients; BMI, body mass index; HCV, hepatitis C virus; IFN, interferon; AST, aspartate aminotransferase; ALT, alanine aminotransferase; SVR, sustained virological response; NR, nonresponse. *P value of age, HCV genotype and virological response (SVR *vs* NR) could not be calculated because there were no data for comparison.

In the present study, 431 patients with PNALT40, identified by an ALT value of ≤40 IU/mL on two to three occasions separated by at least a month over a period of 6 months, were examined for HCC incidence. Univariate analysis showed that older age (≥65 years), male gender, lower platelet counts (<12 × 10^4 per μL) and SVR to combination therapy were significant risk factors for HCC incidence, which were very similar to those for NALT patients; the difference was only that being in relapse was not a significant risk factor for HCC incidence in patients with PNALT40. A longer observation period may reveal a reduction in HCC incidence among relapse patients with PNALT40.

If the definition of PNALT were an ALT value of ≤30 IU/mL on two to three occasions separated by at least a month over a period of 6 months, then 176 of our patients would fit this classification. There were no significant risk factors for HCC incidence among patients with PNALT30, and only one patient with relapse developed HCC during the observation period. No patients with SVR and NR developed HCC. Therefore, little or no liver inflammation among patients with HCV infection and PNALT30 might indicate a fairly low risk of HCC incidence; that is, it may suggest that patients with PNALT30 can be followed up without antiviral therapy from the viewpoint of reducing the risk of HCC incidence. On the other hand, significant risk factors, including male gender and viral response, were found among patients with PNALT30–40 from the univariate analysis. Considering the high risk of HCC incidence, this suggests that patients with HCV infection and PNALT30–40 should be candidates for antiviral therapy.

The limitation of this study was that the HCC incidence was not compared between the treatment and nontreatment groups. HCC incidence was significantly lower in patients with SVR or relapse compared with those with NR as a control group among patients with Peg-IFN plus ribavirin combination therapy. This does not show a clear suppressive effect of antiviral therapy for HCC incidence in NALT patients with HCV infection. However, patients with NR treated with antiviral therapy are reported to have about the same HCC incidence as patients without antiviral therapy [24]. Moreover, Peg-IFN plus ribavirin combination therapy in NALT patients is not effective if a successful virological response does not lead to the reduction in HCC incidence. Our findings that patients with SVR or relapse had significantly lower HCC incidence compared with those with NR suggest that patients with HCV infection and NALT should be candidates for Peg-IFN plus ribavirin combination therapy. A prospective randomized study should be performed to verify the suppressive effect of antiviral therapy for HCC.

In conclusion, among patients with chronic HCV infection and NALT, the cumulative incidence of HCC was significantly lower in patients with SVR and relapse than those with NR to Peg-IFN plus ribavirin combination ther-

apy in the same manner as any EALT patient with HCV infection. Therefore, patients with chronic HCV infection and NALT should be considered as candidates for antiviral therapy from the viewpoint of reducing HCC incidence, especially aged patients (≥65 years) and/or male patients. However, the indication of antiviral therapy for PNALT (ALT ≤ 30 IU/mL) should be carefully examined.

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Using early viral kinetics to predict antiviral outcome in response-guided pegylated interferon plus ribavirin therapy among patients with hepatitis C virus genotype 1

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Abstract

Background HCV kinetics during treatment demonstrated strong association with the antiviral outcome of patients treated with pegylated interferon (Peg-IFN) plus ribavirin. However, the relationship between HCV kinetics and pre-treatment factors remains unclear.

Methods Of 547 patients with HCV genotype 1 treated with Peg-IFN alfa-2b plus ribavirin, 401 completed the response-guided therapy and were assessed for per protocol analysis.

Results The sustained virologic response (SVR) rate was 53 % for all patients, 60 % for those with genotype TT, and 19 % for those with genotype TG/GG according to IL28B (rs8099917) single nucleotide polymorphisms. The SVR rates increased with HCV decrease at week 4; 4 % (2/56) with <1 $\log_{10}$ decrease, 13 % (7/56) with 1–2 $\log_{10}$ decrease, 51 % (44/87) with 2–3 $\log_{10}$ decrease, 64 % (56/87) with 3–4 $\log_{10}$ decrease, 88 % (72/82) with more than 4 $\log_{10}$ decrease but with detectable HCV RNA and 100 % (33/33) with undetectable HCV RNA ($p < 0.001$). Similarly, SVR rates increased step-by-step in proportion to HCV decrease in both IL28B TT and TG/GG groups, showing almost the same SVR rates for the same conditions. In multivariate analysis, age ($p = 0.005$) and the magnitude of HCV decrease at week 4 ($p < 0.001$) but not IL28B were associated with SVR. Advanced liver fibrosis ($p = 0.004$) and the magnitude of HCV decrease at week 4

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($p < 0.001$) but not IL28B were associated with non-response.

Conclusions The magnitude of the HCV decrease at week 4 seems to be the most reliable marker for predicting antiviral outcome after starting Peg-IFN plus ribavirin therapy.

Keywords Chronic hepatitis C · Pegylated interferon plus ribavirin · HCV decrease · IL28B

Introduction

Triple therapy with pegylated interferon (Peg-IFN), ribavirin plus telaprevir (TVR) can improve the antiviral effect for patients infected with hepatitis C virus (HCV) genotype 1 [1–8]. However, this triple therapy is not indicated for some patients due to adverse effects such as severe anemia progression, severe eruption and deterioration of renal function. On the other hand, 48–55 % of genotype 1 chronic hepatitis C (CH-C) patients have been shown to attain sustained virologic response (SVR) by response-guided Peg-IFN plus ribavirin combination therapy [9, 10]. Accordingly, naïve patients for whom there is concern about the possibility of enhanced adverse effects due to the addition of TVR, especially elderly patients, should first be given dual therapy with Peg-IFN plus ribavirin [11]. However, if patients having a high risk for hepatocellular carcinoma show poor antiviral response to this dual therapy, starting them on the triple therapy with Peg-IFN, ribavirin plus TVR should be considered. What is important is the ability to predict the antiviral outcome early during treatment in response-guided Peg-IFN plus ribavirin combination therapy.

The magnitude of the HCV RNA decrease or the timing of HCV RNA negativiation during the treatment has been reported to be strongly associated with SVR rates among genotype 1 CH-C patients treated with Peg-IFN plus ribavirin combination therapy [12–16]. During a 48-week treatment, SVR was attained in 70–80 % of patients with complete EVR (c-EVR), defined as undetectable HCV RNA at 12 weeks of treatment, and in about 90 % of patients with rapid virological response (RVR), defined as undetectable HCV RNA at 4 weeks of treatment [14–16], while those with less than a 2 $\log_{10}$ decrease in the HCV RNA level at 12 weeks mostly were non-SVR [12, 13, 16]. Currently, the magnitude of the HCV RNA decrease at 4 weeks of treatment has been attracting attention as a predictive factor for the antiviral outcome to this combination therapy [16, 17]. However, the aforementioned study was based on an existing method (lower limit 50 IU/ml) [17]. It is now possible to more precisely predict antiviral outcome thanks to progress in measurement of HCV RNA levels, which can now be quantitated with a higher degree of accuracy (lower limit 15 IU/ml) and for a broader range with 2 $\log_{10}$ differences.

A genetic polymorphism near the interleukin 28B (IL28B) gene has been reported to be a strong factor associated with antiviral outcome to the Peg-IFN plus ribavirin combination therapy [18–24]. Patients with the TT genotype of IL28B single nucleotide polymorphism (SNP) (rs8099917) can attain a higher virologic response during treatment (RVR, c-EVR) and a higher SVR rate in comparison with those with non-TT genotype [19, 23].

In the present study, we investigated the magnitude of impact of the early HCV RNA kinetics on predicting the antiviral outcome of response-guided Peg-IFN plus ribavirin combination therapy among genotype 1 CH-C patients. We also examined the relationship between the early viral kinetics and pre-treatment factors.

Patients and methods

Patients

The current study was a retrospective, multicenter trial conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum. A total of 547 patients with CH-C treated with a combination of Peg-IFN alfa-2b plus ribavirin between January 2008 and August 2010 were enrolled in this study. Of the 547 patients, 401 completed the response-guided therapy and were assessed for per protocol analysis.

Eligible patients for this study were those who were infected with HCV genotype 1 and had a viral load of more than 10^5 IU/ml, but were negative for hepatitis B surface antigen and anti-human immunodeficiency virus. Patients were excluded from this study if they had decompensated cirrhosis or other forms of liver disease (alcohol liver disease, autoimmune hepatitis). Informed consent was obtained from each patient included in this study. This study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki amended in 2002.

Treatment

All patients received Peg-IFN alfa-2b (PEGINTRON; Merck & Co. Inc., Whitehouse Station, NJ, USA) plus ribavirin (REBETOL; Merck & Co. Inc.). Peg-IFN alfa-2b was given subcutaneously once weekly at a dosage of 60–150 μg/kg based on body weight (body weight 35–45 kg, 60 μg; 46–60 kg, 80 μg; 61–75 kg, 100 μg; 76–90 kg, 120 μg; 91–120 kg, 150 μg) and ribavirin was given orally twice a day at a total dose of 600–1000 mg/day based on body weight (body weight <60 kg, 600 mg;

60–80 kg, 800 mg; >80 kg, 1000 mg), according to a standard treatment protocol for Japanese patients.

Dose reduction

Dose modification followed, as a rule, the manufacturer's drug information according to the intensity of the hematologic adverse effects. The dose of Peg-IFN alfa-2b was reduced to 50 % of the assigned dose if the white blood cell (WBC) count declined to $<1500/mm^3$, the neutrophil count to $<750/mm^3$, or the platelet count to $<8 \times 10^4/mm^3$, and was discontinued if the WBC count declined to $<1000/mm^3$, the neutrophil count to $<500/mm^3$, or the platelet count to $<5 \times 10^4/mm^3$. Ribavirin was also reduced from 1000 to 600, or 800 to 600, or 600 to 400 mg if the hemoglobin (Hb) level decreased to <10 g/dl, and was discontinued if the Hb level decreased to <8.5 g/dl.

Histological evaluation

Pre-treatment liver biopsies were conducted within 6 months before the start of the combination therapy. Histopathological interpretation of the specimens was done by experienced liver pathologists who had no clinical, biochemical and virological information. The histological appearances, activity and fibrosis, were evaluated according to the METAVIR histological score [25].

IL28B genotyping

Human genomic DNA was extracted from a whole blood sample for each patient. Genetic polymorphism in SNPs located near the IL28B gene (rs8099917) was determined by a real-time PCR system. Each extracted DNA was used for PCR with primers and probes from a commercial kit (Taqman SNP Genotyping Assays, Applied Biosystems). The SNP of IL28B rs8099917 was amplified, and the results were analyzed by real-time PCR in a thermal cycler (7900 Real-time PCR System, Applied Biosystems). Homozygosity for TT genotype was defined as having the IL28B TT genotype, whereas homozygosity for GG or heterozygosity for TT (TG) genotype was defined as having the IL28B non-TT genotype.

Virologic assessment and definition of viral response

Serum HCV RNA level was quantified with the COBAS Taqman HCV test, version 2.0 (detection range 1.2–7.8 log IU/ml; Roche Diagnotics, Branchburg, NJ, USA). Serum HCV RNA level was assessed before treatment, every 4 weeks during treatment and 24 weeks after the therapy. The patients were divided into six groups according to the magnitude of the decrease in HCV RNA from baseline at treatment week 4, 8 and 12: <1 $\log_{10}$ decrease, 1 to <2 $\log_{10}$ decrease, 2 to <3 $\log_{10}$ decrease, 3 to <4 $\log_{10}$ decrease, ≥4 $\log_{10}$ (detectable), and undetectable. The case of serum HCV RNA level lower than 1.2 log IU/ml but positive for the amplification signal was defined as ≥4 $\log_{10}$ (detectable). A RVR was defined as undetectable serum HCV RNA at week 4, a c-EVR as undetectable serum HCV RNA at week 12, and a late virologic response (LVR) as detectable HCV RNA at week 12 but undetectable at week 36. SVR was defined as undetectable serum HCV RNA at 24 weeks after discontinuation of treatment. The treatment duration followed the response-guided therapy, i.e., patients with c-EVR were treated for 48 weeks and those with LVR for 72 weeks. Treatment was stopped for patients with detectable HCV RNA at week 36; they were considered to have experienced treatment failure (non-response, NR).

Definition of PPV and NPV

The positive predictive value (PPV) was defined as the probability that a certain outcome would occur in subjects on implementing the prediction criterion of interest, and a negative predictive value (NPV) was defined as the probability that a certain outcome would not occur in subjects if the prediction criterion of interest were not implemented.

Factors associated with SVR or NR on multivariate analysis

Factors associated with SVR or NR were assessed by multivariate analysis using two models; model 1 used the pre-treatment factors while model 2 also included the virologic response, i.e., the magnitude of the decrease in HCV RNA from baseline at treatment week 4, in addition to the factors used for model 1.

Statistical analysis

Baseline data for various demographic, biochemical, and virologic characteristics of the patients are expressed as mean ± SD or median. Viral response was evaluated using per protocol analysis. The difference between the two groups was assessed by Chi square test or t test and the significance trend was determined with the Mantel–Haenszel Chi square test. The factors associated with the virologic response were assessed by univariate and multivariate analyses using logistic regression analysis. A p value <0.05 was considered significant. Statistical analysis was conducted with SPSS version 19.0J (IBM, Armonk, NY, USA).

Results

The baseline characteristics of the 401 patients who completed the response-guided regimen are summarized in Table 1. All were assessed for the HCV RNA level at baseline and treatment weeks 4, 8 and 12. The IL28B genotype was assessed for 174 patients. The RVR rate was 8 % (33/401), the c-EVR rate was 41 % (164/401) and the LVR rate was 33 % (133/401). The NR rate was 26 % (104/401) and the SVR rate was 53 % (214/401).

Patient prevalence and SVR rates according to the magnitude of the decrease in HCV RNA from baseline at treatment week 4, 8 and 12

The patient prevalence for the different magnitudes of decrease in HCV RNA from baseline at treatment week 4, 8 and 12 are shown (Fig. 1a). The SVR rate on response-guided therapy was assessed for the six groups classified according to the magnitude of decrease in HCV RNA from the baseline (Fig. 1b). At week 4, the SVR rate significantly increased with the magnitude of the decrease in HCV RNA from the baseline; while all patients achieved SVR among those with undetectable HCV RNA (33/33), the SVR rates were very low among patients with <1 $\log_{10}$ decrease (4 %, 2/56). At week 8, none of the patients achieved SVR among those with <1 $\log_{10}$ decrease (0/32) and the SVR rates were very low among patients with 1 to <2 $\log_{10}$ decrease (6 %, 2/36). At week 12, none of the patients achieved SVR among those with <1 $\log_{10}$ decrease (0/32) or 1 to <2 $\log_{10}$ decrease (0/23) and the SVR rates were very low if HCV RNA did not decrease more than 4 $\log_{10}$ compared to the baseline (among patients with 2 to <3 $\log_{10}$ decrease, 8 %, 2/25, 3 to <4 $\log_{10}$ decrease, 7 %, 2/27).

Next, the relationship between the virologic response during the treatment (c-EVR, LVR and NR) and the magnitude of the decrease in HCV RNA from baseline were assessed (Fig. 2). The timing of HCV RNA negativiation tended to shift to an earlier time during the treatment with an increase in the magnitude of the decrease in HCV RNA from the baseline at any week (week 4, $p < 0.0001$, week 8, $p < 0.0001$, week 12, $p < 0.0001$, respectively). At week 4, 89 % of the patients with a <1 $\log_{10}$ decrease resulted in NR; none of those with a <2 $\log_{10}$ decrease achieved c-EVR. At week 8, all patients resulted in NR among those with a <1 $\log_{10}$ decrease (32/32, PPV for NR = 100 %); none of those with a < 3 $\log_{10}$ decrease achieved c-EVR. At week 12, all the patients with a <2 $\log_{10}$ decrease resulted in NR (55/55, PPV for NR = 100 %).

Table 1 Baseline characteristics of patients

Factor	Number or mean ± SD
Number	401
Age (y.o)	56.0 ± 10.6
Sex: male/female	190/211
Past history of IFN therapy[a]: naïve/experienced	316/66
Liver histology (METAVIR)[b]	
Activity, A0–1/2–3	188/120
Fibrosis, F0–2/3–4	265/42
IL28B SNP[c]: TT/TG/GG	131/40/3
HCV RNA (log IU/ml)	6.5 ± 0.6
White blood cells (/mm^3)	5152 ± 1486
Hemoglobin (g/dl)	13.8 ± 1.4
Platelets (×10^4/mm^3)	16.9 ± 5.5
ALT (IU/l)	65 ± 47
γGTP (IU/l)	62 ± 68

ALT alanine aminotransferase, *γGTP* γ-glutamyl transferase

[a] Past history of IFN was unknown in 19 patients

[b] 94 missing

[c] Single nucleotide polymorphism of IL28B gene determined for rs8099917, 227 missing

Patient prevalence and virologic responses according to IL28B genotype

Among the patients who were assessed for their IL28B genotype, 131 had the IL28B TT genotype and 25 had the IL28B non-TT genotype (Fig. 3A). The SVR rate for response-guided therapy was significantly higher in patients with the IL28B TT genotype than those with the non-TT genotype ($p < 0.001$) (Fig. 3B). The treatment responses distributing c-EVR, LVR, and NR between the two groups were assessed. The c-EVR rate was significantly higher in patients with the IL28B TT genotype and the NR rate was significantly higher in those with the IL28B non-TT genotype ($p < 0.001$) (Fig. 3C).

Patient prevalence and SVR rates according to the magnitude of the decrease in HCV RNA from baseline at treatment week 4, 8 and 12 among patients with IL28B genotype TT or non-TT genotype

The patient prevalence and SVR rates according to the magnitude of the decrease in HCV RNA from baseline at treatment week 4, 8 and 12 are shown by IL28B genotype (Figs. 4a, 5a). The magnitudes of the decrease in HCV RNA from baseline at treatment week 4, 8 and 12 were significantly higher in patients with the IL28B TT genotype than in those with the IL28B non-TT genotype. The SVR rate for response-guided therapy was assessed according to

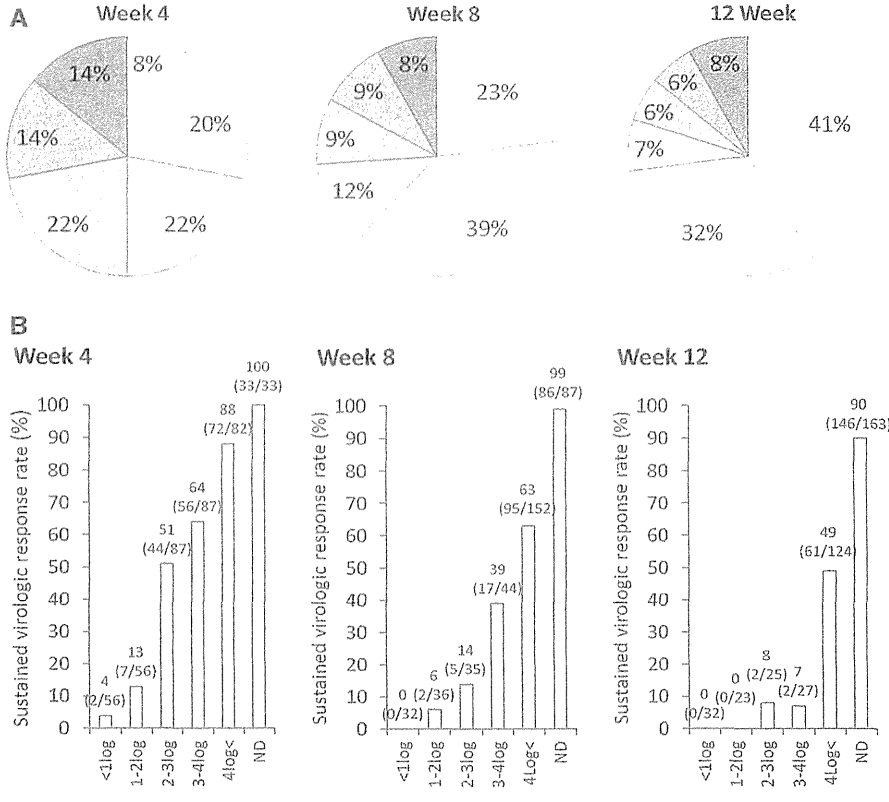

Fig. 1 a Patient prevalence according to the magnitude of HCV RNA decrease from baseline. ■ <1 log_{10} decrease. ▨ 1 to <2 log_{10} decrease. ▩ 2 to <3 log_{10} decrease. ▦ 3 to <4 log_{10} decrease. ▤ ≥4 log_{10} (detectable). □ undetectable. b SVR rate according to the magnitude of the decrease in HCV RNA from baseline

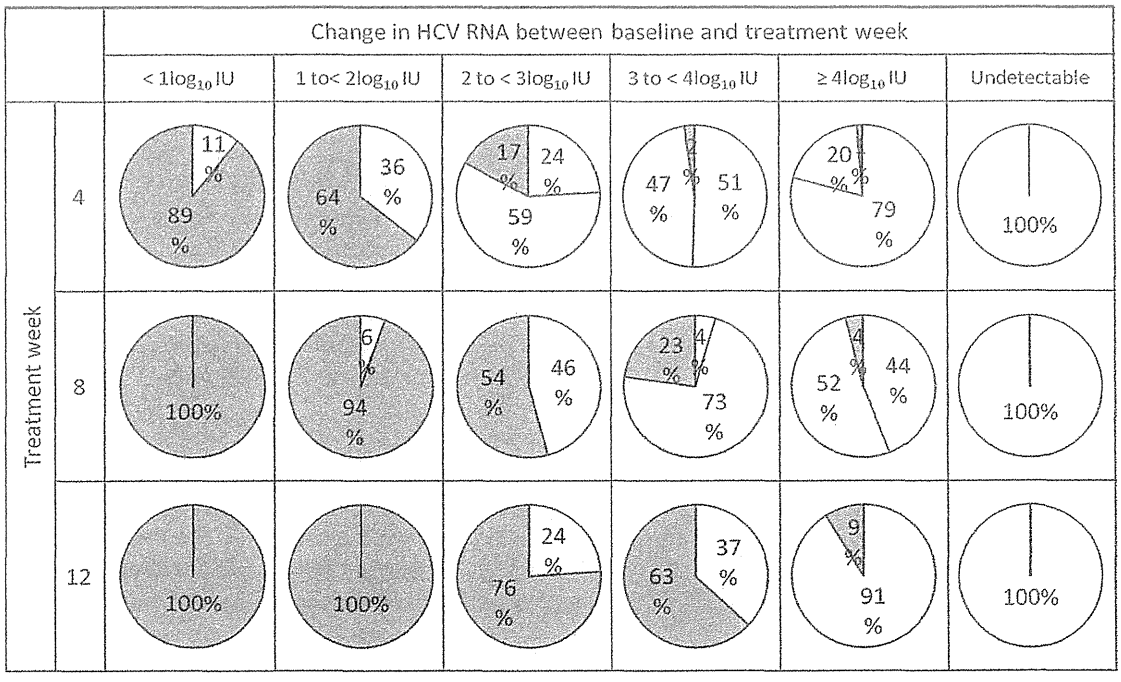

Fig. 2 Relationship between the virologic response during treatment and the magnitude of the decrease in HCV. RNA from baseline. □ early virologic response. ▨ late virologic response. ■ non-response

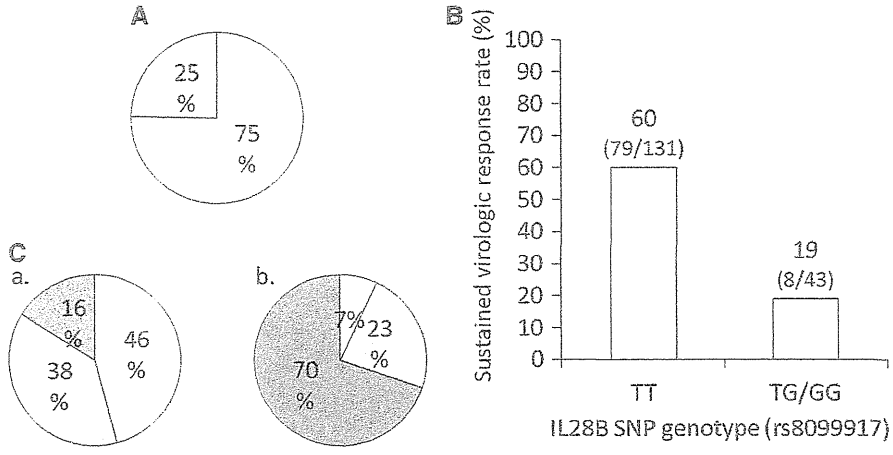

Fig. 3 A Patient prevalence according to IL28B genotype. □ TT genotype. ▨ non-TT genotype (TG of GG genotype). B SVR rate according to IL28B genotype. C Relationship between the virologic response during treatment and IL28B genotype. *a* Patients with IL28B TT genotype. *b* Patients with IL28B non-TT genotype. □ early virologic response. ▨ late virologic response. ■ non-response

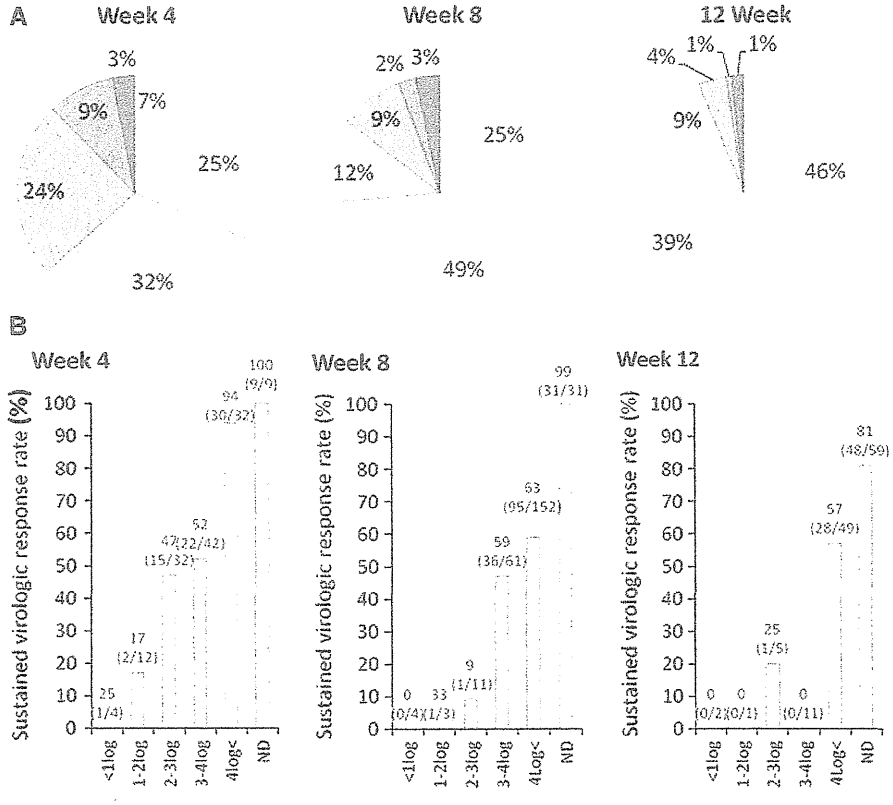

Fig. 4 a Patient prevalence according to the magnitude of HCV RNA decrease from baseline among patients with IL28B TT genotype. ■ <1 $\log_{10}$ decrease. ▨ 1 to <2 $\log_{10}$ decrease. ▧ 2 to <3 $\log_{10}$ decrease. ▩ 3 to <4 $\log_{10}$ decrease. ▩ ≥4 $\log_{10}$ (detectable). □ undetectable. b SVR rate according to the magnitude of the decrease in HCV RNA from baseline among patients with IL28B TT genotype

the six-group classification of the magnitude of the decrease in HCV RNA from baseline (Figs. 4b, 5b). The SVR rate increased step-by-step with the magnitude of the decrease in HCV RNA from baseline at 4, 8 and 12 weeks, irrespective of the IL28B genotype.

Factors associated with SVR

The factors associated with SVR were assessed for the variables shown in Table 1 and the magnitude of the decrease in HCV RNA from baseline at week 4 (Table 2). The factors selected as significant by univariate analysis (age, stage of liver fibrosis, platelet count, IL28B genotype and the magnitude of the decrease in HCV RNA from baseline at week 4) were evaluated by multivariate logistic regression analysis. In model 1, which consisted of only the pre-treatment factors, the IL28B genotype was the most powerful independent factor for SVR (Odds ratio, OR; 6.92, $p < 0.001$), apart from age (OR; 0.96, $p = 0.048$) and the stage of liver fibrosis (OR; 0.23, $p = 0.021$). However, in model 2, which included the parameter of viral kinetics at week 4, the magnitude of the decrease in HCV RNA

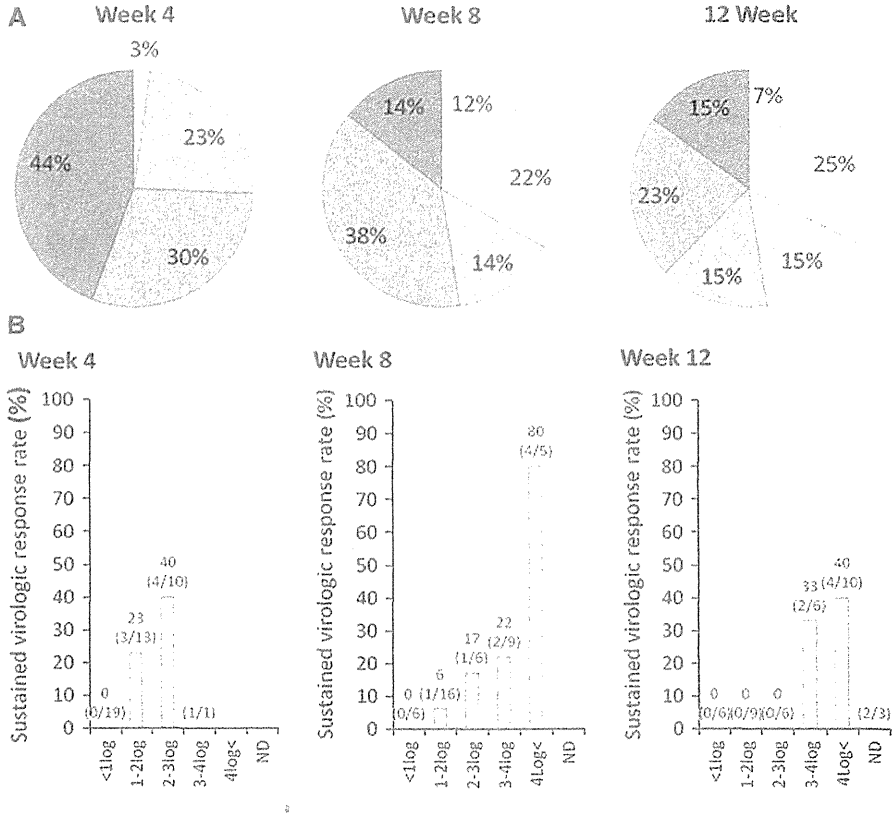

Fig. 5 a Patient prevalence according to the magnitude of HCV RNA decrease from baseline among patients with IL28B non-TT genotype. ■ <1 log_{10} decrease. ▨ 1 to <2 log_{10} decrease. ▩ 2 to <3 log_{10} decrease. ▦ 3 to <4 log_{10} decrease. ▤ ≥4 log_{10} (detectable). □ undetectable. b SVR rate according to the magnitude of the decrease in HCV RNA from baseline among patients with IL28B non-TT genotype

Table 2 Factors associated with SVR

Factor	Category	Univariate analysis			Multivariate analysis					
					Model 1			Model 2		
		OR	95 % CI	*p* value	OR	95 % CI	*p* value	OR	95 % CI	*p* value
Age		0.967	0.95–0.99	0.001	0.963	0.93–1.00	0.048	0.936	0.89–0.98	0.005
Sex	M/F	0.679	0.46–1.01	0.055						
Activity	A0–1/A2–3	0.671	0.42–1.06	0.090						
Fibrosis	F0–2/F3–4	0.301	0.15– 0.62	0.001	0.231	0.07–0.80	0.021	–	–	NS
HCV-RNA	By 1 log	0.657	0.53–1.03	0.075						
WBC		1.000	1.00–1.00	0.167						
Hb		1.080	0.94–1.24	0.272						
PLT		1.069	1.03–1.11	0.001	–	–	NS	–	–	NS
ALT		0.999	0.96–1.00	0.756						
γGTP		0.998	0.99–1.00	0.160						
IL28	TG or GG/TT	6.647	2.86–15.5	<0.001	6.924	2.60–18.4	<0.001	–	–	NS
Change of HCV RNA at 4 weeks	By 1 log increase	3.472	2.74–4.40	<0.001				3.338	2.20–5.06	<0.001

Model 1 consisted of pre-treatment factors. Model 2 consisted of pre-treatment factors and virologic response, that is the magnitude of the decrease in HCV RNA from baseline at treatment week 4

OR Odds ratio, *SVR* sustained virologic response, *WBC* white blood cells, *Hb* hemoglobin, *PLT* platelets, *ALT* alanine aminotransferase, *γGTP* γ-glutamyltransferase

from baseline at week 4 was the most powerful independent factor for SVR (OR; 3.4, $p < 0.001$) apart from age (OR; 0.936, $p = 0.005$), and the factor of IL28B was not a significant independent factor.

Factors associated with NR

The factors associated with NR were assessed in the same manner as SVR (Table 3). The factors selected as significant by the univariate analysis were evaluated by multivariate logistic regression analysis: grade of liver activity, stage of liver fibrosis, WBC count, platelet count, serum γ-glutamyltransferase level, IL28B genotype and the magnitude of the decrease in HCV RNA from baseline at week 4. In model 1, IL28B genotype was the most powerful independent factor for NR (OR; 39.75, $p < 0.001$), apart from the degree of liver fibrosis (OR; 10.31, $p = 0.021$) and platelet count (OR; 0.84, $p = 0.01$). However, in model 2, the magnitude of the decrease in HCV RNA from baseline at week 4 was the most powerful independent factor for NR (OR; 9.29, $p < 0.001$) apart from the degree of liver fibrosis (OR; 14.48, $p = 0.004$). IL28B was not selected as a significant independent factor.

SVR rate according to IL28B genotype and the timing of HCV RNA negativiation during treatment

Lastly, the SVR rate according to the IL28B genotype and the timing of HCV RNA negativiation during the treatment were assessed (Supplementary Figure 1). RVR was attained in nine patients with the IL28B TT genotype, and SVR was attained by all, but none of those with the IL28B non-TT genotype attained RVR. Among the patients without RVR, the SVR rate was significantly higher in the patients with the IL28B TT genotype than those with the non-TT genotype (57 %, 70/122 vs. 19 %, 8/43, $p < 0.001$). Among the patients with c-EVR, the SVR rate was 81 % (48/59) in the patients with the IL28B TT genotype and 67 % (2/3) in those with the non-TT genotype ($p = 0.48$). On the other hand, among the patients without c-EVR, the SVR rate was significantly higher in the patients with the IL28B TT genotype than those with the non-TT genotype (43 %, 30/70 vs. 16 %, 6/37, $p < 0.01$). Among the patients with LVR, the SVR rate was 60 % in both patients with the IL28B TT genotype (30/50) and the non-TT genotype (6/10) ($p = 1.00$). Next, the HCV RNA decrease among the patients without RVR or c-EVR was examined. The results revealed a significantly greater HCV RNA decrease in the IL28 TT group compared with the non-TT group among the patients without RVR (3.3 ± 1.2 $\log_{10}$ IU/ml vs. 1.3 ± 0.9 $\log_{10}$ IU/ml, $p < 0.001$) and those without c-EVR (4.7 ± 1.5 $\log_{10}$ IU/ml vs. 2.6 ± 1.5 $\log_{10}$ IU/ml, $p < 0.001$). The mean level of HCV RNA was significantly lower in the IL28 TT group compared with the non-TT group among the patients without RVR (3.3 ± 1.3 vs. 5.1 ± 1.2 $\log_{10}$ IU/ml, $p < 0.001$) and those without c-EVR (2.0 ± 1.4 vs. 3.9 ± 1.8 $\log_{10}$ IU/ml, $p < 0.001$).

Table 3 Factors associated with NR

Factor	Category	Univariate analysis			Multivariate analysis					
					Model 1			Model 2		
		OR	95 % CI	*p* value	OR	95 % CI	*p* value	OR	95 % CI	*p* value
Age		1.015	0.99–1.04	0.185						
Sex	M/F	1.187	0.76–1.86	0.455						
Activity	A0–1/A2–3	2.186	1.23–3.71	0.004	–	–	NS	–	–	NS
Fibrosis	F0–2/F3–4	5.079	2.53–10.0	<0.001	10.306	2.17–48.9	0.003	18.482	2.5–136.2	0.004
HCV-RNA	By 1 log	1.371	0.93–2.02	0.111						
WBC		1.000	1.00–1.00	0.004	–	–	NS	–	–	NS
Hb		0.947	0.81–1.10	0.498						
PLT		0.887	0.85–0.93	<0.001	0.844	0.74–0.96	0.01	–	–	NS
ALT		0.985	0.99–1.01	0.985						
γGTP		1.003	1.00–1.01	0.035	–	–	NS	–	–	NS
IL28	TT/TG or GG	12.088	5.4–26.9	<0.001	39.750	10.3–153.5	<0.001	–	–	NS
Change of HCV RNA at 4 weeks	By 1 log decrease	6.717	4.56–9.88	<0.001				9.292	3.95–21.8	<0.001

Model 1 consisted of pre-treatment factors. Model 2 consisted of pre-treatment factors and virologic response, that is the magnitude of the decrease in HCV RNA from baseline at treatment week 4

OR Odds ratio, *SVR* sustained virologic response, *WBC* white blood cells, *Hb* hemoglobin, *PLT* platelets, *ALT* alanine aminotransferase, *γGTP* γ-glutamyltransferase

Discussion

The importance of the timing of HCV RNA negativiation for the prediction of SVR has been well recognized [12–16]. In this study, all of the patients with RVR achieved SVR and those with c-EVR achieved 90 % of SVR by 48 weeks of treatment. However, only 8 % of the patients achieved RVR and about 40 % achieved c-EVR. This means that there is a need for another predictor of the effects of the response-guided therapy for the remaining population in which RVR or c-EVR were not attained. At present, triple therapy with Peg-IFN, ribavirin plus TVR, which can improve the antiviral outcome, is an option and the next generation of direct-acting antivirals is coming [1–8, 26–29]. Thus, the decision of "to treat or not to treat" during Peg-IFN plus ribavirin combination therapy should be carefully made with consideration of predictable antiviral outcomes and the degree of the adverse effect. In the present study, we focused on the early HCV RNA dynamics during the first 12 weeks of Peg-IFN plus ribavirin combination therapy.

First, we assessed the relationship between the magnitude of the decrease in HCV RNA from the baseline and SVR. At week 4, the patients without RVR were categorized into five groups, almost equally (Fig. 1a). The SVR rate increased step-by-step in proportion to the magnitude of the HCV RNA decrease at week 4, 8 and 12 (Fig. 1b). The SVR rates were very low (0–8 %) in patients with <1 $\log_{10}$ decrease at week 4, <2 $\log_{10}$ decrease at week 8 or <4 $\log_{10}$ decrease at week 12; NR rates were very high (63–100 %) in these patient groups (Fig. 2). All patients with <1 $\log_{10}$ decrease at week 8 or with <2 $\log_{10}$ decrease at week 12 were NR. These results could be useful for preparing guidelines on when to stop the response-guided therapy. Similar results were obtained from stratified analysis according to the IL28B genotype; SVR rates increased step-by-step in proportion to HCV RNA decrease in both the IL28B TT group and the non-TT group (Figs. 4, 5). Both groups showed almost the same SVR rates with the same level of HCV RNA decrease over the same number of weeks after the start of treatment, although few patients with non-TT showed a good response (marked HCV RNA decrease) to the treatment. This means that early HCV RNA decrease can predict SVR irrespective of the IL28B status.

In order to examine in more detail whether early HCV RNA dynamics can predict antiviral outcome irrespective of IL28B status, we investigated the predictive factors for treatment response before and after the start of treatment by multivariate logistic regression analysis. As a result, the IL28B genotype was found to be the strongest predictive factor for pre-treatment prediction in response-guided therapy (Table 2, model 1). However, on analysis including the HCV RNA decrease from the baseline at week 4, the magnitude of the HCV RNA decrease at week 4 was shown to be the best predictor for SVR (Table 2, model 2). Multivariate analysis for NR gave similar results; the IL28B genotype was the strong predictive factor for pre-treatment prediction and the HCV RNA decease at week 4 for post-treatment (Table 3). These results indicate that the information of the IL28B genotype is very useful for predicting the antiviral response before treatment, however, once the treatment is initiated, that of the HCV RNA decrease at week 4 can replace the IL28B status for predicting the antiviral outcome.

Recently, the relationship between the timing of HCV RNA negativiation and IL28B status for predicting the antiviral effect has been reported [21, 24]. No significant difference of the SVR rates was shown among patients with the same on-treatment virologic response, such as RVR or c-EVR, regardless of the IL28B genotype. However, the IL28B status was shown to affect the antiviral outcome among patients who could not attain RVR or c-EVR; higher SVR rates were obtained for patients with a favorable IL28B genotype. With the cohort enrolled in this study, we assessed stratified analysis according to the IL28B genotype and the timing of HCV RNA negativiation in order to determine whether the IL28B genotype directly affects the antiviral outcome or whether the difference of HCV RNA decrease caused by IL28B status affects the antiviral outcome (Supplementary Figure 1). The results showed that the IL28B genotype was associated with SVR among the patients without RVR or c-EVR, while nearly equal SVR rates were attained regardless of the IL28B genotype among the patients with HCV RNA negativiation over the same period of time. These results correspond to those of a previous study [21, 24], and the IL28B genotype was concluded to affect the antiviral outcome among the patients without RVR or c-EVR. However, examination of the HCV RNA decrease among the patients without RVR or c-EVR in our cohort showed that HCV RNA significantly decreased in the IL28 TT group compared with the non-TT group. These results suggest that the IL28B status does not directly affect the antiviral outcome and that the difference of HCV RNA decrease caused by the IL28B status affects the antiviral outcome among the patients without RVR or c-EVR. In sum, the HCV decrease at week 4 can be used in place of the IL28B status for predicting the antiviral outcome after the start of treatment, as shown by the multivariate analysis in the present study.

The limitation of this study was that pre-treatment viral factors were not assessed due to the small numbers of patients. Among the patients enrolled in this study, the amino acid (aa) sequences at position 70 in the HCV core protein were examined in some cases (126 patients). The analysis for SVR and NR including the factor of aa

substitution of core 70, the same results were obtained. That is, the results of the factor of IL28B in model 1 and the HCV RNA decrease at week 4 in model 2 were the significant predictive factors for SVR and NR but the factor of aa substitution of core 70 was not significantly associated with SVR by univariate analysis and NR by multivariate logistic regression analysis. Further examination is needed to clarify the usefulness of the factor of aa substitution of core 70 as a predictor for SVR or NR in this combination therapy.

In conclusion, the IL28B genotype is a very strong predictive factor in pre-treatment prediction. After the start of treatment, the magnitude of the HCV RNA decrease at week 4 seems to be the most reliable marker for predicting the antiviral outcome among patients treated with response-guided Peg-IFN plus ribavirin combination therapy.

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Research

DNA methylation at hepatitis B viral integrants is associated with methylation at flanking human genomic sequences

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Integration of DNA viruses into the human genome plays an important role in various types of tumors, including hepatitis B virus (HBV)–related hepatocellular carcinoma. However, the molecular details and clinical impact of HBV integration on either human or HBV epigenomes are unknown. Here, we show that methylation of the integrated HBV DNA is related to the methylation status of the flanking human genome. We developed a next-generation sequencing-based method for structural methylation analysis of integrated viral genomes (denoted G-NaVI). This method is a novel approach that enables enrichment of viral fragments for sequencing using unique baits based on the sequence of the HBV genome. We detected integrated HBV sequences in the genome of the PLC/PRF/5 cell line and found variable levels of methylation within the integrated HBV genomes. Allele-specific methylation analysis revealed that the HBV genome often became significantly methylated when integrated into highly methylated host sites. After integration into unmethylated human genome regions such as promoters, however, the HBV DNA remains unmethylated and may eventually play an important role in tumorigenesis. The observed dynamic changes in DNA methylation of the host and viral genomes may functionally affect the biological behavior of HBV. These findings may impact public health given that millions of people worldwide are carriers of HBV. We also believe our assay will be a powerful tool to increase our understanding of the various types of DNA virus-associated tumorigenesis.

[Supplemental material is available for this article.]

Hepatitis B virus (HBV) infects more than two billion people worldwide, and 400 million chronically infected individuals are at high risk of developing active hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (Gatza et al. 2005; Lupberger and Hildt 2007). HBV carriers with chronic liver disease are at a 100-fold greater risk of developing HCC, which is the third leading cause of cancer-related death worldwide. The HBV genome is integrated into the host genome in 90% of patients with HCC (HBV-HCC) (Gatza et al. 2005; Lupberger and Hildt 2007). HBV-HCCs have been analyzed by comprehensive genome sequencing and high-resolution genome mapping (Kan et al. 2013; Li and Mao 2013; Nakagawa and Shibata 2013). Moreover, the recent deep sequencing of HBV DNA in patients with HCC revealed increased integration events, structural alterations, and sequence variations (Ding et al. 2012; Fujimoto et al. 2012; Jiang et al. 2012; Sung et al. 2012; Toh et al. 2013). A recent study identified a viral-human chimeric fusion transcript, HBx-LINE1, that functions like a long noncoding RNA to promote HCC (Lau et al. 2014). However, the molecular details and clinical impact of HBV integration on the epigenomes of human cells and HBV remain to be defined.

Methylation of exogenous DNA (including viral DNA) that is integrated into the human genome has been studied over the past decade (Doerfler et al. 2001). Within the human genome, cytosine methylation in CpG dinucleotides (CpG sites), which cluster into islands associated with transcriptional promoters, is an important mechanism for regulating gene expression. Additionally, host cells use methylation as a defense mechanism against foreign agents (e.g., viral DNA) (Doerfler 2008; Doerfler et al. 2001). DNA methylation suppresses the expression of viral genes and other deleterious elements incorporated into the host genome over time. Establishment of de novo patterns of DNA methylation is char-

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acterized by the gradual spread of methylation (Orend et al. 1991). Another attractive possibility is that DNA methylation camouflages the virus from the immune system (Tao and Robertson 2003; Hilleman 2004), resulting in a DNA methylation–related blockade of viral antigen presentation that allows the virus to escape immune control (Fernandez et al. 2009).

The DNA methylome of HBV in human cells may undergo dynamic changes at different stages of disease (Fernandez et al. 2009). For example, DNA methylation at the *HBVgp2* locus, which codes for the S viral proteins, reportedly increases during the progression from asymptomatic lesions to benign lesions, to premalignant disease and malignant tumors. However, because of the significant deletions of the integrated HBV genome detected in this previous study (Fernandez et al. 2009), the DNA methylome of HBV needs to be further characterized. Moreover, the molecular mechanisms involved and the clinical impact of the integration of HBV on the human and HBV epigenomes are unknown. To address these issues, we developed a next-generation sequencing (NGS)–based method for methylation analysis of integrated viral genomes (denoted G-NaVI) and applied this method to the integrative genomic and epigenomic analysis of human hepatoma cell lines and tissues with integrated *HBV* genomes.

Results

DNA methylation levels in PLC/PRF/5 cells and cancerous tissues obtained from HBV-HCC patients

Methylated CpG island (CGI) amplification (MCA) coupled with microarray (MCAM) analysis (Toyota et al. 1999; Oishi et al. 2012) was performed to detect methylated genes in the human PLC/PRF/5 cell line and in six paired specimens of primary HBV-HCC and adjacent tissues. Compared with the DNA methylation of CGIs in the healthy peripheral blood leukocytes of volunteers or the noncancerous tissues, levels of DNA methylation were not remarkable in the PLC/PRF/5 cells and the cancerous tissues obtained from HBV-HCC patients (Supplemental Fig. 1). These results were confirmed by bisulfite pyrosequencing of candidate tumor-related genes.

DNA methylation of CGIs of *HBx*

We then focused on epigenetic changes in the viral genome. Based on the hidden Markov models for sequence analysis performed on the CpG plugin of bioinformatics software Geneious 5.5.8 (see Methods section), a CpG island was found in only the promoter region of the *HBx* gene in the HBV genome (Fig. 1A; Supplemental Fig. 2; Durbin et al. 1998; Kearse et al. 2012). Host signal transduction pathways and gene expression are disrupted by the expression of *trans*-activating factors derived from the HBV genome, such as the HBx protein and PreS2 activators (Gatza et al. 2005; Lupberger and Hildt 2007). Moreover, transgenic mice expressing high levels of HBx in the liver develop HCC (Kim et al.1991; Koike et al. 1994). The DNA methylation levels of the CGIs of *HBx* were analyzed in 10 HBV-HCC samples and 10 adjacent samples, as well as samples of PLC/PRF/5 cells by bisulfite pyrosequencing (Fig. 1A; Supplemental Fig. 2). We performed advanced methylation quantification in long sequence runs by pyrosequencing on PyroMark Q24 Advanced and PyroMark Q24 instruments. Methylation levels of *HBx* varied across samples (Fig. 1B,C) and were generally lower in HCC tissues than in the adjacent tissues (Fig. 1B). This finding is consistent with a previous report that most HBV genomes, although globally methylated to a greater extent in malignant samples than in premalignant lesions, retain *HBx* in an unmethylated state (Fernandez et al. 2009). Because the pyrosequencing results represent the genome-wide average of DNA methylation levels at the particular CpG *site, the results could be affected by the HBV integration site.* Therefore, genome-wide methylation analysis of the integrated HBV sequence is necessary in relation to the methylation state of the adjacent human genome. We did not detect an association between *HBx* methylation levels and those of the LINE1 and *Alu*Yb8 repeats (Fig. 1B).

Fluorescence in situ hybridization (FISH) and *Alu* PCR analyses of HBV integration

We developed a FISH technique for detecting HBV DNA in the genome of PLC/PRF/5 cells (Supplemental Figs. 3, 4). Twelve specific primer pairs (FISH probes 1–12) were designed based on the HBV sequences integrated into the genome of PLC/PRF/5 cells; amplification from all primer pairs was confirmed (Supplemental Fig. 4A). These results suggest full-length or partial HBV sequences that are covered by the 12 primer pairs were integrated into the genome of the PLC/PRF/5 cells. The FISH probes were labeled with digoxigenin, and FISH was performed using Carnoy-fixed chromosomal and nuclear specimens. Multiple HBV fluorescent signals (green) were detected in the nuclei (Supplemental Fig. 4B) using probes for *HBx* and its CGI sequences (probes 5 and 6), but not with probes 1–4 or 7–12 (Supplemental Fig. 4C–E). *Alu*-PCR identified one *HBx* integration site in PLC/PRF/5 (Supplemental Fig. 5). The integrated *HBx* sequence was 213 bp and included a promoter region. The *HBx* gene body was located only 13 bases (ATG GCT GCT AGG T) from the transcription start site and was integrated into a noncoding region of the host genome. There were 200 bases of viral DNA sequence upstream of the *HBx* transcription start site. According to the human genome reference sequence (GRCh38) published by the Genome Reference Consortium, this integration site was identified as a noncoding region of host Chromosome 5 1,350,106–1,350,478 that is near the telomerase reverse transcriptase (*TERT*) gene (Supplemental Fig. 5).

NGS analysis of HBV DNA integration site sequences

We developed an NGS analysis technique for sequencing the HBV DNA integration sites (Supplemental Fig. 6A). For efficient genome analysis, we synthesized 12,391 custom baits based on the sequences of the HBV genotypes A to J and on those sequences present in the HBV-transformed PLC/PRF/5 cells that were not related to the human genome sequence (Supplemental Fig. 6B). The average read length was 333.14 bp with a modal length of ~500 bp (Supplemental Fig. 6C). The average read quality was 31.91, corresponding to >99.9% accuracy. We did not detect a common HBV integration site (Fig. 2). The integration sites in the PLC/PRF/5 genome included intergenic (39%), intronic (39%), promoter (8%), and divergent promoter (15%) regions but not exonic (0%) sequences (Fig. 2). HepG2.2.15 cells, which stably express and replicate HBV in a culture system, are derived from the human hepatoblastoma cell line HepG2 (Sells et al. 1987). In the HepG2.2.15 genome, the integration sites included intergenic (29%), intronic (57%), and other (14%) regions but not promoter (0%), divergent promoter (0%), or exonic (0%) sequences (Fig. 2).