blood flow in the left gastric vein increased in direct correlation with enlargement of the size of the varices. These findings suggest a relationship between the size of gastrointestinal vessels and portal blood volume and/or portal vein pressure. In fact, the diameter of inflow vessels improved in our study in 17 of 35 (49%) patients, did not change in 13 of 35 (37%) patients, and deteriorated in five of 35 patients (14%) (Table 2). We classified the cases into two groups according to the interval of endoscopic examination (≤1 year and >1 year) and evaluated the relationship between LDLT and EV recurrence. In the >1 year group, there were significant differences in the rate of reduction of major inflow vessel diameters, Child-Pugh score, and PV thrombosis between patients who showed improvement of their varices after LDLT and those who did not. Based on these results, we recommend checking EVs by endoscopy to predict any risk of bleeding associated with lack of improvement in the rate of reduction of inflow vessel diameter 1 year after LDLT.

Endoscopic examination showed improvement of EVs in 30 of 35 (86%) patients. On the other hand, narrowing of the inflow vessel to EV was seen in only 17 of 35 (49%) patients. There is a discrepancy between these two post-LDLT findings, which occurred because the blood pressure of EVs was more likely to decrease as a result of LDLT compared with that of the inflow vessels to EVs. Since EVs are located more distal to the portal vein compared with the inflow vessels to EVs, we think that EVs are more likely to narrow than inflow vessels as a result of the influence of decreased PV pressure and decreased blood flow.

The present study showed no improvement in EV after LDLT in five patients. Hirata et al.[19] reported that nine of 77 recipients developed gastrointestinal bleeding after transplantation, and variceal bleeding subsequently occurred in four patients with PV thrombosis. In our study, PV thrombosis was seen in one of five patients who did not show improvement in EVs after LDLT. In addition, Tabasco-Minguillán et al.[20] reported that the cumulative incidence of gastrointestinal bleeding was 8.9% and the patient and graft survival rates were significantly lower in a group with gastrointestinal bleeding group compared with the control group. In our study, a patient who showed recurrence of HCV infection, which subsequently led to liver cirrhosis (case 33), may be an example of this. We think that the complication of PV and graft failure led to recurrence of portal hypertension.

In conclusion, we demonstrated in the present study improvement of EVs after LDLT; EVs improved after LDLT in 86% of the patients, although 14% of the patients showed no improvement in EVs after LDLT. Measurement of RRGV with MDCT is a good tool for prediction of EV improvement after LDLT.

Acknowledgment. We thank M. Ishifuro for excellent assistance in the assessment of inflow vessel diameter by MDCT examination.

References

1. Moriwaki H, Miwa Y, Tajika M, Kato M, Fukushima H, Shiraki M. Branched-chain amino acids as a protein- and energy-source in liver cirrhosis. Biochem Biophys Res Commun 2004; 313:405–9.
2. Gundling F, Teich N, Strebel HM, Schepp W, Pehl C. Nutrition in liver cirrhosis. Mediz Klin 2007;102:435–44.
3. Grace ND. A hepatologist's view of variceal bleeding. Am J Surg 1900;162:26–31.
4. Escorsell A, Banares R, Garcia-Pagan JC, Gilabert R, Moitinho E, Piqueras B, et al. TIPS versus drug therapy in preventing variceal rebleeding in advanced cirrhosis: a randomized controlled trial. Hepatology 2002;35:385–92.
5. Brandenburger LA, Regenstein FG. Variceal hemorrhage. Current treatment options. Gastroenterology 2002;5:73–80.
6. Seewald S, Seitz U, Thonke F, Sriram PVJ, He XK, Soehendra N. Interventional endoscopic treatment of upper gastrointestinal bleeding: when, how, and how often. Langenbecks Arch Surg 2001;386:88–97.
7. Nevens F, Broeckaert L, Rutgeerts P, Van Steenbergen W, Fevery J. The long-term morbidity and mortality rate in a cohort of patients with liver cirrhosis and oesophageal varices. Hepatogastroenterology 1995;42:979–84.
8. McCormack G, McCormick PA. A practical guide to the management of esophageal varices. Drugs 1999;57:327–35
9. Iwatsuki S, Starzl TE, Todo S, Gordon RD, Tzakis AG, Marsh JW, et al. Liver transplantation in the treatment of bleeding esophageal varices. Surgery 1988;104:697–705.
10. Sudan DL, Shaw BW Jr. The role of liver transplantation in the management of portal hypertension. Clin Liver Dis 1997;1: 115–20.
11. Klupp J, Kohler S, Pascher A, Neuhaus P. Liver transplantation as ultimate tool to treat portal hypertension. Dig Dis 2005;23: 65–71
12. Reyes J, Iwatsuki S. Current management of portal hypertension with liver transplantation. Adv Surg 1992;25:189–208.
13. Henderson JM. Liver transplantation for portal hypertension. Gastroenterol Clin North Am 1992;21:197–213.
14. Idezuki Y; Japanese Research Society for Portal Hypertension. The general rules for recording endoscopic findings on esophagogastric varices: revised edition. Kanzou (Acta Hepatol Jpn) 1991;33:277–81.
15. Sugawara Y, Makuuchi M, Kaneko J Ohkubo T, Matsui Y, Imamura H, et al. Living-donor liver transplantation in adults: Tokyo University experience. A single institution's experience with 335 consecutive cases. J Hepatobiliary Pancreat Surg 2003;10: 1–4.
16. Liver Transplantation in Japan—Registry by the Japanese Liver Transplantation Society. Transplantation 2005;40:518–26.
17. Morioka D, Egawa H, Kasahara M. Outcomes of adult-to-adult living donor liver transplantation. Ann Surg 2007;245:315–25.
18. Matsutani S, Furuse J, Ishii H, Mizumoto H, Kimura K, Ohto M. Hemodynamics of the left gastric vein in portal hypertension. Gastroenterology 1993;105:513–8.
19. Hirata M, Kita Y, Harihra Y, Hisatomi S, Sano K, Mizuta K, et al. Gastrointestinal bleeding after living-related liver transplantation. Dig Dis Sci 2002;47:2386–8.
20. Tabasco-Minguillán J, Jain A, Naik M, Weber KM, Irish W, Fung JJ, et al. Gastrointestinal bleeding after liver transplantation. Transplantation 1997;63:60–7.

doi:10.1111/j.1440-1746.2008.05650.x

HEPATOLOGY

Dose comparison study of pegylated interferon-α-2b plus ribavirin in naïve Japanese patients with hepatitis C virus genotype 2: A randomized clinical trial

Tomokazu Kawaoka,* Yoshiiku Kawakami,* Keiji Tsuji,† Hiroyuki Ito,‡ Mikiya Kitamoto,§ Shiomi Aimitsu,¶ Hiroiku Kawakami,** Soo Cheol Jeong,* Michio Imamura,* Hiroshi Aikata,* Shoichi Takahashi* and Kazuaki Chayama*

*Department of Medicine and Molecular Science, Hiroshima University, †Department of Internal Medicine, Hiroshima City Asa Hospital, ‡Department of Internal Medicine, Saiseikai Kure Hospital, §Department of Internal Medicine, Prefectural Hiroshima Hospital, ¶Second Department of Internal Medicine, Hiroshima Red Cross and Atomic Bomb Survivors' Hospital, and **Kawakami Clinic, Hiroshima, Japan

Key words
hepatitis C virus genotype 2, low-dose pegylated interferon, ribavirin, side-effect, sustained virological response.

Accepted for publication 4 August 2008.

Correspondence
Dr Yoshiiku Kawakami, Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Email: kamy4419@hiroshima-u.ac.jp

Abstract

Background and Aim: To compare the efficacy and safety of pegylated interferon (PEG-I) at 1 and 1.5 μg/kg, and in combination with ribavirin (RBV) for 24 weeks in naïve Japanese patients infected with hepatitis C virus genotype 2.

Methods: The present study was an open-label, randomized trial of 55 patients receiving PEG-I (1 or 1.5 μg/kg body weight [BW], subcutaneously, once a week) and RBV for 24 weeks. The patients were followed up for 24 weeks without treatment.

Results: The intention-to-treat analyses showed that the proportion of patients with a sustained virological response (SVR) in the 1-μg/kg PEG-I–RBV group (38.5%, 10/26) was lower than that of the 1.5-μg/kg PEG-I–RBV group (74.1%, 20/27; $P = 0.013$). The PEG-I dose was reduced in two of the 26 patients of the 1-μg/kg PEG-I–RBV group (one because of thrombocytopenia at 2 weeks, and one because of generalized fatigue at 20 weeks), and four of the 27 patients of the 1.5-μg/kg PEG-I–RBV group (one because of neutropenia at 20 weeks, and three because of generalized fatigue at 1, 5, and 8 weeks). The multivariate analysis identified age (< 60 years) and dose of PEG-I (1.5 μg/kg) as significant determinants of SVR.

Conclusion: The dose of PEG-I to be used at the start of therapy should be 1.5-μg/kg BW in naïve Japanese patients infected with hepatitis C virus genotype 2.

Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, with an estimated 170 million chronic carriers worldwide.[1] Chronic HCV infection is causally associated with liver cirrhosis (LC) and hepatocellular carcinoma (HCC).[2–6] In Japan, 60–70% of patients with HCC or LC are HCV carriers.[7]

Pegylated interferon (PEG-I)-α plus ribavirin (RBV), the current standard treatment for chronic HCV infection, can increase the sustained virological response (SVR) rate.[8–15] In this regard, a small scale, non-randomized study by the Hepatitis C Intervention Therapy Group on the use of PEG-I-α2b plus RBV reported that the SVR rate of patients infected with HCV genotype 2 treated for 24 weeks at a dose of 1.4 μg/kg once per week (83%) was equivalent to that of patients (100%) treated with 0.7 μg/kg once per week.[16] Moreover, Lindsay *et al.*[12] reported that the SVR rate of patients infected with HCV genotype 2 on 48-week PEG-I-α2b monotherapy (dose: 1.5 μg/kg once per week, 41%) was similar to that of patients (42%) treated with 1 μg/kg once per week. Meyer-Wyss *et al.*[17] reported that SVR rates in patients infected with HCV genotype 2 or 3 were similar to those if patients treated with 1 or 1.5 μg/kg PEG-I body weight [BW], that is, SVR rates were achieved in 39 of 55 (71%) and 29 of 36 (81%) patients, respectively (P = ns). Mangia *et al.*[18] reported that the SVR rate of patients infected with HCV genotypes treated with PEG-I at 1 μg/kg BW was 80%.

However, there are no reports on whether 1 and 1.5 μg/kg doses of PEG-I plus RBV for 24 weeks have similar efficacies and safety in Japanese patients infected with HCV genotype 2. It is important to study the response to such low-dose interferon because some Japanese patients who receive treatment are older than 60 years. In addition, it is possible that the SVR rate to interferon is better in patients infected with HCV genotype 2.

The aim of the present study was to determine whether 1 and 1.5 μg/kg doses of PEG-I plus RBV for 24 weeks have similar efficacies and safety in naïve Japanese patients infected with HCV genotype 2. For this purpose, we conducted a randomized clinical trial to evaluate the efficacy and safety of 1 μg/kg versus 1.5 μg/kg

PEG-I combined with RBV for 24 weeks in naïve-infected patients with HCV genotype 2.

Methods

Patients and study design

This study was an open-label, randomized clinical trial conducted in six centers across Japan. Enrolment spanned from February 2006 to October 2007. The inclusion criteria were male and female patients with chronic hepatitis C who were than 20 years. Naïve cases were infected with HCV genotype 2.

The exclusion criteria were as follows: (i) patients treated with Shosaiko-to, a Japanese herbal medicine considered to improve liver function; (ii) patients with autoimmune hepatitis; (iii) patients with a history of hypersensitivity to PEG-I-α-2a or other interferons; (iv) patients with a history of hypersensitivity to biological products, such as vaccines; (v) patients with decompensated liver cirrhosis (LC); (vi) patients with hepatocellular carcinoma (HCC) or malignant tumors in other tissues; (vii) patients with or without a history of severe psychosis, such as being severely depressed and/or suicidal; (viii) women who were pregnant or lactating or who were suspected of being pregnant; and (ix) patients judged by the investigator not to be appropriate for inclusion in this study.

The patients were randomly allocated (1:1, groups of four, central randomization) to one of the following two parallel treatment groups: the 1-μg/kg PEG-I–RBV group and the 1.5-μg/kg PEG-I–RBV group. The patients of the former group received 1 μg/kg BW PEG-I subcutaneously once a week. The RBV dose was adjusted according to BW: 600 mg for ≤ 60 kg BW, 800 mg for > 60 kg BW, but ≤ 80 kg BW and 1000 mg for > 80 kg BW, based on the drug information for RBV supplied by the manufacturer. These durations and dosages are those approved by the Japanese Ministry of Health, Labor and Welfare.

A lower dose of RBV was selected by the Japanese Ministry of Health, Labor and Welfare. Patients of the latter group were treated with 1.5 μg/kg BW PEG-I subcutaneously once a week. The RBV dose was also adjusted according to BW as described earlier. The daily dose of RBV was reduced by 200 mg when hemoglobin (Hb) fell below 10 g/dL, there was an acute decrease followed by the stabilization of Hb concentrations at more than 3 g/dL from baseline, or the appearance of clinical symptoms of anemia (e.g. palpitation, dyspnea on efforts, 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 when 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. After the end of the 24-week active treatment, the patients were followed up for a 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 of the participating centers. Written informed consent was obtained from all participating patients.

Data collection

Visits were scheduled at baseline, after 1, 2, 3, 4, and 8 weeks of treatment, at 4-week intervals until the end of treatment, and finally, 4 and 24 weeks after the completion of treatment. At each visit, blood samples were analyzed for hematology and blood chemistry at the local hospital laboratory using standard methodology. Serum HCV-RNA was determined at baseline, after 4, 8, 12, 16, and 20 weeks of treatment, at the end of treatment, and at the end of the 24-week, drug-free follow-up period. HCV-RNA was centrally assessed by qualitative reverse transcription–polymerase chain reaction. The histopathological stage was conducted before treatment and determined based on the histological scoring system of Desmet *et al.*[19]

At each visit, information on possible side-effects was obtained by questioning the patients in a structured manner about specific, commonly observed, and expected side-effects of the study medication, such as flu-like symptoms, fatigue, nausea, vomiting, diarrhea, dizziness, depression, and hair loss.

Data management and statistical analysis

The primary objectives of the study were to show the efficacy and safety of PEG-I at 1 μg/kg versus 1.5 μg/kg. The primary study end-point was SVR, defined as HCV-RNA below the detection limit at the end of the follow-up period, that is, 24 weeks after the completion of treatment. The secondary end-points were initial and end-of-treatment virological responses at weeks 4 and 24, and virological breakthrough and relapse, that is, the reappearance of HCV-RNA during therapy and follow up, respectively. Safety and tolerability, as reflected by clinical and laboratory side-effects, were analyzed descriptively.

Non-parametric tests were used to compare variables between groups (Mann–Whitney *U*-test, two-tailed test, and Fisher's exact probability test). Missing HCV-RNA values were treated on a worst-case basis, that is, they were treated as if they would have remained above the detection limit. Thus, patients with missing values and those who abandoned the study prematurely were classified as treatment failures at the time points following withdrawal, regardless of the reason for discontinuation. The intention-to-treat (ITT) analyses for efficacy and safety were performed based on the patients who received at least one dose of the study medication.

Univariate and multivariate logistic regression analyses were used to determine the predictors of SVR. We also calculated the odds ratios and 95% confidence intervals (95%CI). All *P*-values less than 0.05 by two-tailed tests were considered significant. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) upon the univariate analysis were entered into the multiple logistic regression analysis to identify significant independent factors. Potential predictive factors associated with SVR included the following variables: sex, age, body mass index, genotype (2a or 2b), aspartate aminotransferase, alanine aminotransferase, platelet count, serum iron, serum ferritin, hyaluronic acid, viremia level, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol (LDL-C), pathological staging, dose of PEG-I, RBV dose/BW, > 80% of RBV total dose, and reaching undetectable levels by week 4. Statistical analyses were performed using SPSS software (SPSS, Chicago, IL, USA).

Results

A total of 55 patients were enrolled. Of these, two patients were excluded from randomization because of decompensated cirrhosis.

A total of 53 patients were randomized. Thus, 53 patients ($n = 26$, for the 1-μg/kg PEG-I–RBV group, and $n = 27$ for the 1.5-μg/kg PEG-I–RBV group) received at least one course of treatment (Fig. 1). Table 1 summarizes the baseline characteristics of the 53 patients. These were similar in the two treatment groups; the majority of patients were males, with a median age of ≥ 50 years and median BW of > 50 kg.

Efficacy

ITT analysis

The proportion of patients in the 1-μg/kg PEG-I–RBV group who exhibited a rapid decrease in HCV-RNA to undetectable levels (HCV-RNA ≤ 100 copies/mL) by week 4 (57.7%, 15/26) was not

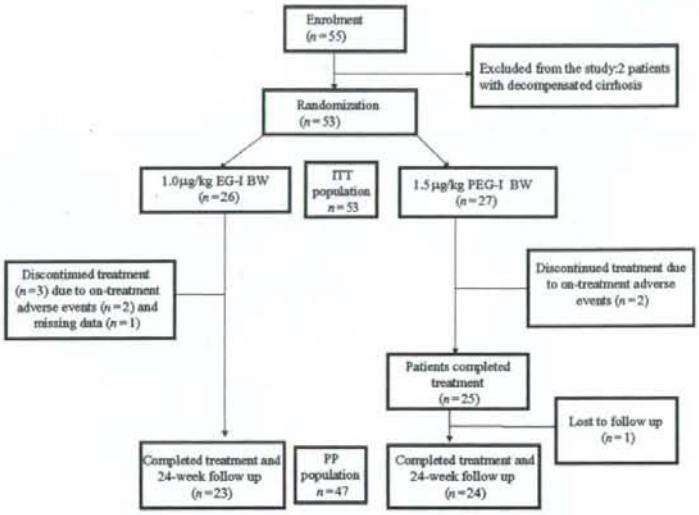

Figure 1 Flow diagram showing the number of patients who enrolled in the study and those who withdrew from the study. BW, body weight; ITT, intention to treat; PP, per protocol.

Table 1 Baseline characteristics

	1 μg/kg pegylated interferon + ribavirin ($n = 26$)	1.5 μg/kg pegylated interferon + ribavirin ($n = 27$)	*P*-value
Age (years)*	57 (31–77)	55 (41–75)	NS
Sex (male/female)	9/17	15/12	NS
Body weight (kg)*	53 (40–85)	61 (38–83)	NS
Body mass index (kg/m²)*	22.1 (16.9–34.0)	23.9 (16.1–28.9)	NS
Genotype (2a/2b)	13/13	13/14	NS
White blood cell (×10³/μL)*	5.8 (4.0–7.2)	5.2 (3.9–6.4)	NS
Neutrophil cell (×10³/μL)*	3.0 (2.0–4.0)	2.8 (1.8–3.5)	NS
Hemoglobin (g/dL)*	13.3 (9.8–16.4)	13.7 (11.3–17.6)	NS
Platelet count (×10⁴/mm³)*	20.9 (15.5–27.9)	20.1 (16.2–26.9)	NS
Serum aspartate aminotransferase (IU/L)*	32 (18–164)	37 (18–203)	NS
Alanine aminotransferase (IU/L)*	31 (16–164)	34 (17–180)	NS
Serum iron (μg/dL)*	129 (12–246)	107 (60–275)	NS
Serum ferritin (μg/dL)*	75.3 (4.9–389.3)	92 (4.9–671)	NS
Total cholesterol (mg/dL)*	198 (134–249)	175 (117–279)	NS
High-density lipoprotein cholesterol (mg/dL)*	55 (25–98)	55 (32–78)	NS
Low-density lipoprotein cholesterol (mg/dL)*	124 (63.8–176.4)	104.6 (44.2–188.2)	NS
Triglycerides (mg/dL)*	93 (12.8–210)	95 (46–210)	NS
Hyaluronic acid*	51 (9–411)	47 (10.3–411)	NS
Hepatitis C virus viremia (KIU/mL)*	1100 (200–> 5000)	1700 (300–> ,000)	NS
Histological stage† (F0/F1/F2/F3)	1/14/8/3	0/13/9/5	NS

*Values are median (range); †as assessed by the local pathologist. NS, not significant.

Table 2 Adherence to therapy

Treatment	1 μg/kg pegylated interferon + ribavirin ($n = 26$)	1.5 μg/kg pegylated interferon + ribavirin ($n = 27$)	*P*-value
> 80% of pegylated interferon dose/ < 80% of pegylated interferon dose†	22/1	24/1	NS
Premature withdrawal of pegylated interferon	3	2	NS
Ribavirin dose (mg/kg)*	11.5 (9.4–15.0)	11.6 (10.0–16.0)	NS
> 80% of ribavirin dose/ < 80% of ribavirin dose†	21/2	22/3	NS
Premature withdrawal of ribavirin	3	2	NS

*Values are median (range); †actual dose was > 80% of prescribed pegylated interferon and ribavirin dose. Patients who received full-length treatment, but required dose reductions (< 80% of the originally assigned dose). NS, not significant.

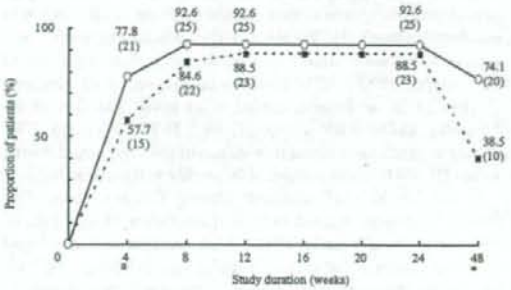

Figure 2 Results of the intention-to-treat analyses. Numbers are percentage and (number) of patients of each treatment group. Data represent the proportion of responders at the end of the indicated week of therapy (0–24 weeks) and those who achieved a sustained virological response (at 48 weeks). With regard to the virological response to combination therapy, patients of both groups exhibited a rapid decrease in HCV-RNA, reaching undetectable levels (HCV-RNA ≤ 100 copies/mL) each week. *$P = 0.13$; *$P = 0.013$. ●, 1 μg ($n = 26$); ◇, 1.5 μg ($n = 27$).

significantly different from that of the 1.5-μg/kg PEG-I–RBV group (77.8%, 21/27; $P = 0.13$). Further analysis showed that 10 of 15 (66.7%) patients of the 1-μg/kg PEG-I–RBV group who exhibited a rapid decrease in HCV-RNA to undetectable levels by week 4 achieved SVR. Twenty of 21 (95.2%) patients of the 1.5-μg/kg PEG-I–RBV who exhibited a rapid decrease in HCV-RNA to undetectable levels by week 4 achieved SVR (Fig. 2).

The proportion of end-of-therapy responders of the 1-μg/kg PEG-I–RBV group (88.5%, 23/26) was similar to that of the 1.5-μg/kg PEG-I–RBV group (92.6%, 25/27) (Fig. 2). The proportion of patients of the 1-μg/kg PEG-I–RBV group who showed SVR (38.5%, 10/26) was significantly lower than that of the 1.5-μg/kg PEG-I–RBV group (74.1%, 20/27; $P = 0.013$). Furthermore, the proportion of patients of the 1-μg/kg PEG-I–RBV group who developed viral relapse after the end of treatment (50%, 13/26) was significantly higher than that of the 1.5-μg/kg PEG-I–RBV group (18.5%, 5/27; $P = 0.047$; Fig. 2).

Tolerance of therapy and adverse events

There was no difference in the proportion of drop-out patients from the 1-μg/kg PEG-I–RBV group (7.7%, 2/26, one for depression, and one for generalized fatigue) and that of the 1.5-μg/kg PEG-I–RBV group (7.4%, 2/27, one for excitability, and one for generalized fatigue).

The dose of PEG-I was reduced in two of the 26 (7.7%) patients of the 1-μg/kg PEG-I–RBV group (one patient for thrombocytopenia at 2 weeks, and one patient for generalized fatigue at 20 weeks), and four of the 27 (14.8%) patients of the 1.5-μg/kg PEG-I–RBV group (one patient for neutropenia at 20 weeks, and three patients for generalized fatigue [one patient at 3 weeks, one patient at 5 weeks, and one patient at 8 weeks]). There was no significant difference in the proportion of patients who required a PEG-I dose reduction ($P = 1.0$).

The changes in leukocyte and platelet counts during the 24-week treatment period were similar between the two groups. However, the neutrophil cell count was significantly different between the two groups at 1 and 24 weeks. The dose of RBV was reduced due to anemia in 15 of the 26 (57.7%) patients of the 1-μg/kg PEG-I–RBV group, and for the same reason in 10 of the 27 (37%) patients of the 1.5-μg/kg PEG-I–RBV group. In addition, 21 (80.7%) patients of the 1-μg/kg PEG-I–RBV group administered > 80% of the prescribed RBV dose, while the > 80% dose was administered by 22 (81.5%) of the 1.5-μg/kg PEG-I–RBV group (P = not significant). There was no significant difference between the two groups with regard to the number of patients who required a RBV dose reduction (Table 2).

Predictors of SVR

The univariate analysis identified nine parameters that influenced SVR: age (< 60 years; $P = 0.001$), Hb (> 13 g/dL; $P = 0.021$), serum iron (< 120 μg/dL; $P = 0.044$), triglycerides (≥ 100 mg/dL; $P = 0.034$), LDL-C (< 120 mg/dL; $P = 0.061$), dose of PEG-I (1.5 μg/kg; $P = 0.009$), total RBV dose (> 80%; $P = 0.003$), and reaching undetectable levels of HCV-RNA (HCV-RNA ≤ 100 copies/mL) by week 4 (≤ 100 copies/mL; $P = 0.028$). The multivariate analysis identified two parameters that independently influenced the SVR: age (< 60 years; odds ratio 11.93, 95%CI 1.75–81.19; $P = 0.011$), and the dose of PEG-I (1.5 μg/kg; odds ratio 5.502, 95%CI 1.248–24.26; $P = 0.024$; Table 3). These results indicated that age and the dose of PEG-I are significant and independent predictors of SVR.

Discussion

Although the number of patients in this clinical trial was relatively small, our results showed a significantly lower SVR in patients of

Table 3 Multivariate analysis of factors associated with sustained virological response to pegylated interferon–ribavirin combination therapy in patients infected with hepatitis C virus

Factors	Category	Odds ratio (95% confidence interval)	*P*-value
Age (years)	1 ≥ 60	1	
	2 < 60	11.93 (1.75–81.19)	0.011
Dose of pegylated interferon	1 μg/kg	1	
	1.5 μg/kg	5.502 (1.248–24.26)	0.024

the 1-μg/kg PEG-I–RBV group than that of the 1.5-μg/kg PEG-I–RBV group in the ITT analysis. Furthermore, the frequency of viral relapse at the end of treatment was higher in the lower PEG-I dose group than in the higher dose group. The cause of the high relapse rate was probably a result of the slower viral response of HCV-RNA in the 1-μg/kg PEG-I–RBV group. Although Meyer-Wyss *et al.*[17] reported that the virological response rates towards the commencement of treatment at week 8 and at the end of treatment at week 48 were not significantly different between the two treatment groups, in the present study, the proportion of patients of the 1-μg/kg PEG-I–RBV group who showed viral response (57.7%, 15/26) at 4 weeks tended to be lower than that of the 1.5-μg/kg PEG-I–RBV group (77.8%, 21/27; $P = 0.13$). This in agreement with the results of Rumi *et al.*,[20] who reported that failure of PEG-I therapy could be predicted by the lack of a rapid virological response in patients infected with HCV genotype 2. Moreover, patients with an early virological response seemed to have a high rate of SVR.[21–23] Accordingly, the time of viral response in the 1-μg/kg PEG-I–RBV group will be achieved later than that of the 1.5-μg/kg PEG-I–RBV group. This suggests that if an early virological response is not evident, treatment with PEG-I should probably be extended to 48 weeks in order to increase viral clearance and improve the SVR rate. In this regard, treatment with PEG-I–RBV for 16 weeks in patients infected with HCV genotype 2 or 3 is reported to achieve a lower overall SVR rate than the standard 24-week regimen.[24]

The SVR rate of the 1-μg/kg PEG-I–RBV group was lower than that reported in previous studies.[17,18,25–27] It is possible that these differences are related to differences in race, age of studied patients, and the dose of RBV. The mean age of our patients was 50 years, but has been reported to be 30–40 years in previous studies.[7,18,25,26] In addition, while previous studies evaluated patients infected with HCV genotypes 2 and 3, the number of patients infected with genotype 2 was small.[17] Although Meyer-Wyss *et al.*[17] reported that although SVR rates in patients infected with HCV genotypes 2 and 3 were similar between patients treated with 1 or 1.5 μg/kg PEG-I BW, were differences between the virological response (85%) at the end of treatment and the virological response (71%) at the end of follow up, in patients treated with 1-μg/kg PEG-I. This means that a high proportion of patients of the 1-μg/kg PEG-I–RBV group developed viral relapse after the end of treatment.

The dose of RBV used in the present study was lower than that used in previous studies.[17,18,25–27] The above durations and dosages are those approved by the Japanese Ministry of Health, Labor and Welfare. A lower dose was selected by the Japanese Ministry of Health, Labor and Welfare. In this regard, 21 (80.7%) patients of the 1-μg/kg PEG-I–RBV group were administered > 80% of the prescribed RBV dose, while the > 80% dose was used by 22 (81.5%) patients of the 1.5-μg/kg PEG-I–RBV group (P = not significant). There was no significant difference between the two groups with regard to the number of patients who required RBV dose reduction. In addition, there was no significant difference between the two groups with regard to the concentration of RBV at 8 weeks (data not shown).

In our study, the proportion of patients of the 1.5-μg/kg PEG-I–RBV group who showed SVR was significantly higher than that of the 1-μg/kg PEG-I–RBV group, despite the lack of a significant difference in the exposure to RBV. The proportion of end-of-therapy responders of the 1-μg/kg PEG-I–RBV group was similar to that of the 1.5-μg/kg PEG-I–RBV group. Although the proportion of patients who exhibited a rapid decrease in HCV-RNA to undetectable levels (HCV-RNA ≤ 100 copies/mL) by week 4 was similar in the two treatment groups, the proportion of patients of the 1-μg/kg PEG-I–RBV group who showed viral response (57.7%, 15/26) at 4 weeks tended to be lower than that of the 1.5-μg/kg PEG-I–RBV group (77.8%, 21/27; $P = 0.13$). This finding suggests the clinical importance of the time period during which HCV-RNA is at undetectable levels (≤ 100 copies/mL).

In the IDEAL (Individualized Dosing Efficacy Versus Flat Dosing To Assess Optimal Pegylated Interferon Therapy) study, the proportion of 1-μg/kg PEG-I–RBV patients who developed SVR was similar to that of the 1.5-μg/kg PEG-I–RBV group.[27] We believe that there was no significant difference in the exposure to PEG-I between the IDEAL study and the present one with respect to SVR. However, no information was provided in the IDEAL study on the rapid virological response and end-of-therapy response. Therefore, we could not compare our rapid virological and end-of-therapy responses with those of that study.

Based on the above results, we believe that the time period during which HCV-RNA is at undetectable levels (≤ 100 copies/mL) is more important than the dosage of PEG-I in 24-week treatment regimens, that is, we prefer to use the 1.5-μg/kg PEG-I–RBV regimen since it is more likely to achieve a rapid virological response than the 1-μg/kg PEG-I–RBV regimen.

Our study showed no significant difference between the two treatment groups in the tolerance of therapy and adverse events. At the start of the study, we believed that the number of patients of the 1.5-μg/kg PEG-I–RBV group who would require a PEG-I dose reduction or termination of such therapy would be greater than that of the 1-μg/kg PEG-I–RBV group. However, the data analysis showed no significant difference in the proportions of such patients between the two groups. Furthermore, there were no significant differences in the rate of change in leukocyte and platelet counts over 24 weeks of therapy between the two groups, and neutrophil cell counts of the 1.5-μg/kg PEG-I BW group at 1 and 24 weeks were significantly lower than those of the 1-μg/kg PEG-I BW group. These results indicated that 1.5 μg/kg PEG-I BW is a safe regimen.

The multivariate analysis showed that SVR was dependent on the age of the patient and the dose of PEG-I. Other studies reported a higher SVR rate for young patients than older patients.[28–30] Interestingly, the dose of PEG-I was a significant and independent predictor of SVR. Furthermore, other studies reported that no or mild hepatocyte steatosis was a significant factor associated with SVR.[31] However, we did not investigate hepatocyte steatosis because of the small sample used in this study.

In conclusion, the dose of PEG-I to be used at start of therapy of naïve Japanese patients infected with HCV genotype 2 should be 1.5 μg/kg BW.

References

1 Alter MJ. Epidemiology of hepatitis c in The West. *Semin Liver Dis.* 1995; **15**: 5–14.
2 Poynard T, Bedossa P, Opolon P, and The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. Natural history of liver fibrosis progression in patients with chronic hepatitis C. *Lancet* 1997; **349**: 825–32.
3 Kiyosawa K, Sodeyama T, Tanaka E *et al.* Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990; **12**: 671–75.
4 Tsukuma H, Hiyama T, Tanaka S *et al.* Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N. Engl. J. Med.* 1993; **28**: 1797–801.
5 Takano S, Yokosuka O, Imazeki F *et al.* Incidence of hepatocellular carcinoma in chronic hepatitis B and C: A prospective study of 251 patients. *Hepatology* 1995; **21**: 650–55.
6 Shiratori Y, Shiina S, Imamura M *et al.* Characteristic difference of hepatocellular carcinoma between hepatitis-B and C-viral infection in Japan. *Hepatology* 1995; **22**: 1027–33.
7 Tomimatsu M, Ishiguro N, Taniai M *et al.* Hepatitis C virus antibody in patients with primary liver cancer (hepatocellular carcinoma, cholangiocarcinoma, and combined hepatocellular-cholangiocarcinoma) in Japan. *Cancer* 1993; **72**: 683–88.
8 McHutchison JG, Gordon SC, Schiff ER *et al.* Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. *N. Engl. J. Med* 1998; **339**: 1485–92.
9 Poynaud T, Marcellin P, Lee SS *et al.* Randomised trial of interferon alpha-2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha-2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. *Lancet* 1998; **352**: 1426–32.
10 Zeuzem S, Feinman SV, Rasenack J *et al.* Peginterferon alfa-2a in patients with chronic hepatitis C. *N. Engl. J. Med.* 2000; **343**: 1666–72.
11 Heathcote EJ, Shiffman ML, Cooksley WG *et al.* Peginterferon alfa-2a in patients with chronic hepatitis C and cirrhosis. *N. Engl. J. Med.* 2000; **343**: 1673–80.
12 Lindsay KL, Trepo C, Heintges T *et al.* A randomized, double-blind trial comparing pegylated interferon alfa-2b to interferon alfa-2b as initial treatment for chronic hepatitis C. *Hepatology* 2001; **34**: 395–403.
13 Reddy KR, Wright TL, Pockros PJ *et al.* Efficacy and safety of pegylated (40-kd) interferon alpha-2a compared with interferon alpha-2a in noncirrhotic patients with chronic hepatitis C. *Hepatology* 2001; **33**: 433–8.
14 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon-2b plus ribavirin compared with interferon-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. *Lancet* 2001; **358**: 958–65.
15 Fried MW, Shiffman M, Reddy KR. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N. Engl. J. Med* 2002; **347**: 975–82.
16 Glue P, Rouzier-Panis R, Raffanel C *et al.* A dose-ranging study of pegylated interferon alfa-2b and ribavirin in chronic hepatitis C. The Hepatitis C Intervention Therapy Group. *Hepatology* 2000; **32**: 647–53.
17 Meyer-Wyss B, Rich P, Egger H. Comparison of two PEG-interferon alpha-2b doses (1.0 or 1.5 microg/kg) combined with ribavirin in interferon-naïve patients with chronic hepatitis C and up to moderate fibrosis. *J. Viral. Hepat.* 2006; **13**: 457–65.
18 Mangia A, Santoro R, Minerva N *et al.* Peginterferon alfa-2b and ribavirin for 12 *vs* 24 weeks in HCV genotype 2 or 3. *N. Engl. J. Med.* 2005; **352**: 2609–17.
19 Desmet VJ, Gerber M, Hoofnagle JH *et al.* Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994; **19**: 1513–20.
20 Rumi MG, Aghemo A, D'Ambrosio R *et al.* Lack of rapid virological response predicts interferon-alpha2b/ribavirin therapy failure in HCV genotype 2 patients: a single-centre study. *Antivir Ther* 2007; **12**: 1033–40.
21 Zeuzem S. Clinical implications of hepatitis C viral kinetics. *J. Hepatol.* 1999; **31** (Suppl 1): 61–4.
22 Bekkering FC, Stalgis C, McHutchison JG *et al.* Estimation of early hepatitis C viral clearance in patients receiving daily interferon and ribavirin therapy using a mathematical model. *Hepatology* 2001; **33**: 419–23.
23 Bjøro K, Bell H, Hellum KB *et al.* Effect of combined interferon-alpha induction therapy and ribavirin on chronic hepatitis C virus infection: A randomized multicentre study. *Scand. J. Gastroenterol.* 2002; **37**: 226–32.
24 Shiffman ML, Suter F, Bacon BR *et al.* Peginterferon alfa-2a and ribavirin for 16 or 24 weeks in HCV genotype 2 or 3. *N. Engl. J. Med.* 2007; **357**: 124–34.
25 Zeuzem I S, Hultcrantz R, Bourliere M *et al.* Peginterferon alfa-2b plus ribavirin for treatment of chronic hepatitis C in previously untreated patients infected with HCV genotypes 2 or 3. *J. Hepatol.* 2004; **40**: 993–9.
26 McHutchison J, Sulkowski M. Scientific rationale and study design of the individualized dosing efficacy *vs* flat dosing to assess optimal pegylated interferon therapy (IDEAL) trial: determining optimal dosing in patients with genotype 1 chronic hepatitis C. *J. Viral. Hepat.* 2008; **15**: 475–81.
27 Sulkowski M, Lawitz E, Shiffman ML *et al.* Final results of the IDEAL (individualized dosing efficacy versus flat dosing to assess optimal pegylated interferon therapy) phase IIIB study. *J. Hepatol.* 2008; **48** (Suppl 2): S370–371.
28 Prati D, Zanella A, Zanuso F *et al.* Sustained response to interferon-a2a monotherapy of young blood donors with minimal-to-mild chronic hepatitis C. *J. Viral. Hepat.* 2000; **7**: 352–60.
29 Casiraghi MA, De Paschale M, Romano L *et al.* Long-term outcome (35 years) of hepatitis C after acquisition of infection through mini transfusions of blood given at birth. *Hepatology* 2004; **39**: 90–6.
30 Uka K, Suzuki F, Akuta N *et al.* Efficacy of interferon monotherapy in young adult patients with chronic hepatitis C virus infection. *J. Gastroenterol.* 2006; **41**: 470–5.
31 Akuta N, Suzuki F, Tsubota A *et al.* Efficacy of interferon monotherapy to 394 consecutive naïve cases infected with hepatitis C virus genotype 2a in Japan: therapy efficacy as consequence of tripartite interaction of viral, host and interferon treatment-related factors. *J. Hepatol.* 2002; **37**: 831–6.

Journal of General Virology (2008), **89**, 2108–2113 DOI 10.1099/vir.0.83658-0

Establishment of an infectious genotype 1b hepatitis C virus clone in human hepatocyte chimeric mice

Takashi Kimura,[1,2] Michio Imamura,[1,2] Nobuhiko Hiraga,[1,2] Tsuyoshi Hatakeyama,[1,2] Daiki Miki,[1,2] Chiemi Noguchi,[1,2] Nami Mori,[1,2] Masataka Tsuge,[1,2] Shoichi Takahashi,[1,2] Yoshifumi Fujimoto,[1,2] Eiji Iwao,[3] Hidenori Ochi,[2,4] Hiromi Abe,[1,2,4] Toshiro Maekawa,[4] Keiko Arataki,[5] Chise Tateno,[2,6] Katsutoshi Yoshizato,[2,6] Takaji Wakita,[7] Toru Okamoto,[8] Yoshiharu Matsuura[8] and Kazuaki Chayama[1,2,4]

Correspondence
Kazuaki Chayama
chayama@hiroshima-u.ac.jp

[1]Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

[2]Liver Research Project Center, Hiroshima University, Hiroshima, Japan

[3]Research Division, Mitsubishi Tanabe Pharma Corporation, Osaka, Japan

[4]Laboratory for Liver Disease, SNP Research Center, Institute of Physical and Chemical Research (RIKEN), Yokohama, Japan

[5]Hirosimakinen-Hospital, Internal Medicine, Hiroshima, Japan

[6]Developmental Biology Laboratory, Department of Biological Science, Graduate School of Science, Hiroshima University, Higashihiroshima, Japan

[7]Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Japan

[8]Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

The establishment of clonal infection of hepatitis C virus (HCV) in a small-animal model is important for the analysis of HCV virology. A previous study developed models of molecularly cloned genotype 1a and 2a HCV infection using human hepatocyte-transplanted chimeric mice. This study developed a new model of molecularly cloned genotype 1b HCV infection. A full-length genotype 1b HCV genome, HCV-KT9, was cloned from a serum sample from a patient with severe acute hepatitis. The chimeric mice were inoculated intrahepatically with *in vitro*-transcribed HCV-KT9 RNA. Inoculated mice developed viraemia at 2 weeks post-infection, and this persisted for more than 6 weeks. Passage experiments indicated that the sera of these mice contained infectious HCV. Interestingly, a similar clone, HCV-KT1, in which the poly(U/UC) tract was 29 nt shorter than in HCV-KT9, showed poorer *in vivo* infectivity and replication ability. An *in vitro* study showed that no virus was produced in the culture medium from HCV-KT9-transfected cells. In conclusion, this study developed a genetically engineered genotype 1b HCV-infected mouse. This mouse model will be useful for the study of HCV virology, particularly the mechanism underlying the variable resistance of HCV genotypes to interferon therapy.

Received 13 December 2007
Accepted 14 May 2008

INTRODUCTION

Hepatitis C virus (HCV), a positive-sense, single-stranded RNA virus, infects and replicates efficiently only in the hepatocytes of humans and chimpanzees. There are many genotypes of HCV distributed worldwide (Simmonds *et al.*, 1993); among them genotype 1b is the major genotype in Asia, including Japan, and is known to be one of the most resistant genotypes to interferon (IFN) therapy (Fried *et al.*, 2002). Until recently, studies of HCV replication have long been hampered by the lack of a virus culture system. The development of HCV replicon systems has allowed the

The GenBank/EMBL/DDBJ accession numbers for the sequences of HCV-KT9 and HCV-KT1 determined in this work are AB435162 and AB426117, respectively.

study of the mechanisms of replication of HCV (Lohmann *et al.*, 1999). However, these replicons lack structural proteins, do not replicate efficiently without adaptive mutations and do not produce infectious virions. Recently, it was reported that the genotype 2a full-length JFH-1 genome replicated efficiently in Huh7 cells without adaptive mutations and produced virions that were infectious for both naïve cells and chimpanzees, as well as for a human hepatocyte-transplanted chimeric mouse (Wakita *et al.*, 2005; Zhong *et al.*, 2005; Lindenbach *et al.*, 2006). To date, five full-length genotype 1b clones, HCV-N (Beard *et al.*, 1999), Con-1 (Bukh *et al.*, 2002), HCV-J4 (Okamoto *et al.*, 1992), HCV-CG1b (Thomson *et al.*, 2001) and HCV-BK (Takamizawa *et al.*, 1991), have been demonstrated to be infectious by intrahepatic inoculation of transcribed HCV RNA into the liver of chimpanzees. Among these, only the HCV-CG1b genome is reported to produce HCV particles when transfected into Huh7 cells (Heller *et al.*, 2005).

Although the chimpanzee is a useful animal model for the study of HCV infection, there are ethical restrictions on the use of this animal. Instead, Mercer *et al.* (2001) developed a useful small-animal model for the study of HCV infection using chimeric urokinase-type plasminogen activator (uPA)/severe combined immunodeficiency (SCID) mice (which are immunodeficient and undergo liver failure) with engrafted human hepatocytes. This HCV-infected mouse model is reported to be useful for evaluating anti-HCV drugs such as IFN-α and anti-NS3 protease (Kneteman *et al.*, 2006). We have previously described methods to improve the replacement levels of human hepatocytes in this mouse model (Tateno *et al.*, 2004) and we have developed a reverse genetics system for hepatitis B virus (Tsuge *et al.*, 2005) and HCV (Hiraga *et al.*, 2007). In the present study, we report the establishment of an infectious genotype 1b HCV clone that infects and replicates efficiently in human hepatocyte chimeric mice.

METHODS

Cloning of infectious genotype 1b HCV isolate. Serum samples were obtained from a 43-year-old physician who developed severe acute hepatitis after needle stick exposure from a patient with chronic hepatitis C. On admission, the serum total bilirubin concentration was 10.0 mg dl^{-1} and the prothrombin time was 40 %. The patient tested positive for HCV antibodies by a third-generation radioimmunoassay (Ortho-Clinical Diagnostics) and for HCV RNA by RT-PCR. Serum HCV RNA was quantified using an Amplicor Monitor HCV test (Roche Diagnostics). The HCV RNA titre was 2.5×10^6 copies ml^{-1} on admission and then decreased gradually. Fig. 1 shows the serial changes in alanine aminotransferase (ALT) as a measure of liver function and HCV RNA levels in this patient. Serum samples obtained in the early phase of infection were used for cloning the full-length genome.

RNA extraction, cDNA synthesis, plasmid construction and RNA transcription. Total RNA was extracted from 100 µl serum samples using SepaGene RV-R (Sanko Junyaku) and reverse transcribed with random hexamers and ReverTra Ace reverse transcriptase (Toyobo) according to the manufacturer's instructions. PCR primers were designed based on the sequence of HCV-Con1 (GenBank accession

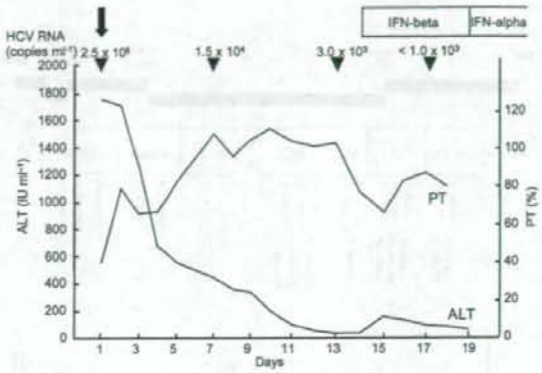

Fig. 1. Clinical course of a patient with severe acute hepatitis C. Alanine aminotransferase (ALT) and prothrombin time (PT) are shown from the day of admission (day 1). The patient was treated daily with 10^6 U IFN-β intravenously for 5 days, followed by 10^6 U IFN-α intramuscularly three times a week for 6 months. HCV RNA was measured on days 1, 7, 13 and 17 (arrowheads). A serum sample was taken on day 1 (arrow) and used to clone the full-length HCV genome.

no. AJ238799; Bukh *et al.*, 2002). Five overlapping cDNA segments (nt 1–2292, 2269–6715, 6696–9094, 7564–9404 and 9361–9605; nucleotide numbers are those of HCV-Con1) were amplified by PCR with TaKaRa LA *Taq* polymerase (Takara Biochemicals) using the above cDNA. Amplified products were separated by agarose gel electrophoresis. Nucleotide sequences were determined using a Big Dye Terminator Mix Cycle Sequencing kit (Applied Biosystems Japan) with an automated DNA sequencer (model 310; PE Biosystems). We corrected the nucleotide sequences of the obtained clones by site-directed mutagenesis and made them identical to the nucleotide sequences obtained by direct sequencing. Naturally occurring restriction enzyme cutting sites were utilized to clone each segment. We utilized the vector pBR322 and created a multiple-cloning site under the control of the T7 promoter by ligating a linker at restriction enzyme cutting sites as they appeared in order from 5′ to 3′ in the HCV sequences (Fig. 2a). Each segment of HCV was cloned into this vector to generate the full-length clones. The HCV-KT9 clone was established using the 3′-terminal fragment with the longest poly(U/UC) tract length (115 nt), which should have a high replication ability (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). A clone with a shorter poly(U/UC) tract length (86 nt), HCV-KT1, was also generated. A polymerase-deficient mutant with an amino acid substitution in the GDD motif (GDD→GND; HCV-KT9-GND) was generated using a Quick Change Site-Directed Mutagenesis kit (Stratagene). After digesting the plasmid with *Xba*I (New England BioLabs) at the 3′ end of the HCV cDNA, HCV RNA was transcribed using T7 RNA polymerase (MEGAscript; Ambion) at 37 °C for 3 h in a 100 µl reaction mixture, according to the manufacturer's instructions. The RNA was analysed using denaturing agarose gel electrophoresis and kept at −80 °C until use.

Construction of a phylogenetic tree. A phylogenetic tree was constructed based on the entire nucleotide sequences of 26 full-length genotype 1b clones plus HCV-KT9. The total number of synonymous and non-synonymous substitutions among the nucleotide sequences was estimated using the method of Gojobori *et al.* (1982) and a phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987).

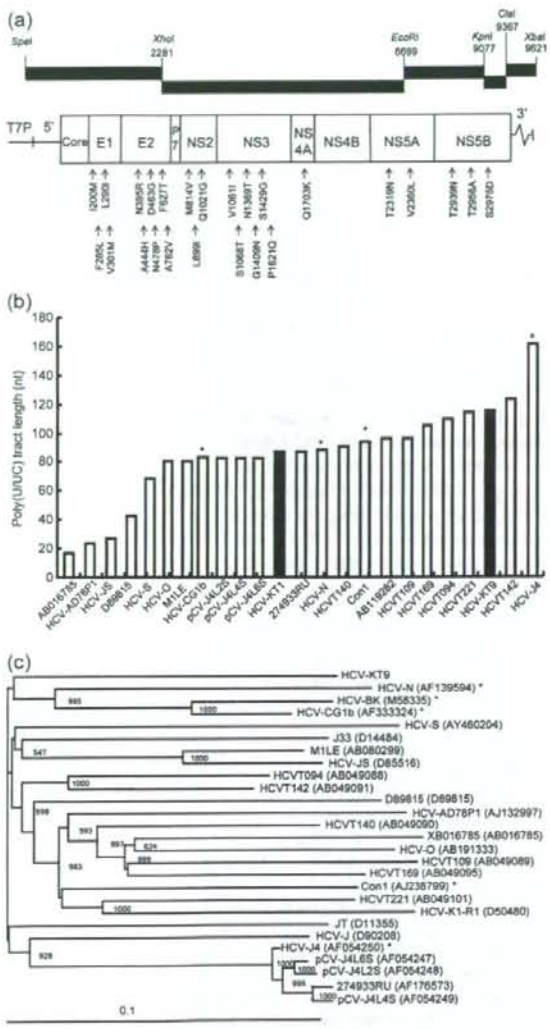

Fig. 2. (a) Schematic diagram of the organization of the cDNA clone HCV-TK9. The T7 RNA promoter (T7P) is located immediately upstream of the HCV genome. Restriction enzyme sites that were used to create clone HCV-KT9 are labelled according to their nucleotide position within the HCV sequence. Amino acid sequences unique to HCV-KT9 compared with 26 other HCV genotype 1b isolates are indicated at the bottom of the figure, with the position of the repaired amino acid residues noted within the polyprotein. (b) Length of the poly(U/UC) tracts of HCV-KT1, HCV-KT9 and 22 other HCV genotype 1b clones reported previously. Asterisks indicate clones confirmed to be infectious by experiments using chimpanzees. (c) Phylogenetic tree constructed with HCV-KT9 and 26 genotype 1b HCV whole-genome sequences. Bar, number of nucleotide substitutions per site. Asterisks indicate clones confirmed to be infectious in experiments using chimpanzees.

Intrahepatic injection experiments in human hepatocyte chimeric mice. We used methods described previously (Tateno *et al.*, 2004) to generate uPA$^{+/+}$/SCID$^{+/+}$ mice and transplant human hepatocytes. All mice used in this study were transplanted with frozen human hepatocytes obtained from the same donor. Mouse serum concentrations of human serum albumin (HSA) correlate with the repopulation index and were measured as described previously (Tateno *et al.*, 2004). Intrahepatic injection of RNA, extraction of serum samples and euthanasia were performed under ether anaesthesia. Briefly, 500 µl RNA solution containing 30 µg transcribed HCV RNA was injected into the liver of anaesthetized chimeric mice through a small abdominal incision. RNA extraction from mouse serum samples, quantification of HCV RNA and nested PCR were performed as described previously (Hiraga *et al.*, 2007). All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments and under the approval of the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University.

Cell culture, RNA transfection and measurement of HCV core antigen. The human hepatoma cell line Huh7 was maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10 % fetal calf serum. RNA transfection and measurement of HCV core antigen in the culture medium were performed as described previously (Wakita *et al.*, 2005).

Statistical analysis. The infectious ratio of chimeric mice was compared and the differences assessed using a χ^2 test. Differences in HCV RNA replication ability *in vitro* were analysed statistically by one-way analysis of variance followed by Scheffe's test. A P value of less than 0.05 was considered statistically significant.

RESULTS

Characteristics of genotype 1b clones HCV-KT9 and HCV-KT1

The entire genome of HCV cDNA was assembled from five DNA fragments (Fig. 2a). We obtained 24 3′-extremity clones with different poly(U/UC) tract lengths. We selected the clone with the longest (U/UC) tract because a previous study indicated that the length of poly(U/UC) tract correlates with HCV replication in an HCV replicon system (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). The length of the poly(U/UC) tract in the longest 3′ clone was 115 nt. The entire genome length of the HCV-KT9 clone using this longest 3′ clone was 9621 nt. We also generated the clone HCV-KT1 with a shorter (86 nt) poly (U/UC) tract to compare the replication abilities of these clones. The lengths of the poly(U/UC) tracts of 22 clones deposited in GenBank are shown in Fig. 2(b). All infectious clones had a poly(U/UC) tract longer than 80 nt. Fig. 2(c) shows a phylogenetic tree constructed using the nucleotide sequences of the 26 full-length genotype 1b clones published to date. Interestingly, the sequence of HCV-KT9 was closest to that of HCV-CG1b (GenBank accession no. AF333324), which has been reported to be infectious, and formed a cluster with two other infectious clones, HCV-N (Beard *et al.*, 1999) and HCV-BK (Takamizawa *et al.*, 1991). We compared the amino acid

sequences of HCV-KT9 with an alignment of the sequences of the 26 other genotype 1b strains. All HCV full-length clones reported from Japan were included in these 26 strains. Based on these comparisons, we identified 25 aa unique to HCV-KT9 (Fig. 2a). We found that the amino acid sequence of the IFN sensitivity-determining region in the NS5A region, which has been suggested to mediate IFN resistance via interaction with the cellular protein kinase R (Enomoto *et al.*, 1996; Gale *et al.*, 1997), was that of the wild-type.

Intrahepatic injection of HCV-KT1 and HCV-KT9 RNAs into human hepatocyte chimeric mice

In the next experiments, 30 μg *in vitro*-transcribed RNA of HCV-KT1, HCV-KT9 or HCV-KT9-GND was injected into the livers of chimeric mice. Eight of 10 (80%) HCV-KT9-injected mice developed measurable viraemia at 2 weeks post-inoculation (Table 1 and Fig. 3), with the HCV RNA titre reaching 1.1×10^6 to 8.8×10^6 copies ml^{-1} at 6 weeks post-inoculation (Fig. 3). To check for the presence of infectious HCV in the serum of HCV-KT9-infected mice, each of five naïve mice was injected with 10 μl serum sample (containing 3.5×10^5 copies of HCV) obtained from an HCV-KT9-infected mouse 6 weeks after inoculation. All five naïve mice became positive for HCV RNA, as confirmed by nested PCR, at 2 weeks post-inoculation and two mice developed persistent viraemia (Fig. 4). These results indicated that the serum of HCV-KT9-injected mice contained infectious HCV. In contrast to HCV-KT9, none of the three mice injected with HCV-KT9-GND RNA developed viraemia (Table 1). These results indicated that HCV-KT9 replicates efficiently in mice livers and produces infectious virus continuously. On the other hand, only one out of seven HCV-KT1-injected mice (14%) developed measurable viraemia (Table 1 and Fig. 3). The level of viraemia was low in this HCV-KT1-infected mouse, HCV RNA was negative by nested PCR at 2 weeks after inoculation and the titre was only 2.2×10^4 copies ml^{-1} at 4 weeks post-inoculation (Fig. 3). These results confirmed the importance of the poly(U/UC) tract length in experimentally induced viraemia.

The nucleotide and amino acid sequences of the viral genome isolated from an HCV-KT9-injected mouse (Fig. 3) at 6 weeks after RNA injection were identical to the injected HCV-KT9 (data not shown). We tried to reclone the poly(U/UC) tract in the HCV-KT1-infected mouse, but it was impossible to reamplify the HCV cDNA using the remaining small amount of serum.

Table 1. Correlation between length of the poly(U/UC) tract and HCV infection

Clone	Length of poly(U/UC) tract	Number of mice			Infection ratio
		Infected	Not infected	Total	
HCV-KT1	86	1	6	7	14%
HCV-KT9	115	8	2	10	80%*
HCV-KT9-GND	115	0	3	3	0%

*P=0.015, compared with HCV-KT1.

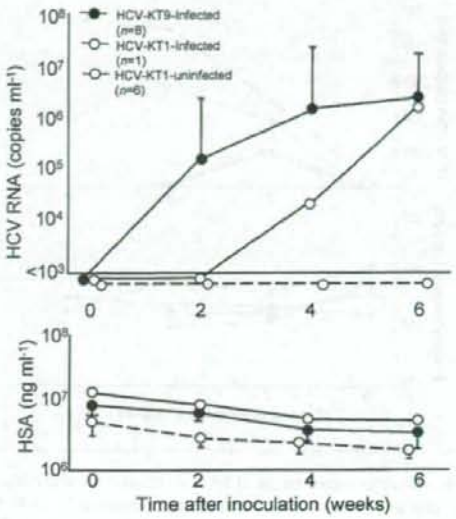

Fig. 3. Changes in HCV RNA levels and HSA concentrations in the sera of mice infected with clonal HCV. Mice were inoculated intrahepatically with 30 μg *in vitro*-transcribed HCV RNA. Eight of the ten HCV-KT9-infected mice (80%), one of the seven HCV-KT1-infected mice (14%) and none of the three HCV-KT9-GND-infected mice became positive for HCV RNA. The results for six HCV-KT1-uninfected mice are also shown. Mice serum samples were obtained every 2 weeks post-infection for analysis of HCV RNA titres. Data are shown as mean ± SD.

Analysis of virus production from HCV-KT9-transfected cells

Next, we evaluated the ability of the HCV-KT9 clone to replicate in transfected Huh7 cells. In these experiments, we used JFH-1 RNA, which is known to replicate efficiently in cell cultures, as control (Wakita *et al.*, 2005). Core protein was secreted efficiently from JFH-1 RNA-transfected Huh7 cells. In contrast, we did not observe any measurable levels of core protein in the supernatant of HCV-KT9-transfected cells (Fig. 5), suggesting a minimal replication ability of HCV-KT9 to produce and release virus into the supernatant.

DISCUSSION

In this study, we described the establishment of a genotype 1b clone, HCV-KT9, that replicated efficiently following injection of the transcribed RNA into chimeric mouse liver.

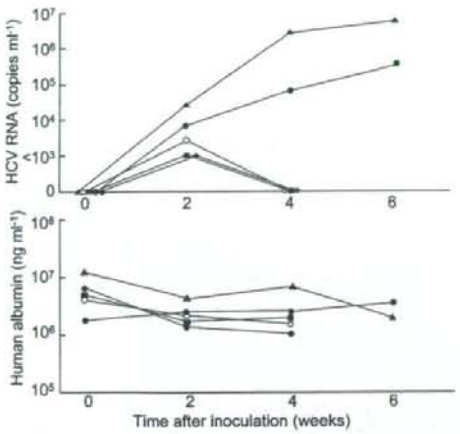

Fig. 4. Passage experiments of HCV in naïve chimeric mice. Five naïve chimeric mice were inoculated intravenously with 10 µl serum sample (containing 3.5×10^5 copies HCV) obtained from an HCV-KT9-infected mouse at week 6 post-inoculation. Serum samples were obtained at the indicated time intervals for the measurement of HCV RNA levels and HSA concentrations. Data represent the changes in five individual mice.

The key factor that determines the infectivity of HCV clones has not yet been established. We previously established a clone from HCV that replicated in a chimeric mouse after injection of serum from a chronically HCV-infected patient. However, we did not observe viraemia after intrahepatic injection of the transcribed RNA from this clone (unpublished results). In contrast, injection of HCV-KT9 RNA in the present study resulted in viraemia in eight out of ten mice (80 %). The fact that the nucleotide and amino acid sequences of the virus recovered from the infected mice were identical to those of the HCV-KT9 clone indicated that no adaptive mutation was necessary for this clone to replicate in the chimeric mouse.

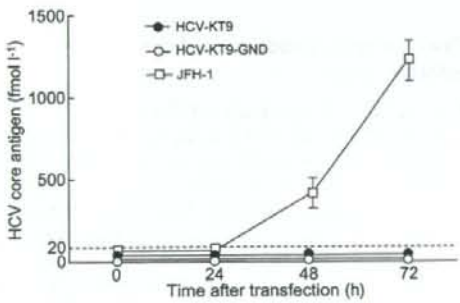

Fig. 5. Time-course studies of HCV core protein secretion into the culture medium of HCV RNA-transfected cells. Huh7 cells were transfected with 10 µg HCV-KT9, HCV-KT9-GND or JFH-1 RNA. HCV core antigen in the culture medium was measured at 24, 48 and 72 h after transfection. Data are shown as mean ± SD of HCV core protein levels obtained from three independent transfection experiments.

Interestingly, the clone was obtained from a patient with severe acute hepatitis. This is similar to JFH-1, an HCV clone with a strong replication ability in cultured cell lines, chimpanzees and chimeric mice, which was cloned from serum samples of a patient who developed acute fulminant hepatitis with a high virus titre (Wakita *et al.*, 2005). A virus that replicates in the early stage of infection may have strong replication ability, which may be lost in the chronic phase of infection.

A key amino acid substitution may be present in one (or some) of the amino acids unique to this clone (Fig. 2a). We also showed that clone HCV-KT1, which differs from HCV-KT9 only in the length of the poly(U/UC) tract, had a poorer replication ability in mice (Table 1 and Fig. 3). However, there is a possibility that a shorter poly(U/UC) tract only slows down the rate of infection, as the HCV RNA titre in the HCV-KT1-infected mouse at 6 weeks after inoculation was similar to that in HCV-KT9-infected mice (Fig. 3). It has been reported that the length and composition of the poly(U/UC) tract is important for the replication of HCV replicons (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). However, no replication advantage of a poly(U/UC) tract longer than 86 bp was revealed in this study. This may be due to differences *in vitro* and *in vivo*, where the innate immune response against the virus may be more robust than in cell culture.

As shown in the present study, reverse genetics of HCV has become available for studies of HCV replication. The important factors for virus replication suggested above can be analysed further using this system.

We also examined the response of HCV-KT9-infected mice to IFN treatment. Three HCV-KT9-infected mice were treated with daily intramuscular injections of 1000 IU IFN-α (g body weight)$^{-1}$ for 2 weeks. This regimen resulted in a reduction in HCV RNA levels of only 1.0 log copies ml^{-1} (data not shown). These results are consistent with our previous study, which showed a similar low-level reduction in HCV RNA in mice infected with a genotype 1a clone, and differ from our previous results in mice infected with HCV genotype 2a, which became negative for HCV RNA following daily treatment with 1000 IU IFN-α (g body weight)$^{-1}$ for 2 weeks (Hiraga *et al.*, 2007). These results are in agreement with our clinical experience that genotype 1 is more resistant to IFN therapy than genotype 2. As shown in the present study and previously (Hiraga *et al.*, 2007), reverse genetics of HCV with three genotypes, 1a, 1b and 2a, is now available. By recombination of these clones or the establishment of mutants with nucleotide and amino acid sequences similar to each other, it may be possible to clarify the mechanism underlying the variability in susceptibility of HCV genotypes to IFN.

In this study, HCV-KT9 showed no virus production ability *in vitro*. Recently, Kato *et al.* (2007) reported that the genotype 1b HCV clone CG1b replicated in Huh7.5.1 cells and produced infectious HCV. It will be of interest to create chimeric viruses of HCV-KT9 and HCV-CG1b, and to determine the mutations that are important for virus production *in vitro*.

In summary, we established an infection model of a genotype 1b HCV clone using human hepatocyte chimeric mice. This model will be useful for studies of HCV replication, particularly the mechanism underlying the variable resistance of HCV genotypes to IFN therapy.

ACKNOWLEDGEMENTS

The authors thank Rie Akiyama and Kana Kunihiro for their technical help and Dr Francis V. Chisari for providing the Huh7.5.1 cells. This work was supported in part by Grants-in-Aid for scientific research and development from the Ministry of Education, Sports, Culture and Technology and the Ministry of Health, Labor and Welfare, Japan.

REFERENCES

Beard, M. R., Abell, G., Honda, M., Carroll, A., Gartland, M., Clarke, B., Suzuki, K., Lanford, R., Sangar, D. V. & Lemon, S. M. (1999). An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. *Hepatology* **30**, 316–324.

Bukh, J., Pietschmann, T., Lohmann, V., Krieger, N., Faulk, K., Engle, R. E., Govindarajan, S., Shapiro, M., St Claire, M. & other authors (2002). Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. *Proc Natl Acad Sci U S A* **99**, 14416–14421.

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

Friebe, P. & Bartenschlager, R. (2002). Genetic analysis of sequences in the 3′ nontranslated region of hepatitis C virus that are important for RNA replication. *J Virol* **76**, 5326–5338.

Fried, M. W., Shiffman, M. L., Reddy, K. R., Smith, C., Marinos, G., Goncales, F. L., Jr, Haussinger, D., Diago, M., Carosi, G. & other authors (2002). Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* **347**, 975–982.

Gale, M. J., Jr, Korth, M. J., Tang, N. M., Tan, S. L., Hopkins, D. A., Dever, T. E., Polyak, S. J., Gretch, D. R. & Katze, M. G. (1997). Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology* **230**, 217–227.

Gojobori, T., Ishii, K. & Nei, M. (1982). Estimation of average number of nucleotide substitutions when the rate of substitution varies with nucleotide. *J Mol Evol* **18**, 414–423.

Heller, T., Saito, S., Auerbach, J., Williams, T., Moreen, T. R., Jazwinski, A., Cruz, B., Jeurkar, N., Sapp, R. & other authors (2005). An in vitro model of hepatitis C virion production. *Proc Natl Acad Sci U S A* **102**, 2579–2583.

Hiraga, N., Imamura, M., Tsuge, M., Noguchi, C., Takahashi, S., Iwao, E., Fujimoto, Y., Abe, H., Maekawa, T. & other authors (2007). Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon. *FEBS Lett* **581**, 1983–1987.

Kato, T., Matsumura, T., Heller, T., Saito, S., Sapp, R. K., Murthy, K., Wakita, T. & Liang, T. J. (2007). Production of infectious hepatitis C virus of various genotypes in cell cultures. *J Virol* **81**, 4405–4411.

Kneteman, N. M., Weiner, A. J., O'Connell, J., Collett, M., Gao, T., Aukerman, L., Kovelsky, R., Ni, Z. J., Zhu, Q. & other authors (2006). Anti-HCV therapies in chimeric scid-Alb/uPA mice parallel outcomes in human clinical application. *Hepatology* **43**, 1346–1353.

Lindenbach, B. D., Meuleman, P., Ploss, A., Vanwolleghem, T., Syder, A. J., McKeating, J. A., Lanford, R. E., Feinstone, S. M., Major, M. E. & other authors (2006). Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. *Proc Natl Acad Sci U S A* **103**, 3805–3809.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L. & Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110–113.

Mercer, D. F., Schiller, D. E., Elliott, J. F., Douglas, D. N., Hao, C., Rinfret, A., Addison, W. R., Fischer, K. P., Churchill, T. A. & other authors (2001). Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* **7**, 927–933.

Okamoto, H., Kojima, M., Okada, S., Yoshizawa, H., Iizuka, H., Tanaka, T., Muchmore, E. E., Peterson, D. A., Ito, Y. & other authors (1992). Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: variability and stability. *Virology* **190**, 894–899.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Simmonds, P., Holmes, E. C., Cha, T. A., Chan, S. W., McOmish, F., Irvine, B., Beall, E., Yap, P. L., Kolberg, J. & other authors (1993). Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. *J Gen Virol* **74**, 2391–2399.

Takamizawa, A., Mori, C., Fuke, I., Manabe, S., Murakami, S., Fujita, J., Onishi, E., Andoh, T., Yoshida, I. & other authors (1991). Structure and organization of the hepatitis C virus genome isolated from human carriers. *J Virol* **65**, 1105–1113.

Tateno, C., Yoshizane, Y., Saito, N., Kataoka, M., Utoh, R., Yamasaki, C., Tachibana, A., Soeno, Y., Asahina, K. & other authors (2004). Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* **165**, 901–912.

Thomson, M., Nascimbeni, M., Gonzales, S., Murthy, K. K., Rehermann, B. & Liang, T. J. (2001). Emergence of a distinct pattern of viral mutations in chimpanzees infected with a homogeneous inoculum of hepatitis C virus. *Gastroenterology* **121**, 1226–1233.

Tsuge, M., Hiraga, N., Takaishi, H., Noguchi, C., Oga, H., Imamura, M., Takahashi, S., Iwao, E., Fujimoto, Y. & other authors (2005). Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology* **42**, 1046–1054.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H. G. & other authors (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* **11**, 791–796.

Yi, M. & Lemon, S. M. (2003). 3′ Nontranslated RNA signals required for replication of hepatitis C virus RNA. *J Virol* **77**, 3557–3568.

You, S. & Rice, C. M. (2008). 3′ RNA elements in hepatitis C virus replication: kissing partners and long poly(U). *J Virol* **82**, 184–195.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T. & other authors (2005). Robust hepatitis C virus infection *in vitro*. *Proc Natl Acad Sci U S A* **102**, 9294–9299.

J Gastroenterol 2008; 43:550–557
DOI 10.1007/s00535-008-2194-5

Journal of Gastroenterology

Clinicopathological features of elderly patients with hepatitis C virus-related hepatocellular carcinoma

Daiki Miki[1], Hiroshi Aikata[1], Kiminori Uka[1], Hiromi Saneto[1], Tomokazu Kawaoka[1], Takahiro Azakami[1], Shintaro Takaki[1], Soo Cheol Jeong[1], Michio Imamura[1], Yoshiiku Kawakami[1], Shoichi Takahashi[1], Toshiyuki Itamoto[2], Toshimasa Asahara[2], Koji Arihiro[3], and Kazuaki Chayama[1]

Departments of [1]Medicine and Molecular Science and [2]Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan
[3]Department of Anatomical Pathology, Hiroshima University Hospital, Hiroshima, Japan

Background. It is well known that the incidence of hepatocellular carcinoma (HCC) in patients with hepatitis C virus (HCV) correlates with progression of liver fibrosis. However, there is little information on the impact of aging on hepatocarcinogenesis. The aim of this study was to elucidate the clinicopathological features of elderly patients with HCV-related HCC. ***Methods.*** The study subjects were 693 consecutive patients newly diagnosed with HCC with anti-HCV. First, we divided them into a younger group (<70 years) and an elderly group (≥70 years) and compared clinicopathological features between the two groups. Next, we selected pure HCV-related HCC patients by excluding the patients with other probable factors for hepatocarcinogenesis (anti-HBc, interferon therapy, and alcohol) and compared the two groups again. ***Results.*** Higher platelet count, lower male/female ratio, lower rate of habitual alcohol consumption, and better Child-Pugh class were recognized in the elderly group thant the younger group, statistically. In 133 cases of hepatic resection, fibrosis stage was lower in the elderly than the younger group. After selection of pure HCV-related HCC patients, in a stepwise multivariate analysis, male sex and platelet count $<10 \times 10^4/mm^3$ were significant variables associated with age <70. Regarding the latency period to HCC development, the patients who received a blood transfusion at an older age developed HCC sooner despite their lower grade of fibrosis. ***Conclusions.*** The elderly patients developed HCC more often, despite their lower grade of fibrosis, compared with the younger patients. In addition to fibrosis, aging could be a factor affecting HCV-related hepatocarcinogenesis.

Key words: hepatocellular carcinoma, hepatitis C virus, elderly patients, aging, fibrosis

Received: September 23, 2007 / Accepted: March 20, 2008
Reprint requests to: H. Aikata

Introduction

Since the discovery of the hepatitis C virus (HCV) RNA in 1989,[1,2] there has been no doubt that HCV is associated with hepatocarcinogenesis based on the high prevalence of HCV infection among hepatocellular carcinoma (HCC) patients who are otherwise negative for hepatitis B virus (HBV) markers.[3–14] In Japan, HCV is considered one of the main causes of HCC.[15,16] Several studies have examined patients with HCV and identified age, the extent of liver fibrosis, male sex, alcohol consumption, positivity for hepatitis B surface antigen (HBsAg), and high alpha-fetoprotein (AFP) level as risk factors for hepatocarcinogenesis.[15–20] The annual incidence of HCC correlates with the severity of liver fibrosis, from 0.5% among patients with stage F0 or F1 fibrosis to 7.9% among patients with stage F4 fibrosis.[21] In patients with chronic hepatitis C, age at infection, alcohol consumption, and male sex are independent factors associated with increased rate of progression of liver fibrosis.[22] Progression of fibrosis is dependent mainly on age and duration of infection. Moreover, the highest rate of progression of liver fibrosis occurs in patients aged ≥50 years.[23] In Japan, we and other groups have reported a recent increase in the number of elderly HCC patients with HCV,[24–26] but the impact of aging on the incidence of HCC has not been investigated carefully.

Thus, we started analyzing HCV-related HCC patients who were positive for anti-HCV but negative for HBsAg to determine the correlation between aging and liver fibrosis in relation to the incidence of HCC with HCV. To date, several factors for affecting hepatocarcinogenesis have been reported, such as antibody to hepatitis B core antigen (anti-HBc), previous interferon therapy, and alcohol consumption. It was reported that previous HBV infection increased the risk for development of HCC in HCV-related liver cirrhosis.[23,27] Also, alcohol consumption also considered a sig-

nificant risk factor for development of HCC with HCV.[15,16,18] Conversely, interferon therapy reduces the risk for development of HCC.[19,21] Therefore, we also analyzed patients without these factors to study them as the nearest condition to the natural history of HCV-related HCC patients and considered them as pure HCV-related HCC patients. In this study, we elucidated the clinicopathological features of elderly patients with HCV-related HCC and examined the impact of aging on hepatocarcinogenesis.

Methods

Patients

A total of 1038 consecutive patients newly diagnosed with HCC were consulted or admitted to Hiroshima University Hospital from January 1988 to December 2005. Of these patients, 693 patients (474 men, 41–92 years of age; 219 women, 41–88 years of age), positive for anti-HCV but negative for HBsAg, were enrolled in the present study. The 693 patients were divided into two groups according to age at initial diagnosis: 440 patients (325 men, 115 women) were <70 years old (younger group), and 253 patients (149 men, 104 women) were ≥70 years old (elderly group). We recorded epidemiological data (age, sex, history of blood transfusion, history of habitual alcohol consumption) and chemical data (serum bilirubin, aspartate aminotransferase, alanine aminotransferase, serum albumin, and prothrombin time) for each patient. A detailed epidemiological questionnaire was administered during face-to-face interviews. Then, we compared the clinical and pathological features of these groups. All patients submitted informed consent for collection and subsequent use of data for research purposes, and the study was carried out in accordance with the Helsinki Declaration.

Selection of pure HCV-related HCC

As already mentioned, we first studied anti-HCV-positive and HBsAg-negative patients with HCC. However, this study included HBsAg-negative and anti-HBc-positive patients. It is recognized that previous HBV infection may be a cofactor for the development of HCC in HCV-related HCC patients.[27] Therefore, we excluded 167 patients (116 males, 51 females) who were positive for anti-HBc, the remainder being 526 patients (358 males, 168 females). Next, we excluded 57 patients (40 males, 17 females) who were negative for HCV-RNA. These patients probably became virus negative as a result of interferon therapy or spontaneously. The remainder included 469 patients (318 males, 151 females). There are some data that interferon reduces HCC development even in patients who do not achieve sustained eradication of HCV[19,21,28]; therefore, we also excluded 84 patients (67 males, 17 females) with history of interferon therapy. The remainder comprised 385 patients (251 males, 134 females). Habitual alcohol consumption is recognized as a risk factor of HCC development,[15,16,18] and in our study the proportion of patients with habitual alcohol consumption was greater in the younger group than in the elderly group. Thus, we excluded 48 patients (46 males, 2 females) with excessive alcohol consumption; the remainder included 337 patients (205 males, 132 females). We decided that 337 patients were appropriate to be the next subjects of our study as pure HCV-related HCC patients. We wanted to elucidate the relationship between the impact of aging and HCV-induced fibrosis for HCC development as the nearest condition to the natural history, so we removed those patients with other probable factors affecting hepatocarcinogenesis.

HCC diagnosis

The diagnosis of HCC was based on hypervascularity, confirmed by dynamic computed tomography (CT), magnetic resonance imaging, angiography, or CT angiography, when the serum levels of HCC-related tumor markers, such as alpha-fetoprotein or protein induced in the absence of vitamin K or antagonist II (PIVKA-II), were increased or a mass lesion was detected by ultrasonography. When a nodule was not proven to be hypervascular, percutaneous biopsy under ultrasonography was performed for confirmation of the diagnosis of HCC. Staging adopted in this study was the revised version of the Liver Cancer Study Group of Japan in 2000.[29]

Blood biochemical tests and viral markers

Routine serum biochemical tests were carried out with automated techniques. Anti-HCV in sera was assayed using the Anti-HCV-EIA Cobas Core Test (Hoffmann La Roche, Basel, Switzerland). All serum samples collected were analyzed with second- or third generation anti-HCV tests. If the index was ≥1.0, anti-HCV was considered positive. HBsAg and anti-HBc in sera were assayed using the HBsAg-EIA Cobas Core Test (Hoffmann La Roche) and the Anti-HBc-EIA Cobas Core Test (Hoffmann La Roche), respectively. HCV-RNA in sera was detected by qualitative reverse transcription-polymerase chain reaction (RT-PCR) (Hoffmann La Roche). For cases before March 1990, serum samples frozen at –20°C were retrieved from storage and tested for anti-HCV using the aforementioned method.

Alcohol consumption

The average quantity of alcohol consumed per day was evaluated regardless of type of alcohol beverage. Habitual alcohol consumption was defined as regular consumption of at least one alcohol drink per week over 10 years. In particular, excessive habitual drinkers were defined as persons consuming more than 86 g ethanol per day.

Platelet count

We evaluated the platelet count, which is known to correlate significantly with the stage of liver fibrosis in patients with chronic hepatitis C; also, platelet count $<10 \times 10^4/mm^3$ has been used as a marker for liver cirrhosis.[30-35]

Stage of liver fibrosis and activity grade of chronic hepatitis

Of 693 patients, 133 patients underwent hepatic resection within 6 months from first diagnosis. The resected specimens were fixed in 10% formalin neutral buffered solution, embedded in paraffin, and processed for staining with hematoxylin and eosin. Staging of fibrosis and grading of liver disease activity was assessed by a pathologist on a blinded basis according to the criteria of Desmet et al.[36]

Statistical analysis

Values are expressed as median and 75th and 25th percentiles. Statistical analysis was performed using the Mann–Whitney U test and Spearman's nonparametric test. Univariate and multivariate logistic regression using the Statistical Package for Social Science (SPSS) version 6.1 for Windows (SPSS Japan, Tokyo, Japan) was performed to evaluate the values of the patients' clinical and laboratory features associated with age at initial diagnosis of HCC. P values less than 0.05 were considered to indicate statistical significance.

Results

Characteristics of patients with HCV-related HCC

Table 1 summarizes the characteristics of HCC patients with HCV, according to age at initial diagnosis. Male/female ratio was significantly higher in the younger group than the elderly group ($P < 0.001$). The median age of male patients (66 years old) was lower than that of female patients (69 years old) ($P < 0.001$). The median of platelet count was 8.6 (25^{th}, 6.1; 75^{th}, 13.3) and 10.0 (25^{th}, 7.3; 25^{th}, 13.4) $\times 10^4/mm^3$ in the younger and the elderly groups, respectively. Platelet count of the elderly group was significantly higher than that of the younger group ($P = 0.005$). The proportion of patients whose platelet count was $\geq 10 \times 10^4/mm^3$, considered as having a noncirrhotic liver, was greater in the elderly group than the younger group ($P = 0.003$). When we compared the platelet count of the younger group and that of the elderly group according to sex, the median of platelet count was 9.2 (25^{th}, 6.2; 75^{th}, 14.2) and 10.5 (25^{th}, 7.8; 25^{th}, 13.7) $\times 10^4/mm^3$ in the younger and the elderly male groups, respectively. The median of platelet count was 7.9 (25^{th}, 5.9; 75^{th}, 10.6) and 8.8 (25^{th}, 7.0; 25^{th}, 12.4) $\times 10^4/mm^3$ in the younger and the elderly female groups, respectively. Platelet count of the elderly group was significantly higher than that of the younger group for both sexes (males, $P = 0.023$; females, $P = 0.017$). The

Table 1. Characteristics of all patients with hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC), according to age at diagnosis

Age at diagnosis	Younger group (<70 years)	Elderly group (≥70 years)	P^a
Sex (male/female)	325/115	149/104	<0.001
Platelet count ($\times 10^4/mm^3$)[b]	8.6	10.0	0.005
(<10/≥10) $\times 10^4/mm^3$	266/174	124/129	0.003
Child-Pugh classification (A or non-cirrhosis/B/C)	271/149/20	184/69/0	0.001
Stage (I/II/III/IV)	88/170/116/66	49/109/73/22	NS
Alpha-fetoprotein (ng/ml)[b]	44.6	46.8	NS
History of blood transfusion (+/−)	100/340	55/198	NS
History of habitual alcohol consumption (Excessive/habitual/none)	64/193/183	23/95/135	0.001
Anti-HBc (+/−)	100/340	67/186	NS
Diabetes mellitus (with/without)	96/344	60/193	NS

NS, not significant
[a]Comparison between patients less than 70 years of age and those older than 70 years
[b]Values are median

Table 2. Significant variables in association with development of HCV-related HCC under 70 years old using univariate logistic analysis

Variables	Odds ratio	95% confidence interval	*P* value
Habitual alcohol consumption*	2.363	1.351–4.133	0.003
Male*	1.973	1.420–2.740	<0.001
Child-Pugh class B or C*	1.663	1.187–2.329	0.003
Platelet count $<10 \times 10^4/mm^3$*	1.575	1.153–2.152	0.004

*Significant variables in multivariate analysis

Child-Pugh class was A or non-cirrhosis, B and C in 62%, 34%, and 4% of the younger group, and 73%, 27%, and 0% of the elderly group, respectively. Child Pugh class were significantly better in the elderly group than the younger group ($P = 0.001$). No significant difference was observed in stage grouping of HCC between the two groups. The median AFP level was 44.6 and 46.8 ng/ml for the younger and the elderly group, respectively. No significant difference was observed in serum AFP level between the two groups. History of blood transfusion was reported by 23% and 22% of the younger and the elderly group, respectively; it was not a significant difference. With regard to alcohol consumption, 14%, 44%, and 42% of the younger group and 9%, 38%, and 53% of the elderly group had history of excessive, habitual, and no drinking, respectively. Significant differences were observed in history of habitual alcohol consumption between the younger and the elderly group ($P = 0.001$). No significant difference was observed in positive rate of anti-HBc and the rate of diabetes mellitus.

Variables associated with age <70 years at diagnosis of HCC

Sex, platelet count, Child-Pugh class, history of blood transfusion, history of habitual alcohol consumption, anti-HBc and diabetes mellitus were selected as independent variables in logistic regression analyses, with the age <70 years at diagnosis as dependent variables. In a univariate analysis, habitual alcohol consumption, male sex, Child-Pugh class B or C, and platelet count $<10 \times 10^4/mm^3$ were significantly correlated with age <70 years at diagnosis (Table 2). In a stepwise multivariate analysis, habitual alcohol consumption (odds ratio, 1.968, 95% confidence interval, 1.107–3.500; $P = 0.021$), male sex (odds ratio, 1.933, 95% confidence interval, 1.373–2.722; $P < 0.001$), platelet count $<10 \times 10^4/mm^3$ (odds ratio, 1.587, 95% confidence interval, 1.144–2.200; $P = 0.006$) and Child-Pugh class B or C (odds ratio, 1.532, 95% confidence interval, 1.080–2.174, $P = 0.017$) were significant variables associated with age <70 years at diagnosis in HCV-related HCC patients.

Table 3. Fibrosis stage and activity grade of liver histology

	<70 years	≥70 years	*P*[a]
Male/female	67/23	29/14	
Fibrosis stage			0.009
F0–1	4 (4%)	2 (5%)	
F2	13 (14%)	14 (38%)	
F3	26 (27%)	8 (22%)	
F4	53 (55%)	13 (35%)	
Activity grade			0.047
A0–1	6 (6%)	5 (13%)	
A2	79 (82%)	31 (84%)	
A3	11 (12%)	1 (3%)	

Data are numbers and percentages (in parentheses) of patients
[a]Comparison between patients less than 70 years of age and those older than 70 years

Fibrosis stage and activity grade

Of the 693 patients, 133 underwent hepatic resection. Their fibrosis staging and activity grading of the liver were evaluated (Table 3). Fibrosis stage F0–1, F2, F3, and F4 were identified in 4%, 14%, 27%, and 55% of the younger group, and in 5%, 38%, 22%, and 35% of the elderly group, respectively. The fibrosis stage of liver histology was significantly lower in the elderly group ($P = 0.009$). The activity grades A0–1, A2, and A3 were recognized in 6%, 82%, and 12% of the younger group, and 13%, 84%, and 3% of the elderly group, respectively. The activity grade of liver histology was significantly lower in the elderly group ($P = 0.047$). Among 133 patients who underwent hepatic resection, both fibrosis stage and activity grade were significantly lower in the elderly patients.

Characteristics of pure HCV-related HCC patients

Table 4 summarizes the characteristics of pure HCV-related HCC patients. Male/female ratio was significantly higher in the younger group than the elderly group ($P = 0.004$). The median age of male patients (67 years) was lower than that of female patients (69 years) ($P = 0.001$). The median platelet count was 8.7 (25^{th}, 6.1; 75^{th}, 12.4) and 9.9 (25^{th}, 7.0; 75^{th}, 13.1) $\times 10^4/mm^3$ in the younger and the elderly group, respectively. The pro-

Table 4. Characteristics of patients with pure HCV-related HCC, according to age at diagnosis

Age at diagnosis	Younger group (<70 years)	Elderly group (≥70 years)	P^a
Sex (male/female)	138/68	67/64	0.004
Platelet count ($\times 10^4/mm^3$)[b]	8.7	9.9	NS
(<10/≥10) $\times 10^4/mm^3$	134/72	69/62	0.024
Child-Pugh classification (A or non-cirrhosis/B/C)	123/72/11	83/48/0	NS
Stage (I/II/III/IV)	48/69/55/34	23/63/33/12	NS
Alpha-fetoprotein (ng/ml)[b]	45.2	60.2	NS
Genotype (1b/2a or 2b or 3a)[c]	127/18	83/24	0.035
History of blood transfusion (+/–)	47/159	32/99	NS
Diabetes mellitus (with/without)	43/163	35/96	NS

NS, not significant
[a]Comparison between patients less than 70 years of age and those older than 70 years
[b]Values are median
[c]Of the patients, 75 were not tested (51 were <70 years, 24 were ≥70 years)

Table 5. Significant variables in association with development of pure HCV-related HCC under 70 years old using univariate logistic analysis

Variables	Odds ratio	95% confidence interval	*P* value
Male[a]	1.924	1.195–3.098	0.007
Platelet count $<10 \times 10^4/mm^3$[a]	1.672	1.070–2.614	0.024
Genotype 1	2.040	1.043–3.990	0.037

[a]Significant variables in multivariate analysis

portion of patients whose platelet count was $\geq 10 \times 10^4/mm^3$ was greater in the elderly group than the younger group ($P = 0.024$). Child-Pugh class also seemed to indicate better liver function in the elderly group than that of the younger group, but it was not statistically significant. Stage grouping of HCC, serum AFP level, history of blood transfusion, and rate of diabetes mellitus showed no significant difference between the younger and the elderly group. The proportion of patients with HCV genotype 1 was greater in the younger group than the elderly group ($P = 0.035$). Next, we selected sex, platelet count, Child-Pugh class, genotype, history of blood transfusion, and diabetes mellitus as independent variables in logistic regression analyses, with the age <70 years at diagnosis as dependent variable. In a univariate analysis, male sex, platelet count $<10 \times 10^4/mm^3$, and genotype 1 were significantly correlated with age <70 years at diagnosis (Table 5). In a stepwise multivariate analysis, male (odds ratio, 2.035, 95% confidence interval, 1.208–3.427; $P = 0.008$) and platelet count $<10 \times 10^4/mm^3$ (odds ratio, 2.105, 95% confidence interval, 1.242–3.570; $P = 0.006$) were significant variables associated with age <70 years at diagnosis in pure HCV-related HCC patients.

Latency period to development of HCC

Of the 337 patients, 79 patients received blood transfusion (BT) and none of these transfusions was provided after the diagnosis of chronic liver disease. These patients were presumed infected with HCV by BT, and thus we considered the interval between BT and diagnosis of HCC as the latency period to development of HCC. Figure 1 shows that the duration to development of HCC was shorter in patients received BT at an older age than those transfused at a younger age. Interestingly, platelet counts of many patients who received BT at an older age were $\geq 10 \times 10^4/mm^3$, which meant that many patients who received BT at an older age develop HCC more rapidly when they had a noncirrhotic liver, in other words, elderly patients might tend to develop HCC despite their liver fibrosis stage and activity grade. Then, we selected sex, platelet count, Child-Pugh class, genotype, diabetes mellitus, and age at BT as independent variables in logistic regression analyses, with the duration to development of HCC <25 years as dependent variable. In a univariate analysis, age when received BT ≥35 years, platelet count $\geq 10 \times 10^4/mm^3$, and Child-Pugh class A were significantly correlated with duration to development of HCC <25 years (Table 6). In a stepwise multivariate analysis, age when received BT ≥35 years (odds ratio, 43.666, 95% confidence interval, 4.950–385.231; $P = 0.001$) and platelet count $\geq 10 \times 10^4/mm^3$ (odds ratio, 6.962, 95% confidence interval, 1.156–41.931; $P = 0.034$) were significant variables associated with the duration to development of HCC <25 years.

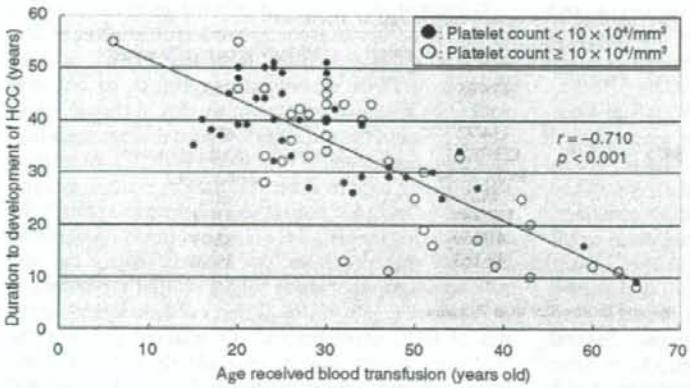

Fig. 1. Interval between age at blood transfusion and age at initial diagnosis in 79 patients with pure hepatitis C virus (HCV)-related hepatocellular carcinoma (*HCC*). *Closed circles*, patients with platelet count $<10 \times 10^4/mm^3$; *open circles*, patients with platelet count $\geq 10 \times 10^4/mm^3$. Statistical analysis by Spearman's nonparametric test

Table 6. Significant variables in association with duration to development of HCC within 25 years using univariate logistic analysis

Variables	Odds ratio	95% confidence interval	*P* value
Age received BT ≥35 years[a]	48.831	5.823–409.514	<0.001
Platelet count $\geq 10 \times 10^4/mm^3$[a]	8.462	1.733–41.306	0.008
Child-Pugh class A	5.173	1.064–25.160	0.042

BT, blood transfusion
[a]Significant variables in multivariate analysis

Discussion

In the present study, we demonstrated the clinicopathological features of elderly patients with HCV-related HCC compared with younger patients. The elderly patients were characterized as lower male/female ratio, better liver function, and less alcohol consumption than the younger patients. At first, we examined all 693 patients with anti-HCV and without HBsAg. Next, we examined 337 patients after excluding patients with factors affecting development of hepatocarcinogenesis. Results of these two examinations were almost the same, as already mentioned. Ultimately, we wanted to emphasize that elderly patients with HCV-related HCC tend to develop HCC despite their low-grade fibrosis stage, and they should be followed more closely.

Several risk factors for the development of HCV-related HCC have been identified to date, such as age, degree of liver fibrosis, male sex, alcohol consumption, positivity for HBsAg, and high AFP level.[15–20] Recently, elderly HCC patients with HCV have increased in number in Japan.[24–26] To improve the survival of elderly cirrhotic patients by increasing the percentage of cancers amenable to effective treatment, it is important to clarify the characteristics of elderly HCC patients with HCV. In the present study, we divided patients with HCV-related HCC into two groups, the younger and the elderly group, according to age at first diagnosis. In previous studies,[16,18,20] the authors divided HCC patients into two groups by age 50, 60, or 70. As we reported previously, elderly patients with HCV-related HCC have recently increased in number,[24] and in this study the median ages of patients were 66 and 69 for men and women, respectively. Because we want to clarify the characteristics of elderly patients in detail, they were divided by age less than or more than 70 years.

The incidence of HCC in HCV-related liver cirrhosis is significantly higher in patients with HBV antibody, such as anti-HBs and anti-HBc, than in those without.[27] First, we analyzed the patients who were positive for anti-HCV but negative for HBsAg. Furthermore, we also excluded anti-HBc-positive patients to reduce the influence of HBV on such analysis, and then we performed the next examination.

Several studies have shown that high AFP value is one of the risk factors of HCC.[15–20] In the present study, there was no significant difference in AFP between the elderly and the younger group.

Alcohol consumption is considered a significant risk factor for HCV-related HCC.[15,16,18] Our results showed significant differences between the elderly and the younger group, which might suggest that habitual alcohol drinkers develop HCC earlier than non-drinkers.

Silini et al.[37] reported a close association between HCV 1b and HCC, but recent studies have failed to

establish a correlation between HCV genotype and HCC.[28,38] In our study, the proportion of patients with genotype 1b was significantly greater in the younger group than the elderly group.

The Child-Pugh class correlates well with liver function. Our results showed that the elderly group tended to have better Child-Pugh class than the younger group. Platelet counts also indicated that the elderly group had better liver function and lower grade of fibrosis stage. The results of the pathological examination might support a tendency for the elderly group to have a lower grade of fibrosis stage, although this result may have a bias for selecting patients with good liver function when elderly patients underwent hepatic resection. Several studies reported that fibrosis stage and aging have major impacts on the incidence of HCC.[15–21] Yoshida et al.[21] suggested that the risk of HCC is strongly associated with the stage of liver fibrosis and that the annual incidence of HCC increased with the degree of liver fibrosis in Japan. In addition, progression of liver fibrosis is also strongly associated with aging.[22,23] As we recently reported, the time interval between blood transfusion and diagnosis of HCC was significantly shorter when patients received blood transfusions at an older age than at a younger age.[24] Therefore, we assumed that fibrosis stage could correlate strongly with aging for hepatocarcinogenesis and that elderly HCC patients should have a higher grade of fibrosis stage than younger patients. However, we found many cases with HCC and low-grade fibrosis stage among the elderly group. Furthermore, patients who received blood transfusions at an older age developed HCC within a shorter period of time than those at a younger age. It should be noted, however, that the former included more patients with a noncirrhotic liver than the latter. Elderly patients seemed to have a tendency to develop HCC earlier despite their low-grade fibrosis stage. Why is aging an independent risk factor for hepatocarcinogenesis? Chiaramonte et al.[39] suggested that increasing age is a risk factor, probably because it reflects a longer duration of cirrhosis, but in our study the majority of elderly patients developed HCC from a noncirrhotic liver. These findings suggest that the shorter duration to development of HCC was not only the result of early progression of liver fibrosis stage but caused by other reasons, e.g., aging. Surprisingly, fibrosis stage seemed to be inversely proportional to age at diagnosis. This finding could mean that the impact of aging is stronger than the degree of liver fibrosis for development of HCV-related HCC. However, the present study is a retrospective study and included only HCC patients while excluding non-HCC patients with HCV. Further studies are needed to determine the correlation between HCV patients with HCC and those without.

Benson et al.[40] suggested that the increase in cancer rates that occurs after the age of 30 is the result of activation of quiescent cells with damaged DNA, or deactivation of DNA surveillance or repair, or impaired apoptosis. We reported previously that in chronic hepatitis C patients, the rate of DNA synthesis becomes high and genomic instability may increase during alternating necrosis and regeneration, resulting in malignant transformation and progression to an aggressive state.[41] We also reported that telomere length in the liver shortened not only with progression of fibrosis staging but also with aging. Another study suggested that the reduction of telomere length in chronic liver disease increased the risk of HCC development.[42] Considered together, the foregoing results suggest that elderly patients might have sufficiently short telomeres for developing HCC even if they had a noncirrhotic liver because the telomeres have shortened in length with aging.

In conclusion, we demonstrated in the present study that elderly patients with HCV developed HCC despite low-grade fibrosis stage. Further studies will be required about the impact of aging on HCV-related hepatocarcinogenesis.

References

1. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 1989;244: 359–62.
2. Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, et al. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 1989;244:362–4.
3. Bruix J, Barrera JM, Calvet X, Ercilla G, Costa J, Sanchez-Tapias JM, et al. Prevalence of antibodies to hepatitis C virus in Spanish patients with hepatocellular carcinoma and hepatic cirrhosis. Lancet 1989;2:1004–6.
4. Colombo M, Kuo G, Choo QL, Donato MF, Del Ninno E, Tommasini MA, et al. Prevalence of antibodies to hepatitis C virus in Italian patients with hepatocellular carcinoma. Lancet 1989;2:1006–8.
5. Saito I, Miyamura T, Ohbayashi, Harada H, Katayama T, Kikuchi S, et al. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc Natl Acad Sci USA 1990;87:6547–9.
6. Hasan F, Jeffers LJ, De Medina M, Reddy KR, Parker T, Schiff ER, et al. Hepatitis C-associated hepatocellular carcinoma. Hepatology 1990;12:589–91.
7. Kew MC, Houghton M, Choo QL, Kuo G. Hepatitis C virus antibodies in Southern African blacks with hepatocellular carcinoma. Lancet 1990;335:873–4.
8. Sbolli G, Zanetti AR, Tanzi E, Cavanna L, Civardi G, Fornari F, et al. Serum antibodies to hepatitis C virus in Italian patients with hepatocellular carcinoma. J Med Virol 1990;30:230–2.
9. Vargas V, Castells L, Esteban JI. High frequency of antibodies to the hepatitis C virus among patients with hepatocellular carcinoma. Ann Intern Med 1990;112:967.
10. Yu MC, Tong MJ, Coursaget P, Ross RK, Govindarajan S, Henderson BE. Prevalence of hepatitis B and C viral markers in black and white patients with hepatocellular carcinoma in the United States. J Natl Cancer Inst 1990;82:1038–41.