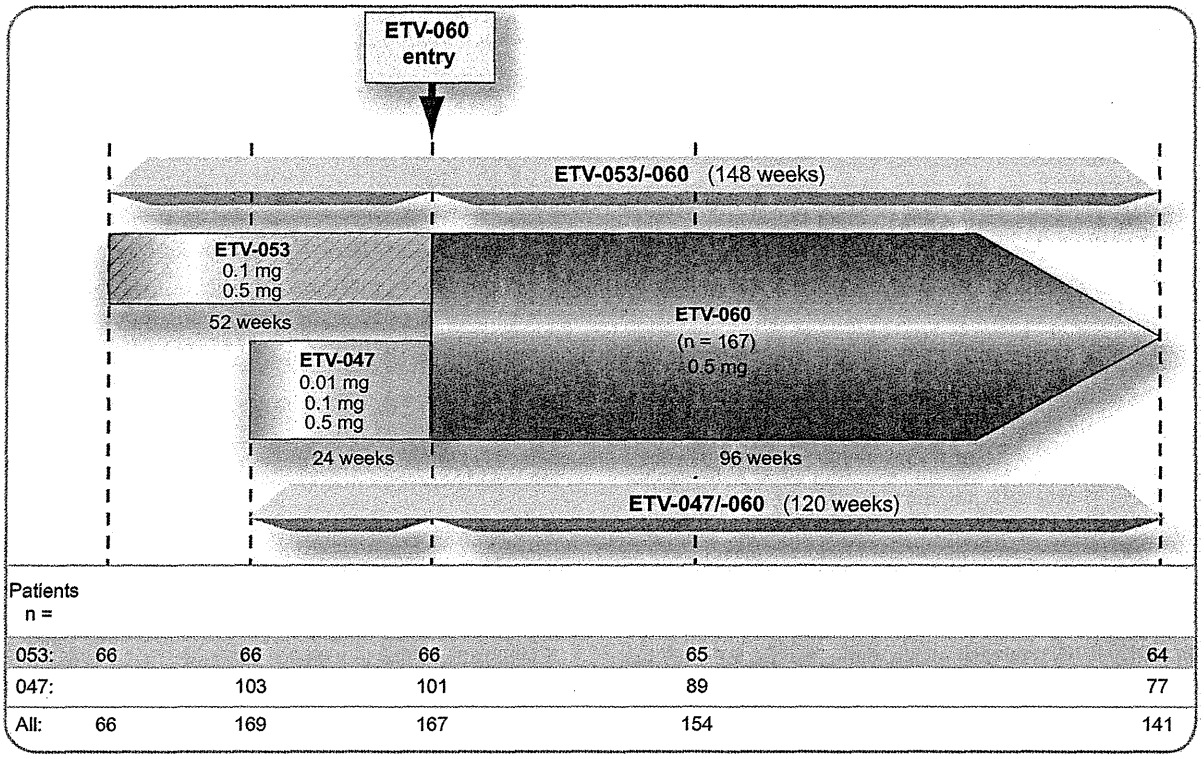

Fig. 1. Entecavir nucleoside-naïve long-term treatment cohort. One hundred and one patients completed 24 weeks of entecavir 0.01 mg, 0.1 mg or 0.5 mg treatment daily in study ETV-047, and 66 patients completed 52 weeks of entecavir 0.1 mg or 0.5 mg treatment daily in study ETV-053. Of these, 167 were enrolled in study ETV-060 with no interruption or gap in treatment. One hundred and forty-four patients remained on entecavir 0.5 mg daily through 96 weeks in study ETV-060 (for a total entecavir treatment time of 120–148 weeks).

Resistance monitoring

During treatment, HBV polymerase/reverse transcriptase substitutions were analyzed for all patients who had HBV-DNA ⩾400 copies/ml at weeks 100 and 120 (from Phase II [pre-treatment] baseline) for patients originating in study ETV-047, and at weeks 100 and 148 for patients originating in study ETV-053. Samples from all patients, who experienced virological breakthrough during ETV-060 (increase in HBV-DNA of ⩾1 $\log_{10}$ copies/ml from nadir in two consecutive measurements), were also analyzed for HBV polymerase/reverse transcriptase substitutions.

Assay methods

Serum HBV-DNA was determined by Roche Amplicor™ PCR assay (LOQ = 400 copies/ml; Roche Diagnostics K.K., Tokyo, Japan) in a central laboratory. Clinical laboratory tests, PCR assays for HBV-DNA, and serological tests were performed at SRL, Inc. (Tokyo, Japan), the central clinical laboratory designated by the trial sponsor. Genotypic analysis of HBV strains was performed using a PCR-based restriction fragment length polymorphism assay (SRL, Inc., Tokyo, Japan). On-treatment testing for resistance was carried out using a direct-sequencing PCR method.

Statistical analysis

Analyses of efficacy and safety end points were based on patients who received at least one dose of study medication in study ETV-060. Only descriptive summaries were performed. Parameters represented by continuous variables were summarized by the mean, median, standard deviation, minimum, and maximum. Analyses of HBV-DNA as a continuous parameter were applied after $\log_{10}$ transformation. In the analysis of binary end points, patients with missing on-treatment measurements were treated as missing (non-completer = missing). An additional sensitivity analysis using the last observation carried forward method was conducted for the end point of HBV-DNA <400 copies/ml at week 96. In this analysis, the last observed HBV-DNA levels were carried forward for patients without week 96 measurements, i.e., patients who either discontinued prior to week 96 or who were still on study but had a missing HBV-DNA measurement at week 96.

Results

One hundred and sixty-seven patients were treated with entecavir in Phase II studies ETV-047 or -053 and entered ETV-060 (Fig. 1). Twenty-three patients discontinued treatment during ETV-060 for the following reasons: adverse event (6), protocol violation (2), withdrawal of consent (4), pregnancy (1), loss to follow-up (4), insufficient effect (1), and complete response (4) or stability of disease condition (1) in the judgement of the investigator. Table 1 shows the baseline (pre-treatment) demographics and disease characteristics for all treated patients ($n = 167$); the cohort of patients who received the approved dose of entecavir (0.5 mg daily) from Phase II baseline through the end of treatment ($n = 66$); and the subset of patients who received 0.5 mg entecavir and had biopsies at baseline, week 48, and weeks 144–148. Among all treated patients, 72% were male, and the mean age was 43 years. Mean HBV-DNA was 7.88 $\log_{10}$ copies/

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Table 1. Baseline demographics and disease characteristics of the entecavir nucleoside-naïve long-term treatment cohort (n = 167), the entecavir 0.5 mg cohort (n = 66), and the subset of patients from the entecavir 0.5 mg cohort with evaluable liver biopsies at baseline, week 48, and week 144–148 (n = 19). Patients were treated with different doses of entecavir in Phase II studies ETV-047 and ETV-053, and subsequently received 0.5 mg daily in rollover study ETV-060. This table describes characteristics at pre-treatment (Phase II) baseline.

Characteristic	Long-term treatment cohort n = 167	Entecavir 0.5 mg cohort n = 66	Entecavir 0.5 mg cohort with long-term liver biopsy n = 19
Male, n (%)	120 (71.9)	48 (72.7)	15 (78.9)
Age (years), mean ± SD	42.5 ± 11.0	43.2 ± 10.5	43.8 ± 10.3
Weight (kg), mean ± SD	65.9 ± 3.5	65.5 ± 12.2	66.8 ± 13.2
Ethnicity Japanese, n (%)	167 (100)	66 (100)	19 (100)
HBV-DNA, mean ± SD			
Log_{10} copies/ml by PCR	7.88 ± 1.01	8.03 ± 0.93	7.61 ± 0.95
HBeAg – positive, n (%)	141 (84.4)	55 (83.3)	13 (68.4)
ALT (IU/L), mean ± SD	151.2 ± 130.8	142.2 ± 87.7	140.5 ± 68.5
Abnormal ALT (ALT >1.0× ULN), n (%)	163 (97.6)	64 (97.0)	19 (100)
HBV genotype, n (%)			
A	4 (2.4)	1 (1.5)	0
B	5 (3.0)	1 (1.5)	0
C	154 (92.2)	63 (95.5)	19 (100)
Others	4 (2.4)	1 (1.5)	0

ml, mean ALT was 151 IU/l, and 84% (141/167) of patients were HBeAg(+). Ninety-two per cent (154/167) of patients were infected with HBV genotype C. Baseline demographics and disease characteristics were similar for all patient cohorts.

Virological response

Mean HBV-DNA levels fell rapidly during studies ETV-047 and ETV-053 [27,28]. For the cohort that entered ETV-060 from the two Phase II studies (n = 167), HBV-DNA fell from a mean of 7.88 log copies/ml at pre-treatment baseline to a mean of 3.41 log_{10} copies/ml at ETV-060 baseline. Viral load was further suppressed during treatment in ETV-060 and was maintained at low levels through 96 weeks (120–148 weeks total entecavir treatment time). Forty-nine per cent (82/167) of patients in the cohort had HBV-DNA <400 copies/ml at ETV-060 entry (Fig. 2A). By week 96 of the study, this proportion had increased to 88% (127/144). Of the 82 patients with HBV-DNA <400 copies/ml at ETV-060 entry, 81 patients (99%) maintained this response to the end of treatment. Eighty-five patients had HBV-DNA >400 copies/ml at ETV-060 entry; 62 (73%) achieved HBV-DNA <400 copies/ml during treatment in ETV-060, and 23 (27%) maintained >400 copies/ml at end of treatment. Among the 23 patients who discontinued treatment during ETV-060, 14 had HBV-DNA <400 copies/ml at the last on-treatment measurement. A sensitivity analysis using the last observation carried forward method was conducted based on the intention-to-treat (ITT) population. The last observed HBV-DNA levels for all subjects who either were still on study but had a missing PCR test at week 96 or discontinued prior to week 96 were carried forward; this maintained the total number of subjects in this cohort intact (n = 167). When the HBV-DNA end point was re-calculated using this method, 85% (142/167) of patients had HBV-DNA <300 copies/ml at week 96.

Biochemical response

Almost all patients (97.6%; 163/167) in the Phase II studies had abnormal ALT (ALT >1.0× ULN) at pre-treatment baseline (Table 1 and Fig. 3A). At the time of entry into study ETV-060, 81.0% (132/163) of those patients demonstrated normalized ALT levels (Fig. 3A). By ETV-060 week 48, that proportion had risen to 86.7%, and by week 96 (120–148 weeks total entecavir treatment time), the rate of ALT normalization was 90.1%.

Serological response

One hundred and forty-one patients (84%) were HBeAg(+) at pre-treatment baseline (Table 1 and Fig. 4A). At the time of entry into study ETV-060, 16.3% (23/141) of those patients had lost HBeAg and undergone HBe seroconversion (Fig. 4A). By week 96 of ETV-060 (120–148 weeks total entecavir treatment time), 38.8% (47/121) of patients had lost HBeAg, and 26.4% (32/121) had undergone HBe seroconversion. Among patients who underwent HBe seroconversion in ETV-060, the majority had achieved HBV-DNA suppression (<400 copies/ml) during treatment in study ETV-047 or ETV-053. One patient lost HBsAg and one patient underwent HBs seroconversion during treatment in study ETV-060.

Resistance

One hundred and sixty-four out of 167 patients were monitored for resistance through the end of treatment in ETV-060 (three patients refused consent for resistance testing). Five patients developed genotypic resistance to entecavir, which emerged during the third year of treatment, for a 3-year cumulative probability of resistance of 3.3%. Four of these five patients had received the lower (non-approved) doses of entecavir (0.01 mg or 0.1 mg) during the Phase II studies prior to ETV-060. Of the five patients with resistance, one patient had achieved HBV-DNA levels <400 copies/ml prior to developing resistance, and four patients experienced virological breakthrough. Fig. 5 provides HBV-DNA and ALT profiles for the patient who received continuous treatment with the approved 0.5 mg dose. This patient had detectable levels of HBV-DNA after 48 weeks of entecavir treatment in ETV-060. Genotypic resistance testing did not reveal any mutations associated with resistance to entecavir. The patient experienced virological breakthrough at week 96, which was associated with development of entecavir resistance (rt L180M, rt S202G, rt M204V).

Safety

Mean exposure to entecavir during study ETV-060 was 103.9 weeks (range: 5.1–140.6 weeks). Adverse events were reported for 99% (166/167) of patients, and most were mild to moderate in severity (Table 2). The most common clinical adverse event was nasopharyngitis (16.1%). Increased serum lactic acid

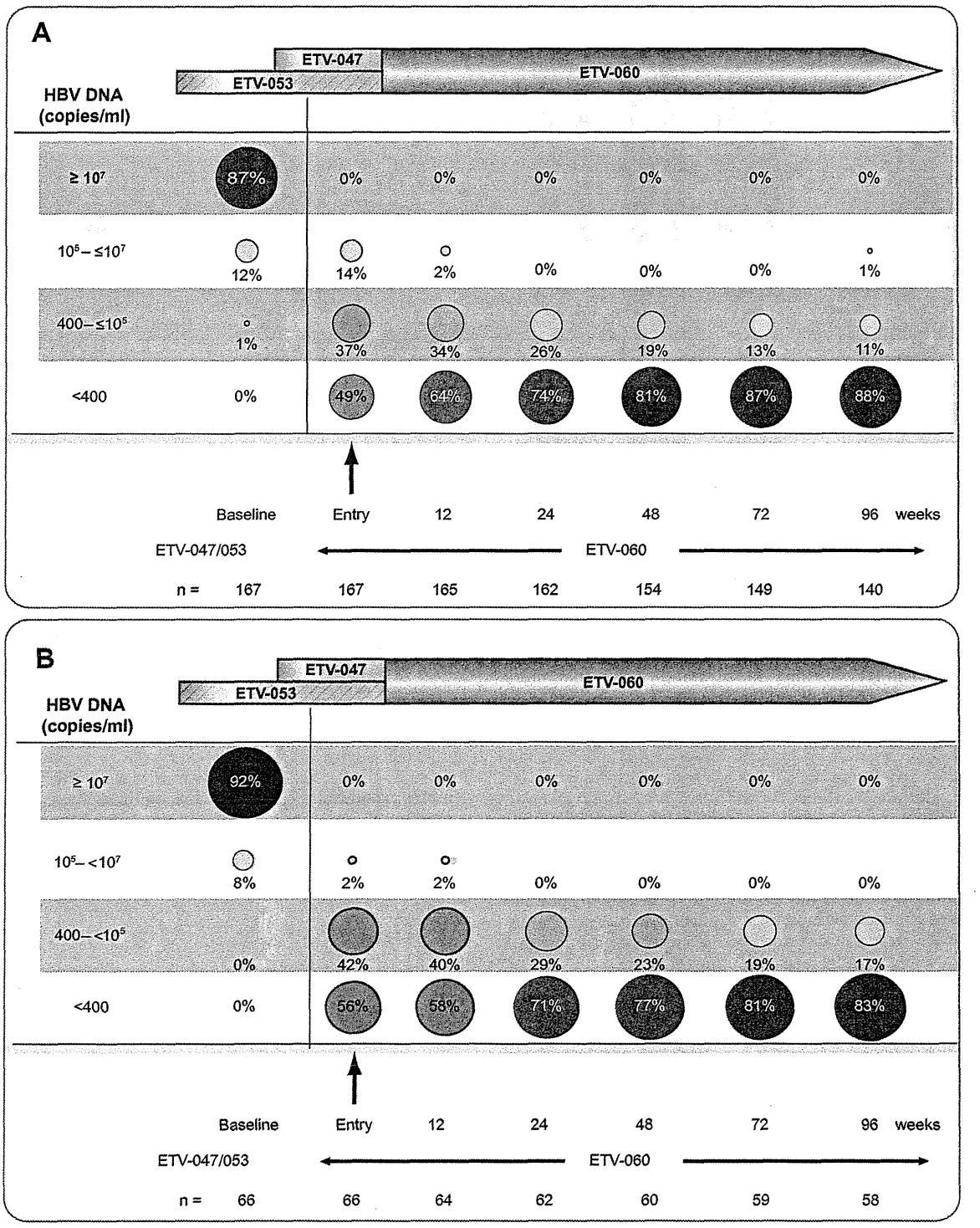

Fig. 2. Distribution of HBV-DNA levels over 96 weeks of treatment in rollover study ETV-060 (total entecavir treatment time, 120–148 weeks) for (A) the entecavir nucleoside-naïve long-term treatment cohort and (B) the entecavir 0.5 mg cohort.

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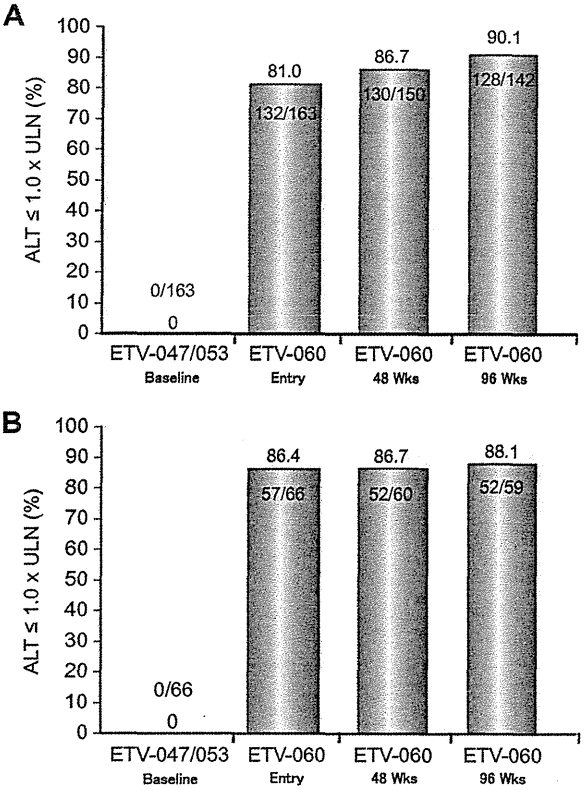

Fig. 3. Proportions of patients with normal ALT (ALT ⩽1.0× ULN) over time in (A) the entecavir nucleoside-naïve long-term treatment cohort and (B) the entecavir 0.5 mg cohort. One hundred and sixty-three patients in the entecavir nucleoside-naïve long-term treatment cohort and 66 patients in the entecavir 0.5 mg cohort had abnormal ALT (>1.0× ULN) at pre-treatment baseline.

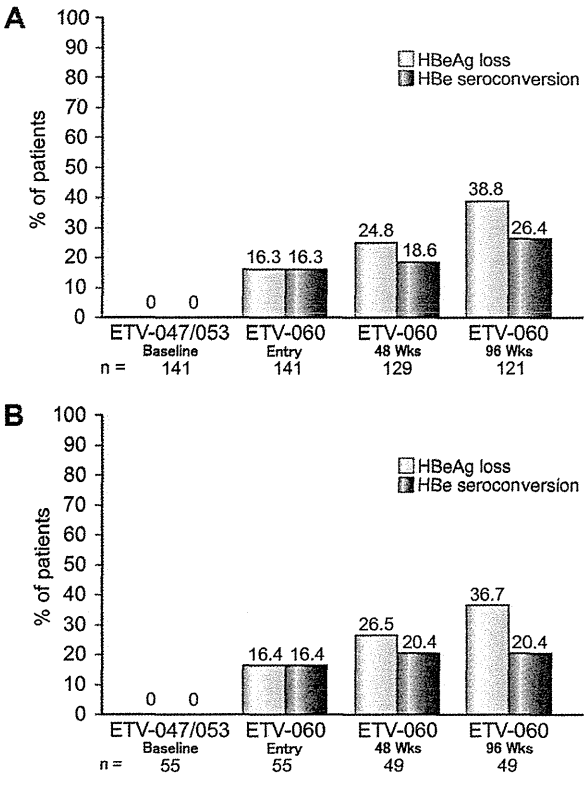

Fig. 4. Proportions of patients with HBeAg loss and HBe seroconversion over time in (A) the entecavir nucleoside-naïve long-term treatment cohort and (B) the entecavir 0.5 mg cohort. One hundred and forty-one patients in the entecavir nucleoside-naïve long-term treatment cohort and 55 patients in the entecavir 0.5 mg cohort were HBeAg(+) at pre-treatment baseline.

(44.3%) and increased lipase (32.3%) were the most common laboratory adverse events. The most common Grade 3–4 adverse event (clinical or laboratory) was increased lipase, which occurred in 6% of patients. The frequency of clinical or laboratory serious adverse events was 13.7% (22/167), the majority of which resolved on continued entecavir treatment. Five patients (3%) discontinued treatment due to adverse events. There were no ALT flares. No deaths were reported during the study.

Entecavir 0.5 mg cohort

A subset of 66 patients (66/167) received the approved dose of entecavir (0.5 mg daily) from Phase II baseline through to the end of ETV-060. For this subset, among patients with available samples, 83% (48/58) had HBV-DNA <400 copies/ml by week 96 (Fig. 2B). When this end point was re-calculated using the last observation carried forward analysis, 80% (53/66) achieved HBV-DNA <400 copies/ml. By week 96 in ETV-060, 88% (52/59) of patients in the 0.5 mg cohort had ALT ⩽1.0× ULN (Fig. 3B), 37% (18/49) had lost HBeAg, and 20% (10/49) achieved HBe seroconversion (Fig. 4B). The mean change in HBV-DNA from pre-treatment baseline through to the end of ETV-060 was $-5.19\ \log_{10}$ copies/ml. Resistance emerged in only one patient in this cohort, for a cumulative 3-year probability of resistance of 1.7%.

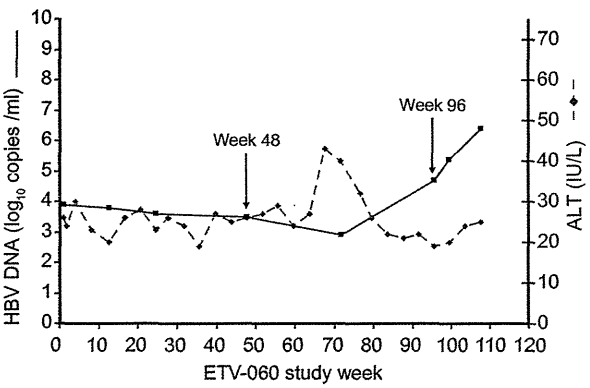

Fig. 5. On-treatment HBV-DNA and ALT profiles for the patient in the 0.5 mg entecavir cohort who developed entecavir resistance during treatment in ETV-060. Following virological breakthrough at week 96, genotypic resistance analysis revealed the presence of entecavir resistance (rt L180M, rt S202G, rt

Table 2. Summary of safety during ETV-060: entecavir nucleoside-naïve long-term treatment cohort.

On-treatment	Number of patients (%) ETV-060 $n = 167$
Any adverse event	166 (99.4)
Clinical adverse events	161 (96.4)
Clinical serious adverse events	22 (13.7)
Grade 3–4 clinical adverse events	8 (4.8)
Most frequent clinical adverse events*	
Nasopharyngitis	102 (61.1)
Headache	34 (20.4)
Diarrhoea	26 (15.6)
Laboratory adverse events	160 (95.8)
Laboratory serious adverse events	0
Grade 3–4 clinical adverse events	16 (9.6)
ALT increased	10 (6)
ALT flare†	0
Discontinuations due to adverse events	5 (3.0)
Deaths	0

* Occurring in at least 15% of patients.
† ALT >2× baseline and >10× ULN.

Twenty-one (21/66) patients in the 0.5 mg cohort, all originating from study ETV-053, had paired evaluable liver biopsies at pre-treatment (Phase II) baseline and either week 100 or week 148 (ETV-060 weeks 48 or 96, respectively). Nineteen (19/21) patients had evaluable biopsies at three time points: baseline, week 48, and week 148. Among this latter subset, 89% (17/19) had HBV-DNA <400 copies/ml at week 148. Histological improvement was observed in 100% (19/19) of these patients from baseline through week 148. There was a marked improvement in the distribution of Knodell necroinflammatory scores with increasing treatment time (Fig. 6A). The two patients who had repeat biopsies at week 100 (but not at week 148) also demonstrated histological improvement from baseline through to week 100. The mean Knodell necroinflammatory score improved from 8.95 at baseline to 1.89 at week 148, and 95% of patients (18/19) exhibited minimal necroinflammation (Knodell NI score ⩽3 points) at week 148 (Fig. 6A). Improvements in Knodell fibrosis scores were demonstrated in 63% (12/19) of patients with evaluable biopsies at baseline, week 48, and week 148 (Fig. 6B). Ten patients in this cohort had advanced fibrosis (Knodell fibrosis score = 3), and three patients had cirrhosis (Knodell fibrosis score = 4) at pre-treatment baseline, and 11 out of these 13 patients (85%) showed improvement at week 148. Among 21 patients with biopsies at baseline and either week 100 or week 148, 12/21 (57%) demonstrated an improvement in Knodell fibrosis scores, and 9/21 showed no change. The mean Knodell fibrosis score improved from 2.53 at baseline to 1.47 at week 148. Assessment of liver histology by the New Inuyama classification system confirmed the results obtained using the Knodell classification system (data not shown).

Discussion

The current long-term study of entecavir presents results for a cohort of patients treated continuously for 3 years. The strengths of this study include its focus on a well-defined cohort followed closely over 3 years, as well as the long-term follow-up liver biopsies on a subset of that cohort enabling a direct assessment of the effect of entecavir therapy on liver disease progression. These results show that long-term treatment with entecavir is well tolerated and achieves histological improvement, durable HBV-DNA suppression, and minimal resistance. Of 167 patients in the cohort, 86% (144) completed 96 weeks in the follow-up study for a total of 2.5–3 years of entecavir therapy, and only one patient discontinued treatment due to resistance emergence. In both global long-term studies of entecavir and in the present study, continuation of therapy beyond 2 years resulted in approximately 90% of patients achieving or maintaining HBV-DNA levels below the PCR assay limit of detection of 300–400 copies/ml [32]. These results were consistent with the results of a sensitivity analysis (last observation carried forward), in which 85% of patients achieved HBV-DNA <400 copies/ml on their last HBV-DNA observation. This method accounts for patient drop-out

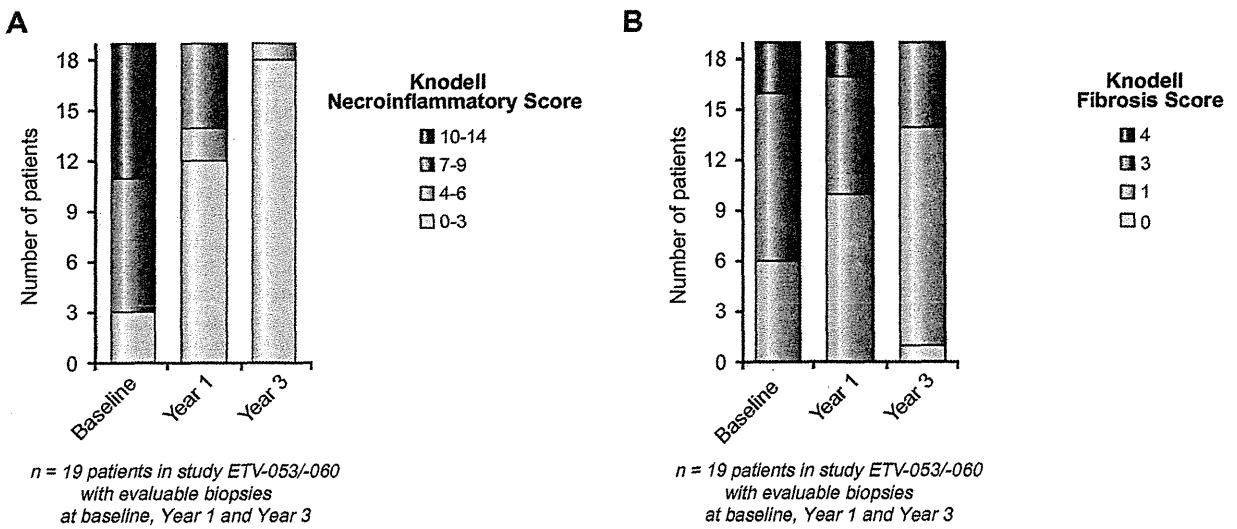

Fig. 6. (A) Distribution of Knodell necroinflammatory scores at pre-treatment baseline, year 1 (48 weeks), and year 3 (148 weeks), for 19 patients in the *entecavir 0.5 mg cohort* with evaluable liver biopsies at all three time points. (B) Distribution of Knodell fibrosis scores at pre-treatment baseline, year 1 (48 weeks), and year 3 (148 weeks), for 19 patients in the *entecavir 0.5 mg cohort* with evaluable liver biopsies at all three time points.

and missing samples, both of which are common occurrences in long-term studies. However, the interpretation of this sensitivity analysis should be approached cautiously, as it assumes: (1) that subjects who discontinued treatment without achieving HBV-DNA <300 copies/ml would not have achieved it with longer treatment; (2) and that patients who achieved this end point prior to discontinuing would have maintained it over time.

The degree of viral suppression reported in this study is higher than that reported for a cohort of HBeAg(+) patients treated with lamivudine for 3 years [33] and higher than that reported for cohorts of HBeAg(+) or HBeAg(−) patients treated with adefovir for 3 years [23,24]. In the current study, 84% of patients were HBeAg(+), and mean baseline HBV-DNA was 7.88 $\log_{10}$ copies/ml, 1 log higher than the baseline viral load in the adefovir study of HBeAg(−) patients. The rate of HBe seroconversion following entecavir treatment for 3 years in this study (26%), is somewhat lower than previously reported for patients treated with adefovir or lamivudine for 3 years (40% and 43%, respectively) [24,33]. This may be related to the large proportion (92%) of HBV genotype C patients enrolled in this study, which has previously been associated with delayed HBe seroconversion [10,34].

The results of long-term epidemiological-outcome studies have demonstrated that CHB patients with persistently detectable HBV-DNA are at highest risk of liver disease progression [12–14]. This suggests that long-term suppression of HBV-DNA should help minimize CHB complications. Liaw et al. demonstrated the value of antiviral therapy in a landmark study of CHB patients with cirrhosis or advanced fibrosis treated with long-term lamivudine [35]. Lamivudine-treated patients experienced lower rates of liver disease progression and HCC compared to those who received placebo, but the benefits were reduced by the emergence of lamivudine resistance.

High rates of histological improvement and improvement in fibrosis were observed in the current study among patients who received entecavir 0.5 mg from baseline. This improvement in liver histology is likely related to effective viral suppression. Long-term suppression of HBV-DNA is a key objective of CHB therapy, with the ultimate aim of preventing or reversing liver disease progression [15,36]. In previous studies, maintenance of virological suppression has been associated with improved liver histology among patients treated with nucleoside antivirals. Dienstag et al. showed that long-term treatment with lamivudine resulted in histological improvement, including reversal of fibrosis and cirrhosis; however, those benefits were lost when lamivudine resistance emerged [37]. Mommeja-Marin et al. showed statistically significant correlations between viral load suppression and histological improvement among HBeAg(+) patients treated with nucleoside analogues [38]. Hadziyannis et al. showed that 5 years of adefovir therapy for a cohort of HBeAg(−) patients resulted in virological suppression along with improvements in necroinflammation and fibrosis [23]. The current study demonstrates that continued entecavir treatment beyond 1 year results in increasing proportions of patients achieving HBV-DNA reduction to <400 copies/ml and further improvements in necroinflammation and fibrosis. At 3 years, all patients in the entecavir 0.5 mg cohort with evaluable biopsy pairs demonstrated histological improvement, and most (57%) showed improvement in fibrosis, including 85% (11/13) of those who had advanced fibrosis or cirrhosis at baseline.

The potent HBV-DNA suppression achieved in the current study, in combination with entecavir's high genetic barrier to resistance, likely contributed to the observed low rate of resistance emergence: 3-year cumulative probability of resistance of 3.3% for all patients and 1.7% for patients who received the approved dose of entecavir (0.5 mg) throughout the treatment period. The rate of 1.7% for patients treated continuously with the approved dose is consistent with that reported in entecavir global studies, in which the cumulative probability of resistance in nucleoside-naïve patients was 1.2% through 5 years [39]. The current study differs from the global studies in its focus on a well-defined cohort who were followed continuously with no dose interruption. In comparison with the consistently low rate of entecavir resistance observed among nucleoside-naïve patients, adefovir resistance emerged at rates of 20% among HBeAg(+) patients treated for 5 years (median of 235 weeks) and 29% among HBeAg(−) patients treated for 5 years [23,24].

In summary, the long-term data presented in the current report demonstrate that continuous entecavir therapy for 3 years is well tolerated in Japanese patients and provides durable clinical benefit. The high antiviral potency and low rate of resistance emergence shown in the current study support entecavir as an appropriate choice of first-line therapy for nucleoside-naïve chronic hepatitis B.

Conflicts of interest

Hiroki Ishikawa, Nobuyuki Masaki and Taku Seriu are employees of Bristol-Myers Squibb. Masao Omata is Member of Advisory Board for Bristol-Myers Squibb.

The other authors have nothing to disclose.

Acknowledgements

This work was sponsored by Bristol-Myers Squibb. Taku Seriu, Hiroki Ishikawa and Nobuyuki Masaki are employees of Bristol-Myers Squibb. Masao Omata serves as an advisor for Bristol-Myers Squibb. In addition to the authors, other study investigators included Joji Toyota, Chitomi Hasebe, Teruaki Kawanishi, Kazuyuki Suzuki, Yoshiyuki Ueno, Satoshi Mochida, Namiki Izumi, Hidetsugu Saito, Naohiko Masaki, Keiko Tatemoto, Yoshiyuki Arakawa, Hiromitsu Kumada, Yasunobu Matsuda, Shunichi Okada, Eiji Tanaka, Yoshiaki Katano, Etsuro Orito, Shinichi Kakumu, Noboru Hirashima, Eiichi Tomita, Takashi Kumada, Takeshi Okanoue, Kazihiro Katayama, Michio Kato, Harumasa Yoshihara, Taizo Hijioka, Kosaku Sakaguchi, Gotaro Yamada, Keisuke Hino, Norio Horiike, Shotaro Sakisaka, Ryukichi Kumashiro, Keisuke Hamasaki, Hiroshi Yatsuhashi, Masataka Seike, Yutaka Sasaki, Katsuhiro Hayashi, Hiroshi Ikeda, Masanori Miyake, Yasuyuki Araki, Kozo Fujio and Masaharu Ando. The study coordinating committee included Kazuyuki Suzuki, Hiromitsu Kumada, Takeshi Okanoue, Yasushi Shiratori, Michio Sata and Hirohito Tsubouchi. The biopsy reading committee included Fumio Imazeki, Mitsuhiko Moriyama and Masayoshi Kage.

References

[1] Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepat 2004;11:97–107.

[2] Merican I, Guan R, Amarapuka D, Alexander MJ, Chutaputti A, Chien RN, et al. Chronic hepatitis B virus infection in Asian countries. J Gastroenterol Hepatol 2000;15:1356–1361.

[3] Chen CJ, Wang LY, Yu MW. Epidemiology of hepatitis B virus infection in the Asia-Pacific region. J Gastroenterol Hepatol 2000;15:E3–E6.
[4] Nakayoshi T, Maeshiro T, Nakayoshi T, Nakasone H, Sakugawa H, Kinjo F, et al. Difference in prognosis between patients infected with hepatitis B virus with genotype B and those with genotype C in the Okinawa Islands: a prospective study. J Med Virol 2003;70:350–354.
[5] Sakugawa H, Ohwan T, Yamashiro A, Oyakawa T, Kadena K, Kinjo F, et al. Natural seroconversion from hepatitis B e antigen to antibody among hepatitis B virus carriers in Okinawa Island. J Med Virol 1991;34:122–126.
[6] Usuda S, Okamoto H, Iwanari H, Baba K, Tsuda F, Miyakawa Y, et al. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the pre S2-region product. J Virol Methods 1999;80:97–112.
[7] Hou J, Liu Z, Gu F. Epidemiology and prevention of hepatitis B virus infection. Int J Med Sci 2005;2:50–57.
[8] Yuen MF, Tanaka Y, Ng IOL, Mizokami M, Yuen JC, Wong DK, et al. Hepatic necroinflammation and fibrosis in patients with genotypes Ba and C, core promoter and precore mutations. J Viral Hepat 2005;12:513–518.
[9] Jang JW, Lee YC, Kim MS, Lee SY, Bae SH, Choi JY, et al. A 13-year longitudinal study of the impact of double mutations in the core promoter region of hepatitis B virus on HBeAg seroconversion and disease progression in patients with genotype C chronic active hepatitis. J Viral Hepat 2007;14:169–175.
[10] Nakashima H, Furusyo N, Kubo N, Kashiwagi K, Etoh Y, Kashiwagi S, et al. Double point mutation in the core promoter region of hepatitis B virus (HBV) genotype C may be related to liver deterioration in patients with chronic HBV infection. J Gastroenterol Hepatol 2004;19:541–550.
[11] Yu MW, Yeh SH, Chen PJ, Liaw YF, Lin CL, Liu CJ, et al. Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: a prospective study in men. J Natl Cancer Inst 2005;97:265–272.
[12] Yuen MF, Yuan HJ, Wong DKH, Yuen JC, Wong WM, Chan AO, et al. Prognostic determinants for chronic hepatitis B in Asians: therapeutic implications. Gut 2005;54:1610–1614.
[13] Iloeje UH, Yang HI, Su J, Jen CL, You SL, Chen CJ. Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. Gastroenterology 2006;130:678–686.
[14] Chen CJ, Yang HI, Su J, Jen CL, You SL, Lu SN, et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA 2006;295:65–73.
[15] Lok ASF, McMahon BJ. Chronic hepatitis B. Hepatology 2007;45:507–539.
[16] Liaw YF, Leung N, Kao JH, Piratvisuth T, Gane E, Han KH, et al. Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2008 update. Hepatol Int 2008;2:263–283.
[17] Keeffe EB, Dieterich DT, Han SH, Jacobson IM, Martin P, Schiff ER, et al. A treatment algorithm for the management of chronic hepatitis B virus infection in the United States: 2008 update. Clin Gastroenterol Hepatol 2008;6:1315–1341.
[18] Lok AS, Wu PC, Lai CL, Lau JY, Leung EK, Wong LS, et al. A controlled trial of interferon with or without prednisone priming for chronic hepatitis B. Gastroenterology 1992;102:2091–2097.
[19] Cooksley WGE, Piratvisuth T, Lee SD, Mahachai V, Chao YC, Tanwandee T, et al. Peginterferon alpha-2a (40 kDa): an advance in the treatment of hepatitis B e antigen-positive chronic hepatitis B. J Viral Hepat 2003;10:298–305.
[20] Lau GK, Piratvisuth T, Luo KX, Marcellin P, Thongsawat S, Cooksley G, et al. Peginterferon alfa-2a, lamivudine, and the combination for HBeAg-positive chronic hepatitis B. N Engl J Med 2005;352:2682–2695.
[21] Chang TT, Lai CL, Chien RN, Guan R, Lim SG, Lee CM, et al. Four years of lamivudine treatment in Chinese patients with chronic hepatitis B. J Gastroenterol Hepatol 2004;19:1276–1282.
[22] Lai CL, Dienstag J, Schiff E, Leung NW, Atkins M, Hunt C, et al. Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. Clin Infect Dis 2003;36:687–696.
[23] Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, Chang TT, Kitis G, Rizzetto M, et al. Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis B for up to 5 years. Gastroenterology 2006;131:1743–1751.
[24] Marcellin P, Chang TT, Lim SG, Sievert W, Tong M, Arterburn S, et al. Long-term efficacy and safety of adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. Hepatology 2008;48:750–758.
[25] Gilead Sciences, Inc. Hepsera Prescribing Information. Foster City, CA, USA; 2007.
[26] Lai CL, Gane E, Hsu CW, Thongsawat S, Wang Y, Chen Y, et al. Two-year results from the GLOBE trial in patients with hepatitis B: greater clinical, antiviral efficacy for telbivudine vs. lamivudine. Hepatology 2006;44:222A, [Abstract 91].
[27] Kobashi H, Takaguchi K, Ikeda H, Yokosuka O, Moriyama M, Imazeki F, et al. Efficacy and safety of entecavir in nucleoside-naïve, chronic hepatitis B patients: phase II clinical study in Japan. J Gastroenterol Hepatol 2009;24:255–261.
[28] Shindo M, Chayama K, Toyota J, Fujiwara K, Sugihara J, Hayashi N, et al. Efficacy, safety of entecavir, lamivudine in Japanese adult patients with chronic hepatitis B infection: a phase 2 clinical trial. J Clin Virol 2006;36:S94, [Abstract P109].
[29] Chang TT, Gish RG, de Man R, Gadano A, Sollano J, Chao YC, et al. A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. N Engl J Med 2006;354:1001–1010.
[30] Lai CL, Shouval D, Lok AS, Chang TT, Cheinquer H, Goodman Z, et al. Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. N Engl J Med 2006;354:1011–1020.
[31] Ichida F, Tsuji T, Omata M, Ichida T, Inoue K, Kamimura T, et al. New Inuyama classification: new criteria for histological assessment of chronic hepatitis. Int Hepatol Commun 1996;6:112–119.
[32] Han S, Chang TT, Chao YC, Yoon SK, Gish RG, Cheinquer H, et al. Four-year entecavir treatment in nucleoside-naïve HBeAg(+) patients: results from studies ETV-022, -901. Hepatology 2007;46:654A, [Abstract 938].
[33] Leung NWY, Lai CL, Chang TT, Guan R, Lee CM, Ng KY, et al. Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: results after 3 years of therapy. Hepatology 2001;33:1527–1532.
[34] Furusyo N, Nakashima H, Kashiwagi K, Kubo N, Hayashida K, Usuda S, et al. Clinical outcomes of hepatitis B virus (HBV) genotypes B and C in Japanese patients with chronic HBV infection. Am J Trop Med Hyg 2002;67:151–157.
[35] Liaw YF, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, et al. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med 2004;351:1521–1531.
[36] Omata M. Treatment of chronic hepatitis B infection. N Engl J Med 1998;339:114–115.
[37] Dienstag JL, Goldin RD, Heathcote EJ, Hann HW, Woessner M, Stephenson SL, et al. Histological outcome during long-term lamivudine therapy. Gastroenterology 2003;124:105–117.
[38] Mommeja-Marin H, Mondou E, Blum MR, Rousseau F. Serum HBV-DNA as a marker of efficacy during therapy for CHB infection: analysis and review of the literature. Hepatology 2003;37:1309–1319.
[39] Tenney DJ, Rose RE, Baldick CJ, Pokornowski KA, Eggers BJ, Fang J, et al. Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naïve patients is rare through 5 years of therapy. Hepatology 2009;49:1503–1514.

Research Article

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Common variation of IL28 affects gamma-GTP levels and inflammation of the liver in chronically infected hepatitis C virus patients

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Background & Aims: A common genetic variation at the IL28 locus has been found to affect the response of peg-interferon and ribavirin combination therapy against chronic hepatitis C virus (HCV) infection. An allele associated with a favorable response (rs8099917 T), which is the major allele in the majority of Asian, American, and European populations, has also been found to be associated with spontaneous eradication of the virus.

Methods: As no studies have yet analyzed the effect of the polymorphism on biochemical and inflammatory changes in chronic infection, we analyzed a cohort of patients with chronic hepatitis C ($n = 364$) for the effect of the IL28 polymorphism on viral, biochemical, and histological findings.

Results: We found that the proportion of HCV wild type core amino acids 70 and 91 was significantly greater ($p = 1.21 \times 10^{-4}$ and 0.034) and levels of gamma-GTP significantly lower ($p = 0.001$) in patients homozygous for the IL28 major allele. We also found that inflammation activity and fibrosis of the liver were significantly more severe in patients homozygous for the IL28 major allele ($p = 0.025$ and 0.036, respectively). Although the higher gamma-GTP levels were also associated with higher inflammatory activity and fibrosis, multivariate analysis showed that only the IL28 allele polymorphism, sex, alcohol consumption, and liver fibrosis were independently associated with gamma-GTP levels ($p = 0.001$, 0.0003, 0.0013, and 0.0348, respectively).

Conclusions: These results suggest that different cytokine profiles induced by the IL28 polymorphism resulted in different biochemical and inflammatory conditions during chronic HCV infection and contribute to the progression of liver diseases.
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Keywords: IL28; SNP; Histological activity; Inflammation; gamma-GTP.
Received 23 November 2009; received in revised form 9 March 2010; accepted 29 March 2010; available online 31 May 2010
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Abbreviations: HCV, hepatitis C virus; SNP, single nucleotide polymorphism; ISDR, interferon sensitivity determining region; BMI, body mass index.

Introduction

Hepatitis C virus infection is one of the major causative agents of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1]. The best current therapeutic regimen is pegylated interferon and ribavirin combination therapy [2,3]. Although the eradication rate of the virus has been improved by extending the treatment period from the standard 48 to 72 weeks for genotype 1b infected patients, active viral replication still remains in nearly half of these patients [4].

Recent studies have identified both host and viral factors predictive of interferon therapy. Among the viral factors, a forty amino acid stretch in the NS5 region has been found to be predictive of response to interferon monotherapy [5,6]. More recently, Akuta et al. identified amino acid substitutions in the core region (core aa70 and 91) that are predictive for the effect of interferon and ribavirin combination therapy [7,8].

Among the host factors, many common polymorphisms in the human genome, including single nucleotide polymorphisms (SNP), have been identified [9–13]. We recently reported that a SNP in the MAPKAPK3 gene is associated with response to interferon therapy [14]. More recently, three groups of researchers found that several SNPs in the IL28 locus are related to the effectiveness of combination therapy [15–17]. We also performed a genome wide association study and confirmed that variation at the IL28 locus is related to the effectiveness of combination therapy (Chayama K, personal communication).

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These viral and host factors must influence the natural course of viral infection. Host immune cells produce interferon and other cytokines in response to viral infection. For RNA viruses such as HCV, cellular sensors such as RIG-I detect the double stranded RNA and activate a pathway to produce cytokines, including alpha and beta interferons that trigger an antiviral response to eradicate the virus [18]. Genetic polymorphism of genes involved in innate immunity is likely to influence the strength and nature of this defense. In fact, a polymorphism in the IL28 locus has been reported to correlate with spontaneous eradication of HCV [19]. However, little is known about how these factors affect the course of chronic infection of the virus. In this study, we focused on histological findings in the liver. We also analyzed viral and biochemical factors in patients chronically infected with HCV. We found that histological aspects of the liver (fibrosis and activity), HCV core amino acid substitutions, and gamma-GTP are associated with the polymorphism.

Materials and methods

Study subjects

We analyzed a cohort of 364 consecutive adult patients with chronic hepatitis C virus infection who visited Hiroshima University hospital and received liver biopsies between December 2002 and November 2008 and who agreed to provide blood samples for the human genome study. All patients included in the study had positive HCV viremia in serum for more than six months, assessed using a commercial quantitative polymerase chain reaction (PCR) assay (COBAS Amplicor HCV Monitor Test, v2.0; Roche Diagnostics, Branchburg, NJ). Patients with decompensated liver disease were excluded, as were patients co-infected with hepatitis B virus, or human immunodeficiency virus and patients with apparent auto-immune hepatitis and alcoholic liver disease. All patients provided written informed consent for the genomic analysis. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved *a priori* by the ethical committees of Hiroshima University and Riken. The patient profiles are listed in Table 1. Using criteria reported by Desmet et al. [20], liver biopsy samples were evaluated by two pathologists. To verify consistency and accuracy, one of the pathologists independently re-evaluated samples analyzed by the other, and both pathologists were blind with respect to the IL28 polymorphism. We excluded insufficient or inconclusive biopsy samples, including those that were less than 10 mm^2 in size and containing less than 10 portal tracts. The amount of alcohol consumed was calculated according to the frequency of consumption and the alcohol concentration of beverages consumed. We estimated alcohol concentrations as follows: 5% for beer, 17% for sake, 25% for Japanese vodka, and 43% for whiskey; 1 ml of alcohol was considered equivalent to 0.886 g. The amount of alcohol consumed was divided into three categories: none, light (0–20 g/day), moderate (21–50 g/day). Heavy drinkers (more than 50 g/day) were excluded from the study.

Table 1. Characteristics of patients.

Characteristics of patients	
Age [median (range)]	59 (20–82)
Sex (male/female)	212/152
BMI [median (range)]	23 (16–39)
Alcohol consumption (Unavailable/none/0–20 g/day/21–50 g/day)	64/110/65/125
Hb [median (range)] mg/dl	14 (8–18)
Platelet [median(range)] × $10^4/mm^3$	14 (4–41)
ALT [median (range)]IU/L	62 (2–611)[c]
gamma-GTP [median (range)] IU/L	50 (7–680)
Genotype (1b/2a or 2b/1b + 2b/undertermined)	260/84/1/19
Fibrosis (F0/F1/F2/F3/F4)	4/116/141/66/37
Activity (A0/A1/A2/A3)	1/102/206/51
Virus titer [median (range)]kIU/l	1400 (<0.5–26,000)
Core 70[a] (wild/mutant/undertermined)	120/77/167
Core 91[a] (wild/mutant/undertermined)	107/88/169
ISDR[b] mutation (0/1/>2/undertermined)	58/70/48/188

[a] Hepatitis C virus core amino acid 70R and 91L are presented as wild type. Substituted amino acids are considered mutants.
[b] Interferon sensitivity determining region. Number of amino acids substituted from the prototype genotype 1b sequence were calculated.
[c] ALT levels of two patients remained around 2 IU/L even though AST and gamma-GTP levels were comparable to other chronic hepatitis C patients (peaking above 100 IU/L and returning to normal following SVR), probably due to deficiency of the ALT enzyme. These values were omitted from analysis of ALT.

Genotyping

Genotyping of some of the samples was performed as part of a genome wide association study using the Illumina HumanHap610-Quad Genotyping BeadChip (Illumina, Inc., CA) at Riken Yokohama Institute. Genotyping of the remaining samples was performed using TaqMan assay or Invader assay as described previously [21,22].

Analysis of amino acid sequences in the core and ISDR region

HCV RNA was extracted from 100 µl serum samples by SepaGene RV-R (Sanko Junyaku Co., Tokyo, Japan) and dissolved in 20 µl of H_2O. The RNA was then reverse transcribed with random primers and MMLV reverse transcriptase (Takara Shuzo, Tokyo, Japan). The resultant cDNA was then amplified by nested PCR. PCR was performed in 25 µl of reaction mixture containing 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 20 pmol of each primer and 1.25 U of LA Taq (Takara Bio Inc.) with a buffer supplied by the manufacturer. One microliter of 10×-diluted products from the first PCR was used as a template for the second PCR. The PCR primer sequences are listed in Table 2. The PCR protocol involved initial denaturation at 95 °C for 5 min, 35 cycles of denaturation for 30 s at 94 °C, annealing of primers for 1 min at 57 °C and extension for 1 min at 72 °C, followed by final extension at 72 °C for 7 min. The amplified DNA fragments were separated onto a 2% agarose gel and purified with the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Nucleotide sequences were determined using the Big-Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., CA).The obtained nucleotide and amino acid sequences were compared with the prototype sequence of genotype 1b HCV-J (GenBank Accession Number D90208) [23]. Amino acids at positions 70 and 91 of the core region that were identical to the prototype (arginine and leucine, respectively) were considered wild type.

Statistical analysis

χ^2 and Mann–Whitney U-tests were applied to detect significant associations. Simple and multiple regression analyses were used to examine the association between serum gamma-GTP levels and the values of other markers. When the data were not normally distributed, Box-Cox power transformation was performed to remove skewness, followed by linear regression analyses. All of the statistical analyses were two sided, and $p < 0.05$ was considered significant. All statistical analysis was performed using the PASW Statistics 18 program (SPSS Inc., IL).

Results

IL28 locus genotypes and viral and biochemical markers

We compared viral and biochemical markers with IL28 genotypes. First we analyzed the relationship between IL28 genotypes

Table 2. Primers used in this study.

Core region	
Outer forward	5′-GCC ATA GTG GTC TGC GGA AC-3′
Outer reverse	5′-GGA GCA GTC CTT CGT GAC ATG-3′
Inner forward	5′-GCT AGC CGA GTA GTG TT-3′
Inner reverse	5′-GGA GCA GTC CTT CGT GAC ATG-3′
ISDR[a]	
Outer forward	5′-TTC CAC TAC GTG ACG GGC AT-3′
Outer reverse	5′-CCC GTC CAT GTG TAG GAC AT-3′
Inner forward	5′-GGG TCA CAG CTC CCA TGT GAG CC-3′
Inner reverse	5′-GAG GGT TGT AAT CCG GGC GTG C-3′

[a] Interferon sensitivity determining region.

Table 3. Amino acid substitutions in the core region of HCV and IL28 genotype.

SNP	Allele (1/2)	Genotype			p value[a]	OR
		11	12	22		(95% CI)[b]
rs8099917 T/G						
	Core aa70					
	Wild	2	17	101	1.21E-04	0.30
	Non-wild	3	28	46		(0.14–0.55)
	Core aa91					
	Wild	3	18	86	0.034	0.50
	Non-wild	2	27	59		(0.26–0.95)
	ISDR					
	0–1	2	37	89	0.120	1.90
	>2	2	7	39		(0.84–4.3)
	HCV genotype					
	1	6	63	190	0.443	0.81
	2	1	25	58		(0.47–1.4)

[a] p value by χ^2 test for the minor allele dominant model.
[b] Odds ratio for the minor allele in a dominant model.

Table 4. Factors associated with higher gamma-GTP levels.

Variable	Simple		Multiple	
	Estimate	p	Estimate	p
Age	−0.00004	0.899436		
Sex (male vs. female)	0.04647	5E-09	0.033	0.0003
BMI	−0.00257	0.044003		
Activity (A2–4 vs. A0–1)	−0.02518	0.004103	−0.015	0.1415
Fibrosis (F2–4 vs.F0–1)	0.03	0.000382	0.021	0.0348
Alcohol consumption	−0.03962	6.81E-06	−0.029	0.0013
IL28 genotype (2/2 vs. 1/2, 1/1)	0.02641	0.003522	0.03	0.001
HCV genotype (1 vs. 2)	−0.0068	0.471293		
Log virus titer (Log IU/ml)	0.00032	0.748826		
Core aa70 (wild vs. others)	−0.01589	0.117424		
Core aa91 (wild vs. others)	−0.01422	0.162341		
ISDR (0–1 vs. ⩾2)	0.00253	0.824685		

Simple and multiple regression analyses were used to examine the association between serum gamma-GTP and the values of other markers. All of the statistical analyses were two sided, and p <0.05 was considered significant.

and substitutions in the HCV core protein amino acids 70 and 91, as well as the HCV genotype and the number of amino acid substitutions in the ISDR. As shown in Table 3, there are significant associations between amino acid substitutions in the core region and the genotype of the rs8099917 SNP at the IL28B locus. In particular, patients homozygous for the major IL28 allele were significantly associated with wild type core amino acid 70 (OR = 0.30; p = 1.21E-04). A similar trend is seen with core amino acid 91 substitutions (OR = 0.50; p = 0.034). Patients with more than one amino acid substitution in the ISDR region also tended to occur in patients homozygous for the major allele, although the difference was not statistically significant (Table 3). There was no correlation between the HCV genotype and the IL28 allele.

We further examined the relationship between IL28 and biochemical markers such as ALT, gamma-GTP, total cholesterol, HDL cholesterol, serum iron, and HCV RNA levels. Only the gamma-GTP level was significantly associated with the IL28 genotype. As shown in Fig. 1A, the gamma-GTP levels were lowest in the IL28 major allele homozygotes and highest in minor allele homozygotes. As drinking alcohol is known to elevate gamma-GTP levels, we examined the effect of alcohol intake in our cohort. As shown in Fig. 1B, there was an association between alcohol and gamma-GTP levels. As we found that the gamma-GTP level is higher in patients with core amino acid 70 substitutions (Fig. 1C), we performed multivariate analysis to examine what factors contribute to higher levels of gamma-GTP. As shown in Table 4, a simple regression analysis revealed that serum gamma-GTP levels were associated with sex, BMI, inflammation activity, liver fibrosis, alcohol consumption, and IL28 genotype, whereas in multiple regression analysis, sex, liver fibrosis, alcohol consumption, and IL28 genotype remained positively associated with serum gamma-GTP levels.

Histological findings and polymorphism in the IL28 locus

We then analyzed the relationship between the IL28 locus polymorphisms and histological findings. We divided patients into mild fibrosis (F0 and F1) and severe fibrosis (F2–4) as well as lower activity (A0 and A1) and higher activity (A2 and A3) and compared these factors against IL28 genotypes. As shown in Table 5, both inflammatory activity and fibrosis were significantly associated with IL28 genotype. Inflammation was more active (A2–3) in patients homozygous for IL28 major alleles

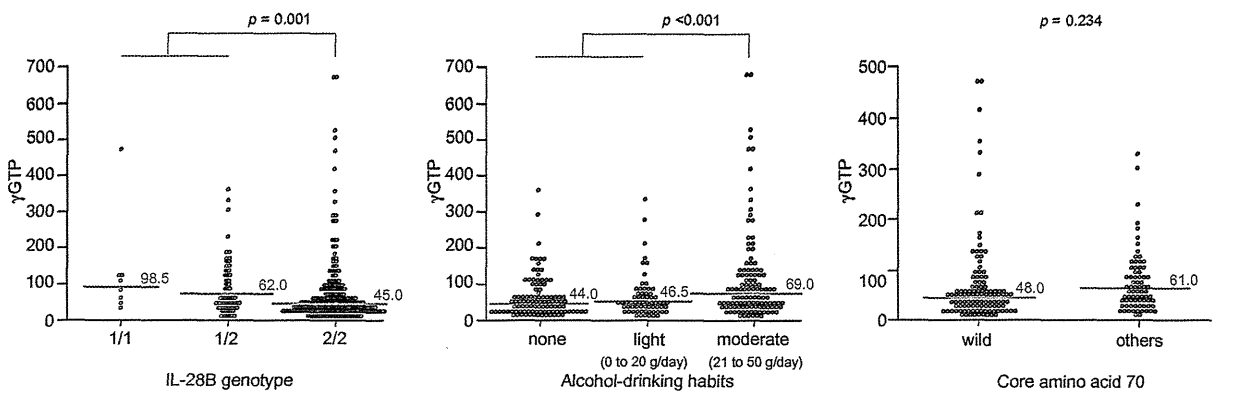

Fig. 1. gamma-GTP levels and IL28 genotype, alcohol intake, and core amino acid substitutions. gamma-GTP levels according to (A) IL28 genotypes, (B) alcohol consumption, and (C) core amino acid 70 substitutions are shown. Horizontal bars represent the median. Mann–Whitney U-test was used to compare gamma-GTP levels.

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Table 5. Histological findings and IL28 genotypes.

SNP	Allele (1/2)	Genotype			p value[a]	OR
		11	12	22		(95% CI)[b]
rs8099917	*T/G*					
	Fibrosis					
	F0–1	3	38	79	0.036	1.66
	F2–4	5	53	186		(1.03–2.69)
	Activity					
	A0–1	2	35	68	0.025	1.75
	A2–3	6	56	199		(1.07–2.86)

[a] p value by χ^2 test for the minor allele dominant model.
[b] Odds ratio for the minor allele in a dominant model.

(OR = 1.75; $p = 0.025$). Similarly, fibrosis was more severe in patients homozygous for IL28 major alleles (OR = 1.66; $p = 0.036$). We also performed analysis of the association of IL28 alleles and histological findings after adjusting for other factors that might influence the activity and fibrosis of the liver, such as age, gender, and alcoholic consumption. The IL28 allele was associated with F and A factors independently with adjustment for these predictive factors related to severity of liver fibrosis and inflammation (data not shown).

Relationship between histological activity, the IL28 allele, and gamma-GTP

As we described above, histological activity is more active in patients homozygous for IL28 major alleles. However, it seems contradictory that IL28 major allele homozygosity was associated with low levels of gamma-GTP, but severe activity was associated with high gamma-GTP. As shown in Fig. 2, however, when we compare the allele and activity the frequency of patients with higher activity (A2 and A3) were statistically more frequent in patients homozygous for major alleles (Fig. 2 and Table 5). When we compare gamma-GTP levels of A2 and A3 patients between patients homozygous for major alleles against the others, the levels were significantly lower in the major allele homozygous patients (Fig. 2). A0 and A1 patients showed similar results (Fig. 2). gamma-GTP levels are also significantly higher in patients with higher activities (A2 and A3) than in patients with lower activities (A0 and A1) (Fig. 2).

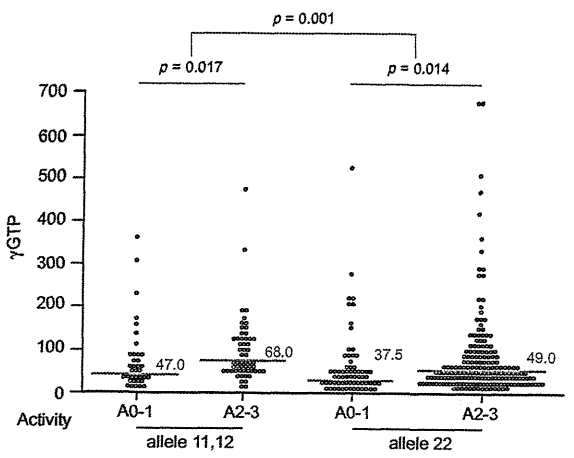

Fig. 2. Relationship between gamma-GTP levels, histological activity and IL28 genotype. *gamma-GTP levels are plotted according to IL28 alleles, and histological activity. Horizontal bars represent the median. Mann–Whitney U-test was used to compare gamma-GTP levels.*

Discussion

Polymorphism at the IL28 locus has been reported to be associated with the effectiveness of interferon and ribavirin combination therapy [15–17]. We have also found that the polymorphism is associated with the effect of interferon monotherapy on genotype 1b infected patients as well as genotype 2a infection in Japanese as well as Taiwanese patients (Chayama K, personal communication). The polymorphism has also been reported to be associated with spontaneous eradication of the hepatitis C virus [19]. As levels of IL28 gene transcripts have been reported to be higher in patients homozygous for the interferon response allele [16,17], we hypothesized that the polymorphism is also associated with inflammation and progression of chronic hepatitis. As expected, there were significant associations between IL28 genotypes and histological inflammatory activity as well as the degree of fibrosis in chronically HCV infected patients (Table 5). It seems reasonable that the inflammation is stronger in patients with elevated IL28 production because this molecule induces expression of interferon stimulated genes, including some inflammatory cytokines. As the polymorphism is associated with the effect of interferon therapy, the interferon therapy performed before biopsy might alter the results. In fact, a part of patients in this study were treated with peg-interferon and ribavirin combination therapy and the treatment outcome was associated with IL28 genotypes and core amino acid substitutions (data not shown). However, when we analyzed the relation between the IL28 allele and histological findings or core amino acid substitutions in only treatment-naïve patients, the results were unchanged, suggesting that the results obtained in this study are applicable without regard to history of interferon therapy.

Interestingly, the IL28 genotype was also associated with gamma-GTP levels and core amino acid substitutions, both of which are known to be predictive of response to interferon and ribavirin combination therapy [7,8,24]. The levels of liver enzymes such as ALT, AST, and gamma-GTP are usually higher in patients with high inflammatory activity. However, we observed that the levels were actually lower in patients with the favorable allele at the IL28 locus (the major allele in the Japanese population) (Figs. 1A and 2). A lower level of gamma-GTP has been reported to be associated with positive response to combination therapy. Further studies are needed to clarify the mechanism underlying the relation between gamma-GTP levels and therapy effectiveness. It would also be interesting to study the relationship between the IL28 allele and steatosis in the liver because gamma-GTP tends to be elevated in patients with steatosis, and steatosis caused by HCV core protein has been reported [25].

Similarly, viral wild type core amino acids 70 and 91 (i.e., core 70R and 91L), which were already known to be associated with positive response to combination therapy [7,8], were also found to be associated with the favorable human IL28 alleles (Table 3). If viruses with wild type core amino acids 70 and 91 are more

susceptible to interferon therapy, such strains should be less frequent in patients with higher cytokine levels. Viruses with wild type core 70 and 91 amino acids must therefore have some survival advantage in order to replicate in cells in which the level of IL28 production is high. Searching for a target molecule in the signaling cascade from sensing of the virus to production of IL28 might help resolve this question.

We also observed an association between high gamma-GTP levels and core amino acid 70 and 91 substitutions (Fig. 1C), although in multivariate analysis only IL28 genotype, liver fibrosis, sex, and alcohol consumption were significant predictors of gamma-GTP. It seems likely that these factors mutually interact in the presence of the virus and cytokines. Understanding these relationships will reveal the mechanism underlying the effective response to combination therapy and may suggest new strategies to cope with the hepatitis C virus.

Financial disclosure

The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Acknowledgments

The authors thank Rie Akiyama, Yoshie Yoshida, Kazuyo Hattori, Mariko Shiota, Hiromi Ishino, Yasufumi Hayashida, and Takako Yokogi for excellent technical assistance, and Junko Sakamiya, Aya Furukawa, Mika Tsuzuno, Sakura Akamatus, Sanae Furuya, and other secretary members for their secretarial assistance. Part of this work was carried out at the Analysis Center of Life Science, Hiroshima University. This work was supported in part by Grants-in-Aid for scientific research and development from the Ministry of Health, Labor, and Welfare and Ministry of Education Culture Sports Science and Technology, Government of Japan.

References

[1] Barrera JM, Bruguera M, Ercilla MG, Gil C, Celis R, Gil MP, et al. Persistent hepatitis C viremia after acute self-limiting posttransfusion hepatitis C. Hepatology 1995;21:639–644.
[2] Hadziyannis SJ, Sette Jr H, Morgan TR, Balan V, Diago M, Marcellin P, et al. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med 2004;140:346–355.
[3] Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 2001;358:958–965.
[4] Jensen DM, Marcellin P, Freilich B, Andreone P, Di Bisceglie A, Brandao-Mello CE, et al. Re-treatment of patients with chronic hepatitis C who do not respond to peginterferon-alpha2b: a randomized trial. Ann Intern Med 2009;150:528–540.
[5] Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 1996;334:77–81.
[6] Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. J Clin Invest 1995;96:224–230.
[7] Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol 2007;46:403–410.
[8] Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Predictive factors of virological non-response to interferon-ribavirin combination therapy for patients infected with hepatitis C virus of genotype 1b and high viral load. J Med Virol 2006;78:83–90.
[9] Welzel TM, Morgan TR, Bonkovsky HL, Naishadham D, Pfeiffer RM, Wright EC, et al. Variants in interferon-alpha pathway genes and response to pegylated interferon-Alpha2a plus ribavirin for treatment of chronic hepatitis C virus infection in the hepatitis C antiviral long-term treatment against cirrhosis trial. Hepatology 2009;49:1847–1858.
[10] Hijikata M, Ohta Y, Mishiro S. Identification of a single nucleotide polymorphism in the MxA gene promoter (G/T at nt −88) correlated with the response of hepatitis C patients to interferon. Intervirology 2000;43:124–127.
[11] Knapp S, Yee LJ, Frodsham AJ, Hennig BJ, Hellier S, Zhang L, et al. Polymorphisms in interferon-induced genes and the outcome of hepatitis C virus infection: roles of MxA, OAS-1 and PKR. Genes Immun 2003;4:411–419.
[12] Matsuyama N, Mishiro S, Sugimoto M, Furuichi Y, Hashimoto M, Hijikata M, et al. The dinucleotide microsatellite polymorphism of the IFNAR1 gene promoter correlates with responsiveness of hepatitis C patients to interferon. Hepatol Res 2003;25:221–225.
[13] Naito M, Matsui A, Inao M, Nagoshi S, Nagano M, Ito N, et al. SNPs in the promoter region of the osteopontin gene as a marker predicting the efficacy of interferon-based therapies in patients with chronic hepatitis C. J Gastroenterol 2005;40:381–388.
[14] Tsukada H, Ochi H, Maekawa T, Abe H, Fujimoto Y, Tsuge M, et al. A polymorphism in MAPKAPK3 affects response to interferon therapy for chronic hepatitis C. Gastroenterology 2009;136:1796–1805, e1796.
[15] Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399–401.
[16] Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 2009;41:1100–1104.
[17] Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 2009;41:1105–1109.
[18] Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, et al. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 2004;5:730–737.
[19] Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 2009;461:798–801.
[20] Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994;19:1513–1520.
[21] Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y. A high-throughput SNP typing system for genome-wide association studies. J Hum Genet 2001;46:471–477.
[22] Suzuki A, Yamada R, Chang X, Tokuhiro S, Sawada T, Suzuki M, et al. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. Nat Genet 2003;34:395–402.
[23] Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA 1990;87:9524–9528.
[24] Bergmann JF, Vrolijk JM, van der Schaar P, Vroom B, van Hoek B, van der Sluys Veer A, et al. Gamma-glutamyltransferase and rapid virological response as predictors of successful treatment with experimental or standard peginterferon-alpha-2b in chronic hepatitis C non-responders. Liver Int 2007;27:1217–1225.
[25] Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4:1065–1067.

Scandinavian Journal of Gastroenterology, 2010; 45: 1488–1496

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ORIGINAL ARTICLE

Prolongation of interferon therapy for recurrent hepatitis C after living donor liver transplantation: Analysis of predictive factors of sustained virological response, including amino acid sequence of the core and NS5A regions of hepatitis C virus

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Abstract

Objective. The aim of the present retrospective study was to evaluate the therapeutic efficacy and predictive factors of prolongation of treatment with peginterferon (PEGIFN) combined with ribavirin (RBV) for recurrent hepatitis C after living donor liver transplantation (LDLT). ***Methods.*** Fifty-three patients underwent LDLT due to HCV-related end-stage liver disease. Sixteen patients were removed from the study as a result of early death (n = 14), no recurrence of HCV (n = 1) and refusal of antiviral therapy (n = 1). Therapy is ongoing in another 10 patients. The remaining 27 patients were available to establish the efficacy of IFN therapy. HCV genotype was 1b in 24 patients. All patients with genotype 1b were treated with IFN therapy for at least 48 weeks after HCV RNA levels had become undetectable. Amino acid substitutions in the HCV core region and NS5A region were analyzed by direct sequencing before LDLT. ***Results.*** The rate of sustained virological response (SVR) was 37.0% (10/27). SVR rate in patients with genotype 1 was 29.2% (7/24) and 100% (3/3) in patients with genotype 2. Most patients with genotype 1b whose HCV RNA reached undetectable levels achieved SVR (87.5%; 7/8). However, mutation of the HCV core region and number of ISDR mutations were not associated with SVR rate in LDLT in our study. ***Conclusions.*** Prolonged IFN therapy for more than 48 weeks after HCV RNA reached undetectable levels might prevent virological relapse of HCV.

Key Words: *Core and NS5A regions, HCV, IFN, LDLT*

Introduction

Hepatitis C virus (HCV)-related end-stage liver disease is currently the leading indication for liver transplantation (LT). Unfortunately, prevention of HCV infection after transplantation is difficult and, unlike the situation with the prevention of hepatitis B virus after transplantation [1], HCV re-infection after LT is almost universal, with histological evidence of chronic hepatitis in approximately 50% of patients within 1 year and cirrhosis in about 30% after 5 years. This in turn yields an excess risk of death or

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(Received 26 April 2010; accepted 26 June 2010)

ISSN 0036-5521 print/ISSN 1502-7708 online © 2010 Informa Healthcare
DOI: 10.3109/00365521.2010.505657

retransplantation for liver failure 10–15 years after transplantation [2]. Given this risk, it seems reasonable to offer antiviral therapy to liver transplant recipients. Many reports have noted rates of sustained viral response (SVR) ranging from only 10% to 30% in liver transplant recipients with recurrent HCV treated for 48 weeks [3–10], indicating the need for new treatment regimens with higher SVR rates. Recent reports have indicated that the extension of treatment with peginterferon and ribavirin (PEGIFN/RBV) from 48 to 72 weeks significantly increases the rate of SVR in immunocompetent patients, particularly slow virological responders [11,12], and one report noted that extended treatment for recurrent hepatitis C infection after liver transplantation (LT) was effective [13,14].

There are many predictive factors of successful treatment with the combination of peginterferon and ribavirin in immunocompetent patients, including the viral factors, such as HCV genotype, pretreatment viral load, amino acid (aa) 70 and/or 91 in the HCV core protein, amino acid substitutions in the HCV NS5A region [15–17]. In their multivariate analyses of predictors of SVR, Akuta and colleagues identified substitutions of aa 70 and 91 in the HCV core region (double-wild-type; odds ratio 5.988) as predictive [18], whereas Enomoto identified substitutions of amino acids of the HCV NS5A region (mutant type; odds ratio 5.3) as predictive [15].

With regard to length of treatment, one study reported that an early viral response at 3 months was useful in predicting a lack of response to antiviral therapy in liver transplant recipients with recurrent hepatitis C [19,20]. To our knowledge, however, no study has analyzed viral factors in extended treatment for recurrent hepatitis C infection after liver transplantation.

The aim of the present study was to evaluate the therapeutic efficacy of peginterferon in combination with ribavirin (PEGIFN/RBV) on long-term treatment for recurrent hepatitis C after LDLT, and predictive factors of virological response to this treatment, particularly viral factors. This study is first report of predictive factors associated with virological response in recurrent hepatitis C patients after LDLT, including amino acid substitutions in the core region and NS5A region.

Material and methods

Patients

A total of 53 patients who underwent LDLT due to HCV-related end-stage liver disease from 2000 to January 2009 were enrolled for this retrospective study. Among them, 14 patients died before the start of therapy, 1 refused treatment with antiviral therapy, and 1 did not become positive for HCV RNA after LDLT. Eventually, leaving 37 patients treated with PEGIFN/RBV in our institution. Of these, 10 patients are currently continuing antiviral therapy.

We introduced all patients to IFN therapy in principle. The efficacy of IFN therapy could thus be established in 27 patients (Figure 1).

Antiviral treatment protocol

Patients received 1.5 μg/kg body weight (BW) PEGIFN (Peg-Intron; Schering-Plough, Segrate, Italy) subcutaneously (s.c.) once weekly and 200 mg RBV (Rebetol; Schering-Plough). PEGIFN/RBV was continued for more than 1 year after serum HCV RNA becomes negative. At the end of active treatment, the patients were followed for further 24 weeks without treatment.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the local Ethics Committees of all participating centers. Written informed consent was obtained from all participating patients.

Safety assessments

Safety was assessed by clinical and laboratory testing, and by evaluating all adverse events reported at each visit. In accordance with the protocol, growth factors were recommended to encourage optimum patient compliance in relation to predictable hematological side effects such as anemia.

Erythropoietin (EPO; Epogin, Chugai) from 6000 IU/week was used to treat anemia (Hb levels <10 g/dl). RBV was administrated from 200 mg. When Hb increased by more than 10 g/dl, 200 mg per day of RBV was added. The daily dose of RBV was reduced by 200 mg when Hb fell below 10 g/dl, an acute decrease was followed by stabilization of Hb concentration at more than 3 g/dl from baseline, or the appearance of clinical symptoms of anemia (e.g. palpitation, dyspnea on effort, and fatigue) associated with a decrease in Hb of >2 g/dl from baseline. Once the RBV dose was reduced, it was maintained at that level throughout the rest of study if patients complained of anemia-related symptoms of fatigue or pallor. However, RBV was discontinued when Hb fell below 8.5 g/dl or when patients manifested more severe anemia, including orthostatic hypotension. PEGIFN was stopped if significant side effects occurred or if cytopenia persisted (neutrophil count <750/mm^3, platelet count <20,000/mm^3).

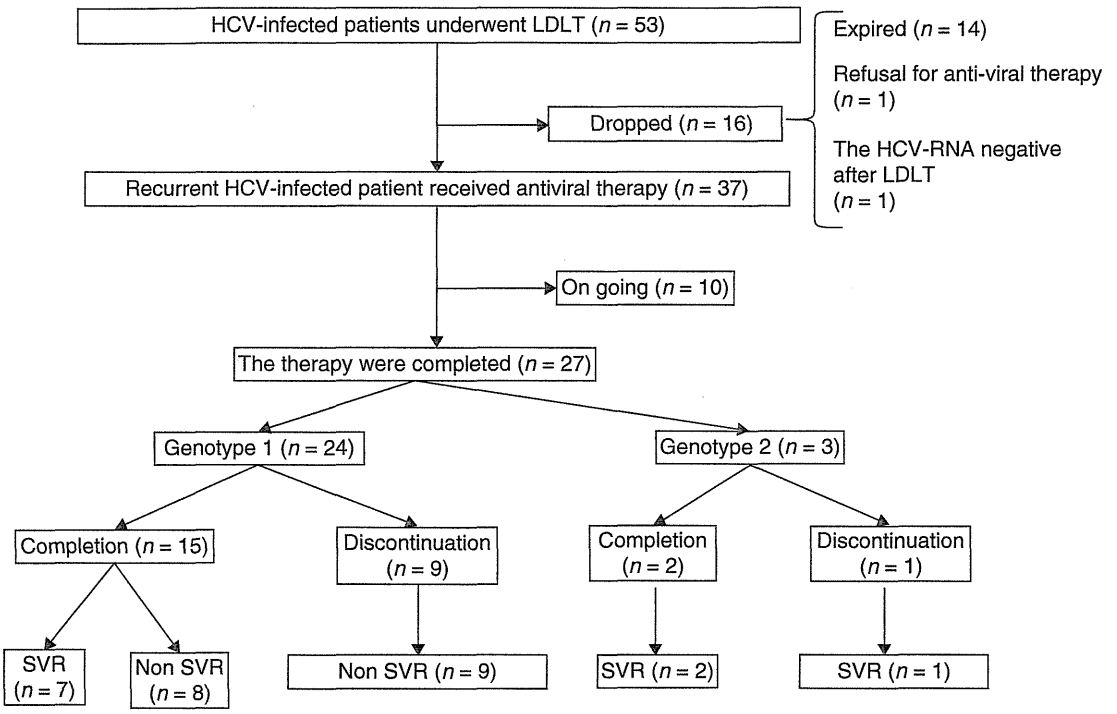

Figure 1. Flow diagram showing the course of HCV-infected patients after living donor liver transplantation. Twenty-seven patients treated with PEGIFN/RBV combination therapy were divided into two groups, namely sustained virological response (SVR) and non-SVR. *n*, number of patients. HCV genotype is shown.

Assessment of efficacy

HCV RNA levels were measured using one of several RT-PCR-based methods (original Amplicor method, high range method, or TaqMan RT-PCR test) at weeks 2 and 4, every 4 weeks of treatment thereafter, and at 24 weeks after the cessation of therapy.

The response was considered to be a SVR after another 6 months of negative serologic results without antiviral treatment. Patients with positive qualitative HCV RNA PCR tests during all examinations were categorized as having a non-virological response. Virological response (VR) was defined as becoming PCR-negative at least once during treatment; early virological response (EVR) as HCV-RNA-positive at 4 weeks after the start of treatment and HCV-RNA-negative at 12 weeks; and late virological response (LVR) as HCV-RNA-negative at more than 13 weeks after the start of treatment.

Analysis of nucleotide sequence of the core and NS5A region

Fifteen patients with genotype 1b completed our protocol. Seven patients achieved SVR whereas eight did not. Using serum obtained before LDLT, we analyzed amino acid (aa) substitutions at aa 70 and aa 91 of the HCV core region (HCV CR) and mutation at the interferon sensitivity-determining region (ISDR) in the nonstructural 5A (NS5A) region of HCV by the direct sequencing method. The core aa 61–110 and NS5A aa 2209–2248 (IFN-sensitive determining region [ISDR]) [15] sequences were determined by direct sequencing using stored serum samples obtained just before therapy. HCV RNA was extracted from serum samples and reverse transcribed with random primers and MMLV reverse transcriptase (Takara Bio Inc., Shiga, Japan). DNA fragments were amplified by PCR using the primers below. Nucleotide sequences of the core region: first-round PCR was performed with primers CC11 (forward, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (reverse, 5′-GGA GCA GTC CTT CGT GAC ATG-3′), and second-round PCR with primers CC9 (forward, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (reverse), as described by Akuta et al. [16,18,21]. After denaturation at 95°C for 5 min, 35 cycles of amplification were set as follows: denaturation for 30 s at 94°C, annealing of primers for 1.5 min at 57°C, and extension for 1 min at 72°C, followed by final extension at 72°C for 7 min. The second PCR was carried out with the same amplification conditions as those used in the first PCR, except that the second PCR primers were used instead of the first PCR primers. Nucleotide sequences of ISDR in NS5A: PCR was performed

with IM11 (forward, 5′-TTC CAC TAC GTG ACG GGC AT-3′) and 5OA2KI (reverse, 5′-CCC GTC CAT GTG TAG GAC AT-3′). After denaturation at 98°C for 30 s, 35 cycles of amplification were set as follows: denaturation for 10 s at 98°C, annealing of primers for 30 s at 66°C, and extension for 15 s at 72°C, followed by final extension at 72°C for 5 min. The amplified PCR products were separated on a 2% agarose gel and purified by GENECLEAN II kit (Q-Bio Gene, Carlsbad, CA). Nucleotide sequences were determined using Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan). Nucleotide and aa sequences were compared with the nucleotide sequences of genotype 1b HCV-J (Gene Bank accession number; D90208) [22].

Statistical analysis

Variables between the SVR and non-SVR groups were compared using non-parametric tests (Mann–Whitney *U* test, two-tailed test and Fisher's exact probability test). Analyses for efficacy and safety were conducted on an intention-to-treat (ITT) basis, performed on patients who received at least one dose of the study medication.

Predictors of SVR were determined using univariate analyses. All p values <0.05 by two-tailed tests were considered significant. Potential predictive factors associated with SVR included sex, age, body mass index (BMI), viremia level, number of mutations in the ISDR, HCV core region (double mutant/non-double mutant), time from transplantation to therapy, duration of treatment, adherence to PEGIFN treatment, and adherence to RBV and EVR treatment. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL).

Results

Patients characteristics

Table I shows the baseline characteristics of the 27 patients with recurrent hepatitis C after LT who were treated with PEGIFN/RBV combination therapy. The median age of patients was 56 years, and 17 were male. Median body mass index was 24.3. Most patients were infected with HCV genotype 1 ($n = 24$) and genotype 2 ($n = 3$). Median time for the initiation of antiviral therapy after transplantation was 4 months, and median pretreatment serum HCV RNA levels were 6.6 log IU/ml. Immunosuppressive therapy included tacrolimus in 22 of 27 patients, and cyclosporine in 5 of 27.

Table I. Characteristics of 27 patients with recurrent hepatitis C after living donor liver transplantation.

Age (years)*	56 (29–69)
Gender (male/female)	17/10
Body mass index*	24.3 (14.8–42.2)
Genotype (1/2)	24/3
Viral load at therapy (log IU/ml)*	6.6 (4.9–7.8)
Time from transplantation to therapy (months)*	4 (1–41)
Immunosuppression (tacrolimus/cyclosporine)	22/5

*Values are median (range).

Efficacy and safety assessment

Among 27 patients who were treated with antiviral therapy, 17 were able to complete our protocol (15 patients with genotype 1, 2 patients with genotype 2), whereas 10 patients had to discontinue the protocol (9 patients with genotype 1, 1 patient with genotype 2). SVR rate with PEGIFN/RBV was 37.0% (10/27). By genotype, SVR rate in patients with genotype 1 was 29.2% (7/24) and 100% (3/3) in those with genotype 2 (Figure 1). Most patients with genotype 1b whose HCV RNA reached undetectable level achieved SVR, at 87.5% (7/8), with only one patient not achieving SVR (Table II) (Figure 2).

Ten patients discontinued treatment, due to liver failure owing to the recurrence of HCV in 5 patients, general fatigue in 2, ALT flare due to acute rejection in 1 patient, anemia in 1, and depression in 1 (Figure 1).

Efficacy of long-term interferon therapy for genotype 1b patients

Table II shows details of patients who were treated with PEGIFN/RBV until HCVRNA had reached undetectable levels and were then further treated for at least more than 1 year.

Seven patients achieved SVR by prolonged PEGIFN/RBV for at least 1 year or more. Seven patients were male.

Eight patients had reached undetectable levels of HCV RNA and 7 patients had never reached undetectable levels of HCV RNA. Although 5 of the 8 patients were classified as LVR, 4 patients of these 5 achieved SVR. One male patient aged 69 years (patient no. 5) who had double mutation of aa 70 and aa 91 in the core region and zero substitutions in ISDR achieved SVR after prolongation of therapy (Figure 3). By contrast, another male aged 51 years (patient no. 9) who had double wild aa 70 and aa 91 in the core region and five substitutions in the ISDR did not achieve SVR after prolongation of therapy (Figure 4).

Table II. Details of 15 patients (genotype 1).

Patient no.	Age (years)	Gender	HCV RNA (log IU/ml)	HCV core region (aa 70/aa 91)	Number of mutations in the ISDR	Time from transplantation to therapy (months)	Time to reach undetectable levels of HCV RNA (weeks)	Treatment duration of VR (weeks)	Treatment duration (weeks)	Adherence to PEGIFN (%)	Adherence to RBV (%)	SVR
1	63	Male	6.1	m/m	4	41	3	103	106	35	29	Yes
2	60	Male	5.8	w/w	1	13	10	48	58	100	62	Yes
3	66	Male	6.6	m/m	3	12	12	60	72	80	11	Yes
4	54	Male	6.5	w/w	3	4	16	56	72	77	54	Yes
5	69	Male	6.3	m/m	0	12	21	57	78	42	14	Yes
6	44	Male	6.6	w/m	0	1	28	76	104	88	47	Yes
7	53	Male	6.1	m/w	1	2	54	125	179	57	25	Yes
8	56	Male	6.6	m/w	0	2	27	52	79	66	7	No
9	51	Male	5.9	w/w	5	6	NR	NR	173	80	25	No
10	47	Female	6.6	m/m	1	3	NR	NR	124	63	15	No
11	59	Female	6.6	m/w	1	7	NR	NR	86	100	65	No
12	64	Female	5	m/m	0	3	NR	NR	81	72	25	No
13	58	Female	6.6	m/m	1	3	NR	NR	79	83	63	No
14	65	Female	5.9	w/m	0	3	NR	NR	79	45	19	No
15	56	Male	7.2	m/w	0	3	NR	NR	58	51	26	No

Abbreviations: m = mutant; w = wild; NR = non-virological responder; VR = virological response; SVR = sustained virological response.

Predictive factors of SVR in genotype 1b patients

Among 15 patients who completed our protocol with genotype 1b, Potential predictive factors associated with SVR were analyzed. Variables were follow up, the age, gender, body mass index, duration for the initiation of antiviral therapy after transplantation, pretreatment serum HCV RNA levels, immunosuppressive therapy, the number of mutations in the ISDR, HCV core region (double mutant/non-double mutant) adherence of PEGIFN and adherence of RBV.

There was no significance difference between the SVR and non-SVR groups among the 15 patients with genotype 1b in our study (Table III). EVR rates in the SVR group tend to be higher than that of the non-SVR group, albeit that the difference was not significant ($p = 0.07$) (Table III). Mutation of aa 70 and aa 91 in the core region of the HCV protein and fewer mutations in its ISDR region did not significantly differ between the SVR and non-SVR groups among the 15 patients with genotype 1b in our study.

Although it has been reported that mutation of aa 70 and aa 91 in the core region of the HCV protein is predictive of a non-virological response [17,18], all three patients who had double mutation of aa 70 and aa 91 in the core region achieved SVR in this study.

Moreover, although it has also been reported that fewer mutations in the ISDR region of the HCV protein is predictive of a non-virological response [15], all four patients with 0 or 1 mutation in the ISDR achieved SVR.

Discussion

The optimal duration of therapy for liver transplant recipients with recurrent HCV is unclear. The treatment period for immunocompetent patients in the majority of published studies is 48 weeks. Among immunocompetent patients, the probability of relapse was greater in those responding later [23,24]. Using a mathematical model, Drusano and Preston reported that genotype 1-infected patients require the continuous absence of detectable HCV RNA in serum for 36 weeks to attain 90% probabilities of an SVR (i.e. relapse rate 10%) [25]. It is recently recommended that 72-week IFN treatment, compared to 48-week standard IFN treatment, was effective for the untransplanted patients with chronic hepatitis C whose HCV RNA does not reach undetectable level within 12 weeks [11,12]. It is also well known that patients with recurrent chronic hepatitis after LDLT are unlikely to achieve SVR, compared to immunocompetent

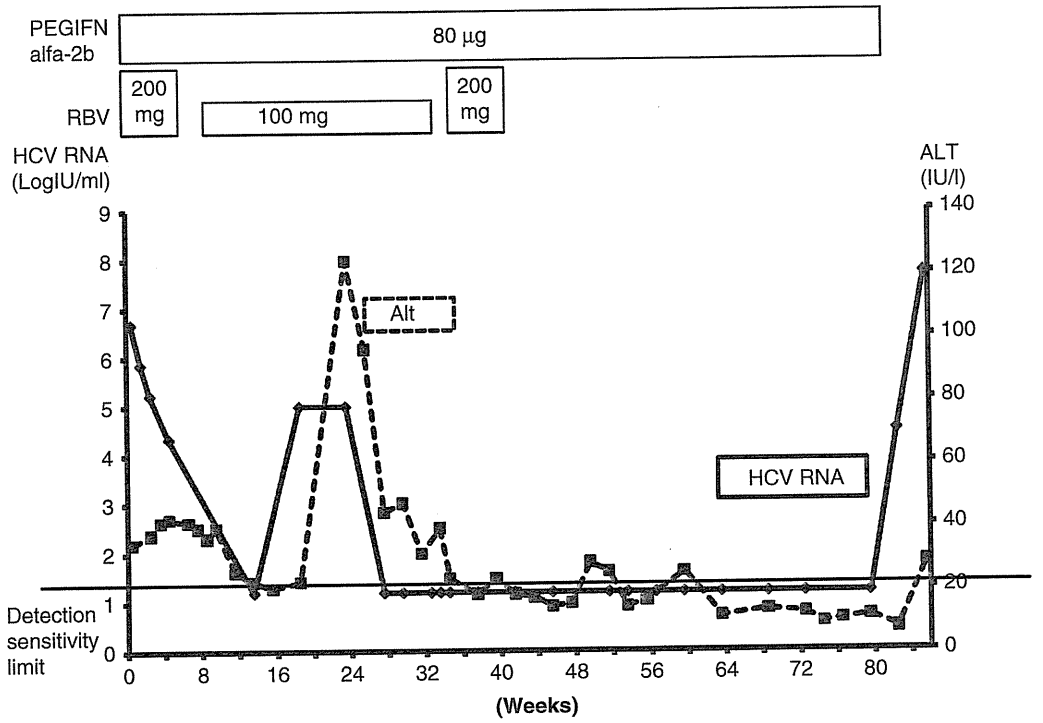

Figure 2. Clinical course in a male patient aged 56 years with genotype 1b, HCV Core 70mutant 91wild, and 0 ISDR mutations. Serum HCV RNA became negative at 27 weeks, after which treatment duration was 52 weeks. However, HCV RNA became positive at 1 week after the cessation of treatment.

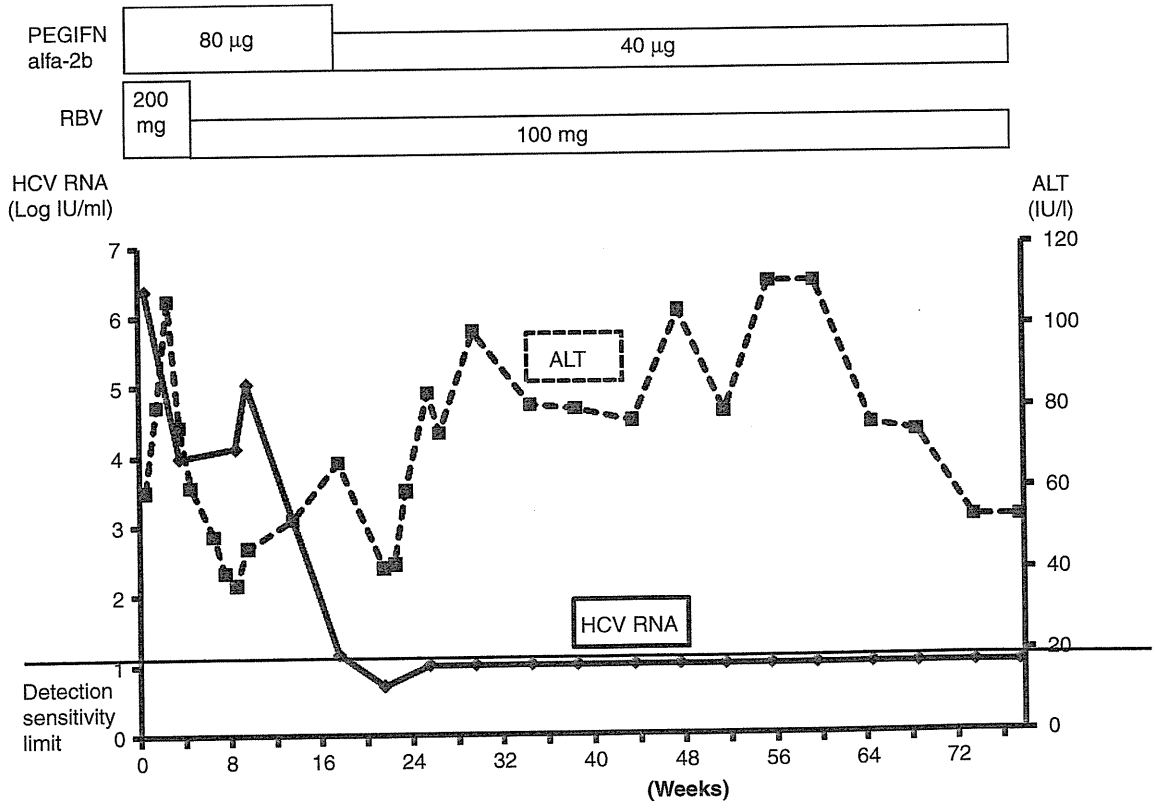

Figure 3. Clinical course in a male patient aged 69 years with genotype 1b, HCV Core 70mutant 91mutant, and 0 ISDR mutations. Virological response occurred at 21 weeks and therapy continued to 78 weeks. Final status was SVR.

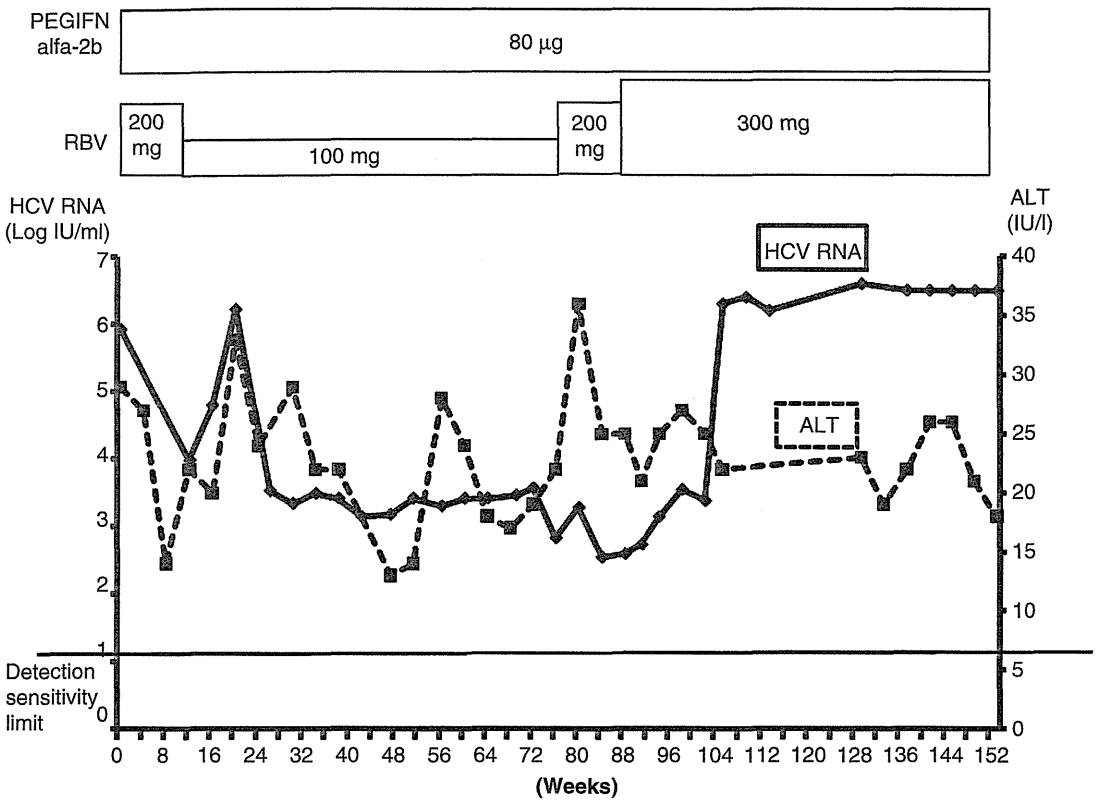

Figure 4. Clinical course in a male patient aged 51 years with genotype 1b, HCV Core 70wild 91wild, and 5 ISDR mutations. He did not develop VR during the dosing period.

untransplanted patients. Consequently, prolonged IFN treatment would be useful and improve the SVR rate for transplanted patients.

We treated recipients with PEGIFN/RBV until HCV RNA had reached undetectable levels and then to continue treatment for at least 1 year. 62.9% of patients (17/27) could complete therapy without severe adverse affects (Figure 1) and relapse rate under this study was 12.5% (Table II), whereas Tamura and Ueda reported a relapse rate of 14% and 3% under the same treatment. As a result, SVR rate was 34% and 50%, respectively [13,14]. With the aid of these results, it might indicate that prolonged PEGIFN/RBV therapy would be useful in eradicating HCV in LDLT patients.

In our study, seven patients achieved SVR by prolonged PEGIFN/RBV for at least 1 year or more. Three of seven patients were EVR and four were LVR.

By contrast, in eight patients who were non-SVR, only one patient had reached undetectable levels of HCV

Table III. Predictive factors associated with SVR in genotype 1b patients.

	SVR (n = 7)	Non-SVR (n = 8)	p-Value
Age (years)*	60 (44–69)	57 (47–65)	0.64
Gender (male/female)	7/0	3/5	0.07
Body mass index*	24.1 (21.4–26.5)	24.2 (18.9–42.2)	0.67
Viral load at therapy (log IU/ml)*	6.3 (5.8–6.6)	6.6 (5.9–7.2)	0.48
Time from transplantation to therapy (months)*	12 (1–41)	3 (3–7)	0.21
Number of mutations in the ISDR (0–1/2–5)	4/3	7/1	0.28
HCV core region (double mutant/non-double mutant)	3/4	3/5	0.6
Duration of treatment (week)*	72 (48–179)	75 (61–133)	0.7
Immunosuppression (tacrolimus/cyclosporine)	6/2	7/1	1
Adherence of PEGIFN (%)*	80 (35.5–100)	71.5 (45.4–100)	0.39
Adherence of RBV (%)*	47.4 (11.2–62.5)	25.5 (15.3–65.9)	0.74
Early virological response (yes/no)	3/4	0/8	0.07

*Values are median (range).

RNA and other seven patients had never reached undetectable levels of HCV RNA. That is, if patients had reached undetectable levels of HCV RNA, they could eradicate HCV RNA in the liver tissue by prolonged IFN therapy for more than 48 weeks after HCV RNA reached undetectable levels. This regimen is similar to that of a recent recommendation that PEGIFN/RBV therapy for 72 weeks is necessary for patients with chronic hepatitis C whose HCV RNA does not reach undetectable levels within 12 weeks.

Recent findings among immunocompetent patients of pretreatment factors that could predict treatment efficacy of 72-week PEGIFN/RBV identified substitution of either or both aa 70 or 91 in the HCV core region, and the number of substitutions in amino acids 2209–2248, the ISDR of NS5A in HCV genotype 1b [26]. By contrast, however, our present results showed that substitution of aa 70 and/or 91 in the HCV core region or the number of ISDR were not predictive of SVR (Table III). All three patients who had double mutation of aa 70 and aa 91 in core region of HCV protein achieved SVR in this study, as did all four patients whose number of mutations in the ISDR was 0 or 1 (Table II).

Recently Fukuhara et al. reported that mutations of the HCV core and NS5A regions of HCV genome were associated with the SVR rates in 50 patients [27]. Although the number of our patients included was less than Fukuhara's, we think that our result is still worth reporting because, in the case of acute hepatitis C, 24-week IFN treatment is enough to eradicate HCV in most cases, suggesting that HCV core mutant and the substitutions of amino acids of the HCV NS5A region are not likely to affect the SVR rate for acute hepatitis C. Since the recurrence of hepatitis C for transplanted patients is another acute hepatitis C, those substitutions might not affect the SVR rate of IFN treatment. Further studies would reveal whether the mutations of the HCV core and NS5A regions of HCV genome were associated with the SVR rates.

Only one patient had HCV relapse after 79 weeks treatment, a male aged 56 years with genotype 1b, HCV Core 70mutant 91wild and number of ISDR mutation 0 (Figure 2). He had a VR at 27 weeks, which lasted for 52 weeks, and continued therapy to 79 weeks. However, he subsequently experienced relapse of HCV. One of many possible reasons was likely low adherence to RBV (7%).

Several reasons may account for the lack of association of HCV core region mutation and number of ISDR mutations with SVR rate. One reason is that it is acute hepatitis after LDLT, which is usually treated as soon as possible: even in those infected with genotype 1, HCV could be eradicated with regular IFN for 24 weeks after acute infection [28–31], meaning that mutation of the core region and NS5A could not be determinants of PEGIFN therapy in LDLT cases. A second reason might be poor adherence to PEGIFN and RBV treatment in patients with LDLT. Among patients who experience severe leucocytopenia, thrombocytopenia and anemia after LDLT, dose reductions in PEGIFN or RBV are therefore inevitable. Therefore, it is reasonable to prolong the duration of PEGIFN/RBV therapy. Taken together, recurrent hepatitis C after LDLT is different from hepatitis C in immunocompetent patients. This might be the reason why any predictive factor but EVR was an only predictive factor of SVR in this study.

There were several limitations in present study. One was that our study is retrospective.

Since it was scheduled that the end point of treatment should be 1 year after serum HCV RNA became negative, it compelled to design the retrospective study as a pilot study. Further prospective study will prove our protocol strongly and help achieving high SVR and low relapse rate. Another limitation was the low number of patients included. Although other institutes also demonstrated good results with similar interferon protocol, as mentioned above [13,14], another study with more number of patients will prove the consistence of our study.

In conclusion, for recurrent hepatitis C after LDLT, our findings indicate that PEGIFN therapy for at least 1 year after HCV RNA reaches undetectable levels might prevent HCV viral relapse. Combination of the new selective inhibitors of HCV, named STAT-C (specifically targeted antiviral therapy for HCV), is expected to further improvements in SVR rates.

Acknowledgements

The authors thank Rie Akiyama and Hiromi Abe for their excellent technical assistance.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Samuel D, Muller R, Alexander G, Fassati L, Ducot B, Benhamou JP, et al. Liver transplantation in European patients with the hepatitis B surface antigen. N Engl J Med 1993;329:1842–7.

[2] Forman LM, Lewis JD, Berlin JA, Feldman HI, Lucey MR. The association between hepatitis C infection and survival after orthotopic liver transplantation. Gastroenterology 2002; 122:889–96.

[3] Ueda Y, Takada Y, Haga H, Nabeshima M, Marusawa H, Ito T, et al. Limited benefit of biochemical response to combination therapy for patients with recurrent hepatitis C