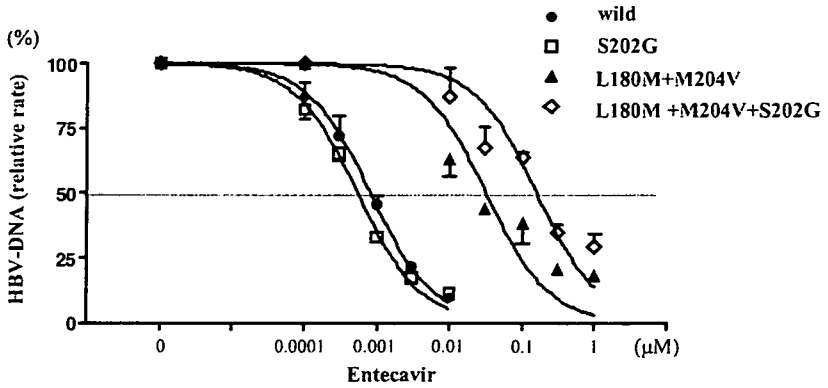

Fig. 3. In vitro analyses of susceptibilities of wild-type HBV and three mutants (rtS202G, rtL180M + M204V, rtL180M + M204V + S202G) to entecavir (ETV) after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4 genome lengths HBV and treated with the indicated amount of entecavir. Data are the dose–response curves of the four HBV strains against entecavir. The strains were used to estimate the entecavir IC_{50} values for each HBV strains. Values are relative to no entecavir treatment controls for each strain. Experiments were performed in triplicates.

TABLE IV. In Vitro Susceptibility of rtS202/rtM204 Mutant to Lamivudine (LAM) and Adefovir (ADV)

	LAM		ADV	
	IC_{50} (μM)	Fold resistance	IC_{50} (μM)	Fold resistance
Wild	0.1	1	0.39	1
L180M + M204V	>100	>1,000**	—	—
L180M + M204V + S202G	>100	>1,000**	0.32	0.82[a]

Experiments were performed in triplicates.
[a]NS, not significant.
**$P < 0.001$ compared with the wild-type.

multiple anti-HBV drugs is real. Therefore, further studies are necessary to develop safes and more useful treatment strategies.

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REFERENCES

Allen MI, Deslauriers M, Andrews CW, Tipples GA, Walters KA, Tyrrell DL, Brown N, Condreay LD. 1998. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. Hepatology 27:1670–1677.

Bruix J, Llovet JM. 2003. Hepatitis B virus and hepatocellular carcinoma. J Hepatol 39:S59–S63.

Colonno RJ, Rose R, Baldick CJ, Leveine S, Pokornowski K, Yu CF, Walsh A, Fang J, Hsu M, Mazzucco C, Eggers B, Zhang S, Plym M, Klesczewski K, Tenney DJ. 2006. Entecavir resistance is rare in nucleoside naive patients with hepatitis B. Hepatology 44:1656–1665.

Delaney WE, Yang H, Westland CE, Das K, Arnold E, Gibbs CS, Miller MD, Xiong S. 2003. The hepatitis B virus polymerase mutation rtV173L is selected during lamivudine therapy and enhances viral replication in vitro. J Virol 77:11833–11841.

Ganem D, Prince AM. 2004. Hepatitis B virus infection–natural history and clinical consequences. N Engl J Med 350:1118–1129.

Gunther S, Sommer G, Von Breunig F, Iwanska A, Kalinina T, Sterneck M, Will H. 1998. Amplification of full-length hepatitis B virus genomes from samples from patients with low levels of viremia: Frequency and functional consequences of PCR-introduced mutations. J Clin Microbiol 36:531–538.

Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF. 1998. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 339:61–68.

Lai CL, Dienstag J, Schiff E, Leung NW, Atkins M, Hunt C, Brown N, Woessner M, Boehme R, Condreay L. 2003. Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. Clin Infect Dis 36:687–696.

Lee YS, Suh DJ, Lim YS, Jung SW, Lee HC, Chung YH, Lee YS, Yoo W, Kim SO. 2006. Increased risk of adefovir resistance in patients with lamivudine-resistant chronic hepatitis B after 48 weeks of adefovir dipivoxil monotherapy. Hepatology 43:1385–1391.

Levine SD, Hernandez G, Yamanaka S, Zhang R, Rose R, Weinheimer S, Colonno RJ. 2002. Efficacies of entecavir against lamivudine-resistant hepatitis B virus replication and recombinant polymerases in vitro. Antimicrob Agents Chemother 46:2525–2532.

Maecellin P, Asselah T. 2005. Resistance to adefovir: A new challenge in the treatment of chronic hepatitis B. J Hepatol 43:920–923.

Nevens F, Main J, Honkoop P, Tyrrell DL, Barber J, Sullivan MT, Fevery J, De Man RA, Thomas HC. 1997. Lamivudine therapy for chronic hepatitis B: A six-month randomized dose-ranging study. Gastroenterology 113:1258–1263.

Ono SK, Kato N, Shiratori Y, Kato J, Goto T, Schinazi RF, Carrilho FJ, Omata M. 2001. The polymerase L528M mutation cooperates

with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. J Clin Invest 107:449–455.

Rapti I, Dimou E, Mitsoula P, Hadziyannis SJ. 2007. Adding-on versus switching-to adefovir therapy in lamivudine-resistant HBeAg-negative chronic hepatitis B. Hepatology 45:307–313.

Sherman MC, Yurdaydin C, Sollano J, Silva M, Liaw YF, Cianciara J, Boron-Kaczmarska, A, Martin P, Goodman Z, Colonno R, Cross A, Denisky G, Kreter B, Hindes R. 2006. Entecavir for treatment of lamivudine-refractory, HBeAg-positive chronic hepatitis B. Gastroenterology 130:2039–2049.

Suzuki Y, Kumada H, Ikeda K, Chayama K, Arase Y, Saitoh S, Tsubota A, Kobayashi M, Koike M, Ogawa N, Tanikawa K. 1999. Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. J Hepatol 30:743–748.

Tenney DJ, Levine SM, Rose RE, Walsh AW, Weinheimer SP, Discotto L, Plym M, Pokornowski K, Yu CF, Angus P, Ayres A, Bartholomeusz A, Sievert W, Thompson G, Warner N, Locarnini S, Colonno RJ. 2004. Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. Antimicrob. Agents Chemother. 48:3498–3507.

Tenney DJ, Rose RE, Baldick CJ, Levine SM, Pokornowski KA, Walsh AW, Yu CF, Zhang S, Mazzucco CE, Eggers B, Hsu M, Plym MJ, Poundstone P, Yang J, Colonno RJ. 2007. Two-year assessment of entecavir resistance in Lamivudine-refractory hepatitis B virus patients reveals different clinical outcomes depending on the resistance substitutions present. Antimicrob Agents Chemother 51:902–911.

Villet S, Ollivet A, Pichoud C, Barraud L, Villeneuve JP, Trepo C, Zoulim F. 2007. Stepwise process for the development of entecavir resistance in a chronic hepatitis B virus infected patient. J Hepatol 46:531–538.

Yim HJ, Hussain M, Liu Y, Wong SN, Fung SK, Lok SF. 2006. Evolution of multi-drug resistant hepatitis B virus during sequential therapy. Hepatology 44:703–712.

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RAPID COMMUNICATION

Effects of a 24-week course of interferon-α therapy after curative treatment of hepatitis C virus-associated hepatocellular carcinoma

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Abstract

AIM: To assess whether a 24-wk course of interferon (IFN) could prevent hepatocellular carcinoma (HCC) recurrence and worsening of liver function in patients with hepatitis C virus (HCV)-infected patients after receiving curative treatment for primary HCC.

METHODS: Outcomes in 42 patients with HCV infection treated with IFN-α, after curative treatment for primary HCC (IFN group), were compared with 42 matched curatively treated historical controls not given IFN (non-IFN group).

RESULTS: Although the rate of initial recurrence did not differ significantly between IFN group and non-IFN group (0%, 44%, 61%, and 67% *vs* 4.8%, 53%, 81%, and 87% at 1, 3, 5, and 7 years, $P = 0.153$, respectively), IFN group showed a lower rate than the non-IFN group for second recurrence (0%, 10.4%, 28%, and 35% *vs* 0%, 30%, 59%, and 66% at 1, 3, 5 and 7 years, $P = 0.022$, respectively). Among the IFN group, patients with sustained virologic response (SVR) were less likely to have a second HCC recurrence than IFN patients without an SVR, or non-IFN patients. Multivariate analysis identified the lack of SVR as the only independent risk factor for a second recurrence, while SVR and Child-Pugh class A independently favored overall survival.

CONCLUSION: Most intrahepatic recurrences of HCV-related HCC occurred during persistent viral infection. Eradication of HCV is essential for the prevention of HCC recurrence and improvement of survival.

Key words: Hepatitis C virus, Hepatocellular carcinoma, Recurrence, Survival, Sustained virological response

Jeong SC, Aikata H, Katamura Y, Azakami T, Kawaoka T, Saneto H, Uka K, Mori N, Takaki S, Kodama H, Waki K, Imamura M, Shirakawa H, Kawakami Y, Takahashi S, Chayama K. Effects of a 24-week course of interferon-α therapy after curative treatment of hepatitis C virus-associated hepatocellular carcinoma. *World J Gastroenterol* 2007; 13(40): 5343-5350

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms worldwide. Chronic infection with hepatitis C virus (HCV) has been causally associated with HCC[1-3]. Recent advances in imaging and treatment have brought about some improvement in prognosis of patients with HCV-related HCC, but outcomes are still unsatisfactory. The 5-year survival rate is only 50%-70%, even after curative treatment such as hepatic resection or local ablation[4]. Reasons for this unfavorable prognosis are considered to include high intrahepatic tumor recurrence rates and sustained hepatic damage, both resulting from HCV infection[5].

Even after curative hepatic resection for HCV-related HCC, the rate of intrahepatic tumor recurrence within 1 year is 20%-40%, rising to about 80% by 5 years[4,6-8]. Intrahepatic recurrence of HCC may result from intrahepatic metastasis originating from the primary HCC, or from ongoing multicentric carcinogenesis related to chronic HCV infection. Underlying HCV-related hepatic damage may also compromise hepatic functional reserve, and worsen clinical outcome. Thus prevention of HCC recurrence and preservation of liver function are both high priorities for improving prognosis of patients with HCV-

related HCC.

Interferon (IFN) therapy for patients with HCV infection is effective in reducing serum alanine transaminase (ALT) activity and in eradicating HCV[9,10], and thus IFN could have value in minimizing hepatic necrosis, inflammation and fibrosis, as well as reducing the incidence of HCC. Several recent studies have reported that IFN therapy, even after curative treatment for HCV-related HCC, could prevent HCC recurrence and improve survival[11-17]. Unfortunately, since these studies are characterized by differing IFN regimens, definitions of IFN responses, and background characteristics of patients, results have varied and no standard IFN regimen has been established for after curative treatment of HCV-related HCC. As well, the mechanisms by which IFN suppresses HCC recurrence, including possible direct anti-tumor and anti-inflammatory effects, remain uncertain.

In the present study, recurrence and survival outcomes in matched historical controls were compared with those in patients receiving a 24-wk course of IFN-α therapy after receiving curative treatment for HCC.

MATERIALS AND METHODS

Patients

We retrospectively reviewed 495 consecutive patients treated for primary HCC associated with HCV infection at Hiroshima University Hospital from March 1992 to March 2004. Of these, 384 with HCC initially underwent therapeutic intervention with curative intent. Curative treatment was defined as complete tumor eradication, with no residual tumor visible by computed tomography, or resection of all evident tumor tissue. Medical treatment included percutaneous radiofrequency ablation (RFA), ethanol injection, and microwave coagulation therapy (MCT). Surgical treatment included hepatic resection and ablation during laparotomy.

Among these 384 patients, we administered IFN therapy to 42 who met the following eligibility criteria: age under 70 years; up to three tumors with none exceeding 30 mm in diameter, or a solitary tumor less than 50 mm in diameter; tumor-node-metastasis (TNM) stage I, II, or III; detectable serum HCV RNA; seronegativity for hepatitis B surface antigen; chronic hepatitis or compensated cirrhosis with a Child-Pugh class of A or B; platelet count above 70000/μL; absence of local recurrence during the follow-up period; and absence of ectopic intrahepatic recurrence within 24 wk after treatment for primary HCC. We used the TNM classification system of the Liver Cancer Study Group of Japan as the staging system for HCC[18]. Underlying liver conditions such as hepatitis or cirrhosis were confirmed by laboratory, pathologic and radiologic examinations. We classified liver function in chronic hepatitis as Child class A because chronic hepatitis is a known pre-cirrhotic condition. There were only a few chronic hepatitis cases: three in the IFN group and four in the non-IFN group.

As historical control subjects, we selected 42 patients with no IFN therapy after treatment for primary HCC (non-IFN group). These 42 patients, who met the eligibility criteria noted above, were matched by age, gender, tumor size, TNM stage of HCC, serum albumin, platelet counts, and Child-Pugh class with patients who received IFN therapy (IFN group).

Table 1 Patient characteristics

	IFN group (n = 42)	Non-IFN group (n = 42)	P
Median age in years (range)	62[1] (45-69)	63[1] (40-69)	NS
Gender (male/female)	36/6	29/13	NS
Alb (g/dL)	3.9[1]	3.9[1]	NS
PLT (× 10000/μL)	12[1]	11.5[1]	NS
ICG R-15 (%)	17[1]	18[1]	NS
CH or Child A/B	35/7	35/7	NS
Size of main tumor (mm)	20[1] (10-50)	15[1] (10-50)	NS
AFP (ng/mL)	26[1]	31.4[1]	NS
No. of HCC (single/two or three)	30/12	36/6	NS
Stage (I / II or III)	14/28	23/19	NS
Treatment of HCC (medical/surgical)	18/24	20/22	NS

IFN: interferon; Alb: albumin; PLT: platelet; ICG-R15: indocyanine green retention at 15 min; CH: chronic hepatitis; AFP: alpha-fetoprotein; HCC: hepatocellular carcinoma. [1]Median.

Thus, a total of 84 patients (42 in the IFN group and 42 in the non-IFN group) were enrolled. All agreed to participate in the research protocol, which was approved by the hospital research ethics board. Table 1 shows the baseline characteristics of the two groups, indicating no significant differences for age, gender, liver function, tumor characteristics, or therapeutic methods used against HCC.

IFN therapy

In the IFN group, patients received 6 MIU of natural IFN-α (human lymphoblastoid IFN, Sumiferon; Dainippon Sumitomo Pharmaceuticals, Osaka, Japan) intramuscularly every day for 2 wk, followed by three times weekly for 22 wk. IFN therapy began within 24 wk after the initial treatment for HCC. All patients were evaluated every week in an outpatient setting during IFN treatment. Qualitative detection of HCV-RNA was performed by a standardized qualitative reverse transcription-polymerase chain reaction (RT-PCR) assay at every 4 wk during and after IFN treatment.

Among the patients who received IFN therapy, 28 were of HCV genotype 1 and 14 were of HCV genotype 2. These 42 patients had various pretreatment viral loads. Twenty patients (genotype 1, n = 11; genotype 2, n = 9) had high viral loads (≥ 100 kIU/mL by PCR), and 22 (genotype 1, n = 17; genotype 2, n = 5) had low viral loads (≤ 100 kIU/mL by PCR). The 42 patients were divided into two subgroups according to virologic response, i.e. patients with *vs* without a sustained virologic response (SVR). SVR was defined as the sustained absence of serum HCV RNA for more than 24 wk after completion of IFN treatment. Absence of SVR included both persistent viremia (no response) and transient viral disappearance (transient response) during or after IFN therapy. Biochemical response was defined as ALT activity declining to a value within the normal reference range in the presence of viremia.

Follow-up

After curative treatment for primary HCC, all patients studied underwent liver function tests, serum tumor marker assays, such as those for α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist II (PIVKA-II) every month, abdominal ultrasonography every 3 mo, and dynamic computed tomography (CT) every 6 mo. If recurrence of HCC was suspected, additional examinations including CT during arteriography or tumor biopsy were performed. Recurrence of HCC was defined as any new nodules indicated by CT as hyperattenuation during hepatic arteriography or by hypoattenuation in CT performed during arterioportography. Hypovascular HCC was confirmed histopathologically after fine-needle aspiration biopsy. Patients with recurrent HCC were treated medically or surgically, with curative intent if possible.

In IFN patients, including those with or without SVR, and in the non-IFN group, we compared both the rate of HCC recurrence and the survival rate. We also sought to identify significant prognostic indicators for survival and recurrence after curative treatment of primary HCC.

Statistical analysis

Chi-squared and Fisher exact tests were used for categorical variables, while Student's *t* test and the Mann-Whitney *U* test were used for continuous and ordinal variables, as appropriate. The Kaplan-Meier method was used to assess cumulative survival and recurrence rates, calculated from the date of diagnosis to the date of disease recurrence or death. Surviving patients and those who died of causes unrelated to the liver were defined as censored cases, while patients who died of causes related to the liver were defined as non-censored cases. The log-rank test was used to compare survival and recurrence curves. Univariate and multivariate predictors of survival or recurrence time were determined using the Cox proportional hazard model. Hazard ratios and their 95% confidence intervals (95% CI) were computed. $P < 0.05$ was considered to indicate statistical significance. The JMP version 5.1 statistical software package (SAS Institute, Cary, NC, USA) was used for analysis of data.

RESULTS

Virologic and biochemical responses to IFN therapy and side effects

The 42 patients receiving IFN therapy included 29 in the SVR group and 13 in the group without SVR (10 transient virological responders, 3 with no virological response). In the group without SVR, 7 biochemical responders who had a normalized ALT included 5 with transient virological responses and 2 with no virological response. Although there was no significant difference in the population of patients with HCV genotype 1 between the SVR and non-SVR group, patients in the former had significantly lower pre-IFN viral loads than patients in the latter group. In the SVR group, 24 patients received full-dose IFN therapy without dose reduction, while five patients received a reduced dose of IFN until completion of treatment. In the group without SVR, one patient with no response discontinued IFN treatment at 16 wk because of a recurrence of HCC, while three patients with a transient response discontinued treatment because of generalized fatigue. The remainder of the group without SVR received the full course of IFN therapy. Thus, most patients were able to complete the 24-wk course.

Recurrence of HCC

In the IFN group, first recurrences of HCC developed in 20 patients after the initial treatment for HCC during a median follow-up period of 32 mo. Of these recurrences, 10 were in patients with SVR (10/29) and 10 in patients without SVR (10/13), including 7 transient virological responders and 3 with no virological response. For the 7 biochemical responders without SVR, HCC recurred in 6 patients, including 5 transient virological responders and 1 with no virological response. Of these 20 patients with recurrence, 18 were treated with local ablation therapy or surgical resection without leaving any residual tumor. The remaining 2 patients developed uncontrolled multiple HCC and were excluded from the subsequent study concerning the next recurrence. One died of HCC, while the other was treated repeatedly with hepatic arterial infusion, and has survived. Three patients in the SVR group and 7 in the group without SVR (5 transient virological responders and 2 with no virological response) had a second recurrence of HCC. Of these 10 patients with a second recurrence, 3 (2 transient virological responders and one with no virological response) developed uncontrolled HCC, while others were treated curatively with hepatic resection or local ablation therapy. In the non-IFN group, a first recurrence of HCC occurred in 30 patients during a median follow-up period of 31 mo. HCC recurred in 11 of the 17 who had a normal ALT level. Among the 30 patients with recurrent HCC, 25 were treated with local ablation therapy or surgical treatment, with no residual tumor. The remaining 5 patients who did not undergo curative therapy were treated repeatedly with transarterial chemoembolization. A second recurrence developed in 15 of the 25 patients who had curative treatment for a first recurrence. Among these 15 patients, 10 were treated curatively (9 with local ablation and 1 with hepatic resection). The remaining 5 patients had uncontrolled multiple HCC as their second recurrence.

Overall cumulative rates for first and second recurrence of HCC were compared between the groups. The 1-, 3-, 5- and 7-year rates for first recurrence in the IFN and non-IFN group were 0% *vs* 4.8%, 44% *vs* 53%, 61% *vs* 81%, and 67% *vs* 87%, respectively (Figure 1A, $P = 0.153$; no significant difference between groups). However, the 1-, 3-, 5-, and 7-year rates for second recurrence in the IFN and non-IFN group were 0% *vs* 0%, 10.4% *vs* 30%, 28% *vs* 59%, and 35% *vs* 66%, respectively (Figure 1B, $P = 0.022$). Thus, the second-recurrence rate was significantly lower in the IFN group than in the non-IFN group.

Next, the recurrence rates of HCC were compared between the SVR group, the non-SVR group and the non-IFN group. The rate of first recurrence was significantly lower in the SVR group than in the non-SVR and non-IFN group (Figure 2A). The rate of second recurrence in the

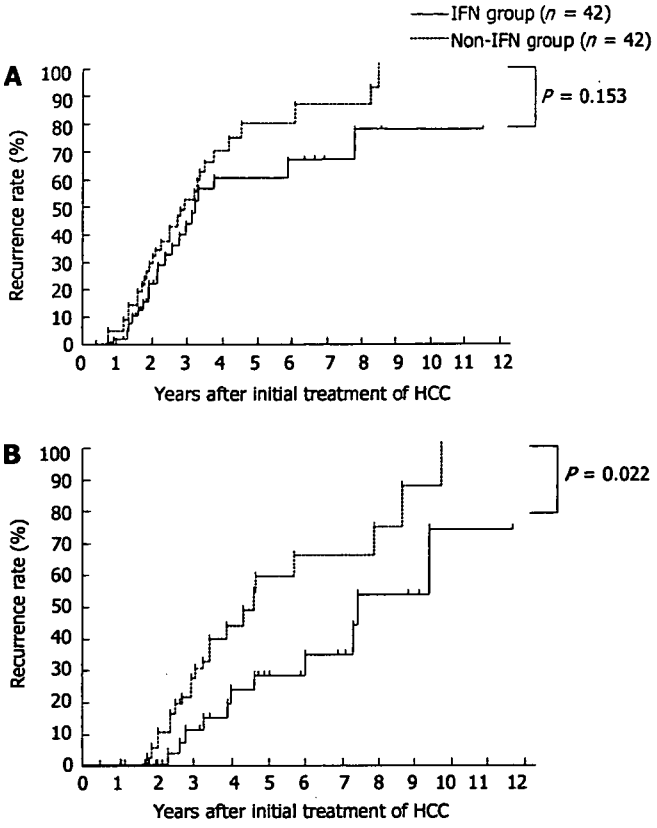

Figure 1 Cumulative recurrence rates after curative treatment of HCC. A: Rates of first recurrence compared between IFN and non-IFN groups, showed no significant difference ($P = 0.153$); B: Rates of second recurrence compared between IFN and non-IFN groups. The second recurrence rate for the IFN group was lower than that for the non-IFN group ($P = 0.022$).

SVR group was also lower than that in the non-SVR and non-IFN groups; this decrease was significantly greater than that for the rate of first recurrence (Figure 2B). No significant difference was seen in cumulative rates for first or second recurrence between the non-SVR and non-IFN groups. We also confirmed that biochemical responders in the non-SVR and non-IFN groups showed similar Kaplan-Meier curves for cumulative recurrence (data not shown). Recurrence curves were similar between the non-SVR group, including biochemical responders, and the non-IFN group, therefore, we defined these two groups as "non-SVR status" for statistical analysis. Factors found to be significantly associated with first recurrence by univariate analysis were tumor size (≥ 20 mm) and non-SVR status ($P = 0.019$, $P = 0.0067$, respectively). Multivariate analysis showed that no independent risk factor was associated with the first recurrence of HCC (data not shown), although non-SVR status tended to be associated with first recurrence ($P = 0.0657$). As shown in Table 2, univariate analysis indicated that non-SVR status, low platelet count (< 100 000) and high indocyanine green retention (≥ 20%) were significantly associated with second recurrence. Multivariate analysis identified only SVR status as a significant independent inhibiting factor for second recurrence of HCC.

Survival of patients

During the observation period, 13 of the total patients studied died of liver disease. Nine died of HCC and 4 of liver failure. When we compared cumulative survival rates between the IFN and the non-IFN groups (Figure 3A), the respective rates were 100% *vs* 95% at 3 years, 100% *vs* 72% at 5 years, and 86% *vs* 63% at 7 years. The cumulative survival rate was significantly higher in the IFN group than in the non-IFN group ($P = 0.039$). Median survival time following the first treatment of HCC was 52.3 mo (range, 12-158) in the IFN group and 51.8 mo (range, 11-126) in the non-IFN group. In the IFN group, 2 patients died of advanced HCC, 1 with an SVR and the other without. No patients in the IFN group died of hepatic failure. In the non-IFN group, 7 patients died of HCC and four of hepatic failure.

Figure 3B shows cumulative survival curves for the SVR, non-SVR and non-IFN groups. The rate of survival in the SVR group was significantly better than that in the non-IFN group ($P = 0.029$), while no significant difference was evident between the non-SVR and non-IFN group ($P = 0.248$).

Pretreatment factors found to be significantly associated with survival by univariate analysis subsequently were evaluated by Cox regression analysis to determine independent factors. Multivariate analysis showed that SVR status and Child-Pugh class A were independent factors favorably associated with long survival (Table 3).

Liver function

Compared with the non-IFN group, patients who received IFN therapy were less likely to have worsening of hepatic dysfunction. For the SVR, non-SVR and non-IFN groups, we compared the average score for Child-Pugh classification at initial treatment of HCC with that at the time of data analysis. Median observation time was 59.8 mo in the SVR group, 45 mo in the non-SVR group, and 51.8 mo in the non-IFN group. There were no significant differences in the Child-Pugh classification score among these three groups at the time of initial treatment of HCC; however, at the time of data analysis, scores in the non-IFN group were significantly worse than in the SVR group ($P = 0.003$). No significant difference was seen between the non-SVR and non-IFN groups (Figure 4).

DISCUSSION

The present study compared historical control subjects with no IFN treatment with other subjects who were treated with IFN. Background characteristics showed no significant difference between the groups. IFN and non-IFN group did not differ significantly in their rate of first recurrence, but did differ significantly in their rate of second recurrence. According to IFN response, the recurrence rate in the SVR group was significantly lower than that in the non-SVR and non-IFN group, while recurrence rates in the non-SVR and non-IFN group did not differ significantly. Thus, SVR (i.e. HCV eradication) was the most important, and only, inhibiting factor for decreasing risk of HCC recurrence, associated with a 24-wk course of IFN-α therapy following HCC treatment.

Although several recent studies have reported the

Table 2 Factors associated with second recurrence

Variables	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
SVR	0.454	0.246-0.728	0.0005	0.457	0.243-0.757	0.0015
PLT > 100000/μ	0.553	0.373-0.814	0.003	0.694	0.445-1.069	0.0973
ICG R-15 (< 20%)	0.667	0.450-0.965	0.032	0.685	0.447-1.035	0.0721

Cox's proportional hazards model was used.

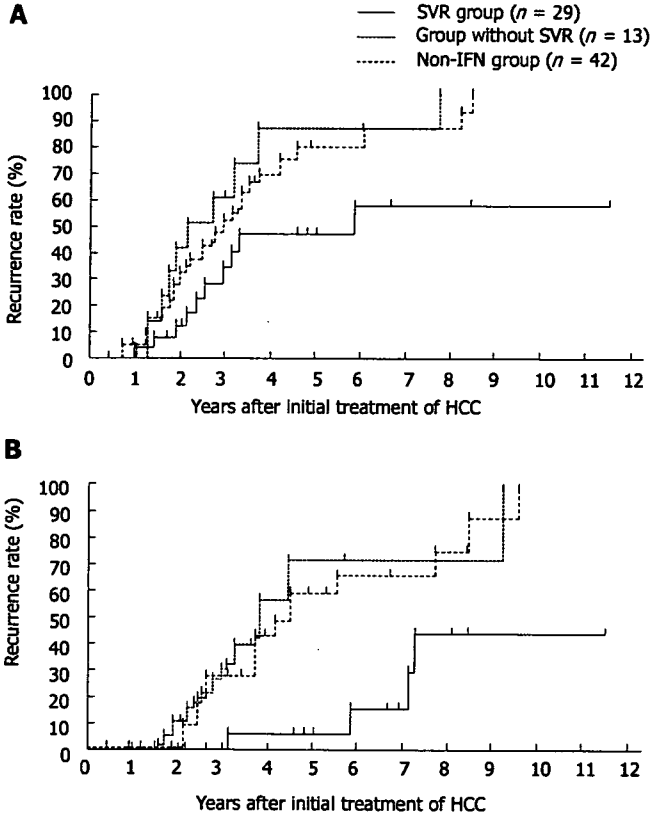

Figure 2 Cumulative recurrence rates according to SVR to IFN therapy after curative treatment of HCC. **A**: Rates of first recurrence compared among SVR, non-SVR and non-IFN groups. The rate of first recurrence of HCC in the SVR group was significantly lower than in the non-SVR and non-IFN groups ($P = 0.002$, $P = 0.016$, respectively). No significant difference in first recurrence rate was seen between the non-SVR and non-IFN groups ($P = 0.381$); **B**: Rates of second recurrence compared among the three groups. Second recurrence of HCC was suppressed in the SVR group compared with the non-SVR and non-IFN groups ($P = 0.0037$, $P = 0.0019$, respectively), and to a more pronounced degree than for the first recurrence rate. No significant difference in second recurrence rate was seen between the non-SVR and non-IFN groups ($P = 0.90$).

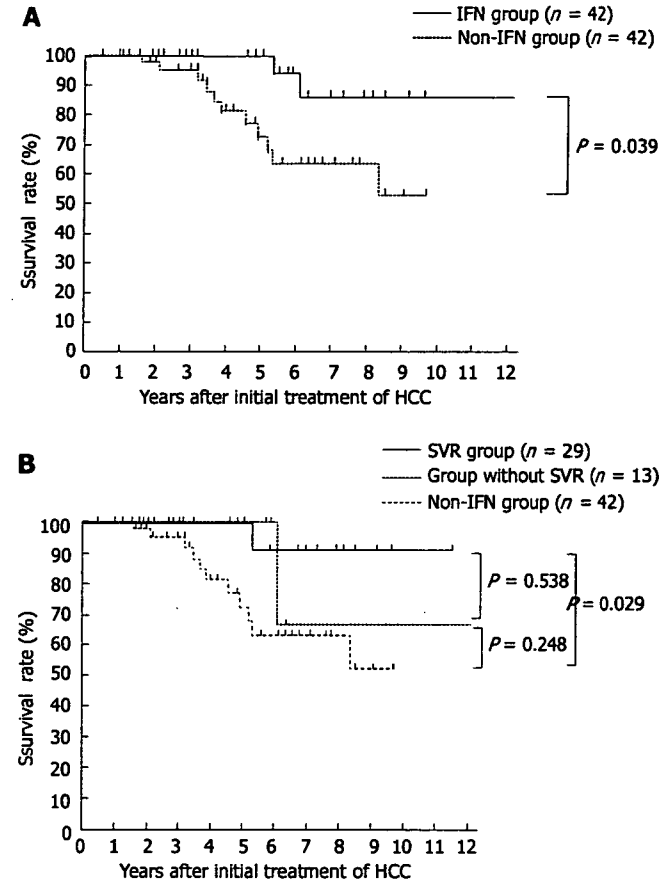

Figure 3 Cumulative survival rates after curative treatment of HCC. **A**: Comparison of cumulative survival rates in the IFN and non-IFN groups. The cumulative survival rate was significantly higher in the IFN group than in the non-IFN group ($P = 0.039$); **B**: Comparison of cumulative survival rates in the SVR, non-SVR and non-IFN groups. Although no significant overall difference was found between the SVR and non-SVR groups ($P = 0.538$), the SVR group had a particularly high survival rate compared with the non-IFN group ($P = 0.029$).

efficacy of chemoprevention with IFN after treatment of HCV-related HCC, the basis of this benefit has not been determined, since IFN has a variety of biologic effects, including antiviral, antiproliferative, immunomodulatory[19-22] and anti-fibrogenic[23,24] activities; growth inhibition through changes in signal transduction[19,25,26]; and activation of natural killer cells[27] and T cells[28,29]. Through these various effects, IFN therapy is thought to suppress tumor recurrence directly and/or indirectly.

Sakaguchi *et al*[15] have reported that low-dose, long-term, intermittent IFN-α therapy can, by a direct anti-cancer effect, inhibit intrahepatic metastasis but not multicentric occurrences. Lai *et al*[29] have reported that IFN-α therapy is effective in advanced HCC. Several experimental studies have shown that IFN inhibits the growth of a human hepatoma cell line[11,15]. In partial disagreement, however, Nishiguchi *et al*[12,14], Suou *et al*[16] and Shiratori *et al*[17] have reported that the rate of HCC recurrence was not different between IFN and non-IFN group during the first few years, but later became significantly lower in the IFN group. They suggested that IFN reduced HCC recurrence in the later period of observation by suppressing multicentric occurrence, as an indirect anti-tumor effect that was related to sustained hepatic inflammation. Although the present study did not have a randomized controlled design, and details of the

Table 3 Factors associated with survival

Variables	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
SVR	0.409	0.096-0.922	0.028	0.329	0.076-0.761	0.006
Child-Pugh class A	0.521	0.299-0.922	0.027	0.463	0.238-0.875	0.019
ICG R-15 (< 20%)	0.551	0.286-0.968	0.038	0.724	0.351-1.429	0.350

Cox's proportional hazards model was used.

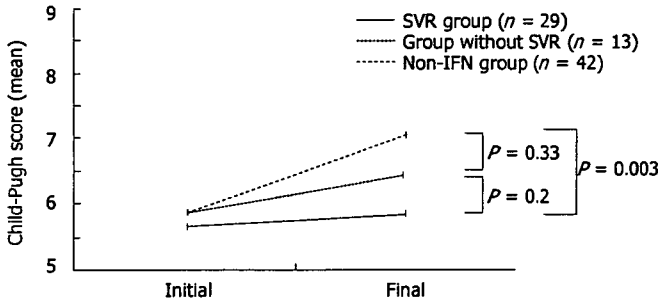

Figure 4 Influence of IFN therapy after curative treatment of HCC on Child-Pugh scores. IFN-treated patients were less likely to show deterioration of hepatic function. In particular, liver function scores in the SVR group were significantly better preserved than in the non-IFN group ($P = 0.003$). Median observation time was 59.8 mo in the SVR group, 45 mo in the non-SVR group, and 51.8 mo in the non-IFN group.

IFN protocol differed from those of others, the long-term results appear to be similar among studies. Recurrence during the first few years might involve undetectable intrahepatic metastasis, or a potential malignant tumor already existing at the time of treatment of the primary HCC; afterward, HCC might recur as multicentric new liver tumor, accompanied by sustained hepatic necrosis and inflammation. Although a direct anti-cancer effect of IFN might to some extent have directly inhibited HCC recurrence, our IFN doses were insufficient to suppress intrahepatic metastatic tumors because there was only a 24-wk treatment. Therefore, in our study, we believe that IFN therapy suppressed HCC recurrence less by a direct anti-tumor effect than by an indirect effect through inhibition of the chronic inflammation associated with HCV infection in the later period of observation.

Several studies have reported that recurrence was suppressed not only in virologic responders to IFN, but also in biochemical responders, even though HCV was not eradicated[12-14]. However, the recurrence rates in our study did not differ significantly between biochemical responders and the non-IFN group. HCV eradication appeared to stand alone as an IFN effect capable of inhibiting recurrence, with eradication having a stronger influence against second recurrence than the first. The differences between the results of the various studies might be due to several reasons. In most previous studies, IFN therapy was given for more than 48 wk, compared with our 24 wk. Differences may also have been present in underlying hepatic inflammatory conditions such as chronic hepatitis and cirrhosis. Although such differences introduce some uncertainty to the conclusions, several recent studies suggest that HCV core protein might directly participate in hepatocarcinogenesis[28,29], which supports the importance of virus eradication.

Although some other recent studies have reported that IFN therapy following HCC treatment also improves liver function and survival of patients with HCV-related HCC, which of the specific IFN actions is important for these benefits remains unknown. We found that overall survival rate and preservation of liver function were significantly better in the SVR group than in the other groups, even including biochemical responders, with all subgroups without SVR resembling non-IFN patients. Favorable independent factors associated with survival by multivariate analysis were SVR and Child–Pugh class A. Thus, with a 24-wk course of IFN-α therapy, HCV eradication appears necessary for prolonging survival, suppressing HCC recurrence, and preserving liver function.

As stated above, effective management of HCV infection is needed, as well as direct treatment of the primary HCC. Although our study had limitations, such as the use of historical controls and a small number of patients, we could demonstrate a clear requirement for HCV eradication to improve survival after a short-course IFN-α therapy. Ribavirin combination or pegylated IFN therapy are considered more effective in HCV eradication than conventional IFN monotherapy[32-34]. Several studies have indicated that pegylated IFN therapy is superior to conventional IFN when administered for 48 wk[34-41]. Pegylated IFN therapy, with or without ribavirin, may improve prognosis in selected patients with no sustained initial response to conventional IFN. For patients who cannot undergo standard-dose IFN therapy because of limited hepatic reserve or thrombocytopenia, low-dose IFN therapy for a longer course might be effective. Nonetheless, further studies with larger controlled groups and long-term follow-up need to be performed to establish what constitutes optimal management of HCV infection after HCC treatment.

COMMENTS

Background

Risk of multicentric recurrence of hepatocellular carcinoma (HCC) and liver function deterioration remains high in hepatitis C virus (HCV)-infected patients even after receiving curative treatment for primary HCC. Most intrahepatic recurrences occurred during persistent viral infection. Although several recent studies have reported the efficacy of chemoprevention with interferon (IFN) therapy after treatment of HCV-related HCC, there was no standard IFN regimen. We investigated whether 24-week course of IFN-α therapy following curative treatment for primary HCC associated with HCV infection could suppress HCC recurrence and improve prognosis.

Research frontiers

To obtain sustained virological response (SVR) was important for suppression of HCC recurrence and for long-term survival in a 24-week course of IFN-α therapy.

Innovations and breakthroughs

Our study demonstrated that only SVR status by a 24-wk IFN-α therapy was the most important factor for decreasing risk of HCC recurrence in the later period of observation including second recurrence.

Applications

This study demonstrated that compared with non-IFN and non-SVR group, SVR group decreased the rate of recurrence, preserved liver function, and prolonged survival time in a 24-wk course of IFN-α therapy.

Peer review

This is a matched historical case controlled study concerning about the effect of 24-week short course IFN-α therapy after receiving curative treatment for primary HCC. The paper is well written and the results show that the most important factor associated with the improvement of prognosis is the SVR status.

REFERENCES

1 **Tsukuma H**, Hiyama T, Tanaka S, Nakao M, Yabuuchi T, Kitamura T, Nakanishi K, Fujimoto I, Inoue A, Yamazaki H. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N Engl J Med* 1993; **328**: 1797-1801

2 **Takano S**, Yokosuka O, Imazeki F, Tagawa M, Omata M. Incidence of hepatocellular carcinoma in chronic hepatitis B and C: a prospective study of 251 patients. *Hepatology* 1995; **21**: 650-655

3 **Shiratori Y**, Shiina S, Imamura M, Kato N, Kanai F, Okudaira T, Teratani T, Tohgo G, Toda N, Ohashi M. Characteristic difference of hepatocellular carcinoma between hepatitis B- and C- viral infection in Japan. *Hepatology* 1995; **22**: 1027-1033

4 **Ikeda K**, Saitoh S, Tsubota A, Arase Y, Chayama K, Kumada H, Watanabe G, Tsurumaru M. Risk factors for tumor recurrence and prognosis after curative resection of hepatocellular carcinoma. *Cancer* 1993; **71**: 19-25

5 **Kubo S**, Nishiguchi S, Shuto T, Tanaka H, Tsukamoto T, Hirohashi K, Ikebe T, Wakasa K, Kuroki T, Kinoshita H. Effects of continuous hepatitis with persistent hepatitis C viremia on outcome after resection of hepatocellular carcinoma. *Jpn J Cancer Res* 1999; **90**: 162-170

6 **Kumada T**, Nakano S, Takeda I, Sugiyama K, Osada T, Kiriyama S, Sone Y, Toyoda H, Shimada S, Takahashi M, Sassa T. Patterns of recurrence after initial treatment in patients with small hepatocellular carcinoma. *Hepatology* 1997; **25**: 87-92

7 **Shimada M**, Takenaka K, Gion T, Fujiwara Y, Kajiyama K, Maeda T, Shirabe K, Nishizaki T, Yanaga K, Sugimachi K. Prognosis of recurrent hepatocellular carcinoma: a 10-year surgical experience in Japan. *Gastroenterology* 1996; **111**: 720-726

8 **Nagasue N**, Uchida M, Makino Y, Takemoto Y, Yamanoi A, Hayashi T, Chang YC, Kohno H, Nakamura T, Yukaya H. Incidence and factors associated with intrahepatic recurrence following resection of hepatocellular carcinoma. *Gastroenterology* 1993; **105**: 488-494

9 **Davis GL**, Balart LA, Schiff ER, Lindsay K, Bodenheimer HC Jr, Perrillo RP, Carey W, Jacobson IM, Payne J, Dienstag JL. Treatment of chronic hepatitis C with recombinant interferon alfa. A multicenter randomized, controlled trial. Hepatitis Interventional Therapy Group. *N Engl J Med* 1989; **321**: 1501-1506

10 **Di Bisceglie AM**, Martin P, Kassianides C, Lisker-Melman M, Murray L, Waggoner J, Goodman Z, Banks SM, Hoofnagle JH. Recombinant interferon alfa therapy for chronic hepatitis C. A randomized, double-blind, placebo-controlled trial. *N Engl J Med* 1989; **321**: 1506-1510

11 **Ikeda K**, Arase Y, Saitoh S, Kobayashi M, Suzuki Y, Suzuki F, Tsubota A, Chayama K, Murashima N, Kumada H. Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumor-A prospective randomized study of hepatitis C virus-related liver cancer. *Hepatology* 2000; **32**: 228-232

12 **Kubo S**, Nishiguchi S, Hirohashi K, Tanaka H, Shuto T, Yamazaki O, Shiomi S, Tamori A, Oka H, Igawa S, Kuroki T, Kinoshita H. Effects of long-term postoperative interferon-alpha therapy on intrahepatic recurrence after resection of hepatitis C virus-related hepatocellular carcinoma. A randomized, controlled trial. *Ann Intern Med* 2001; **134**: 963-967

13 **Kubo S**, Nishiguchi S, Hirohashi K, Tanaka H, Shuto T, Kinoshita H. Randomized clinical trial of long-term outcome after resection of hepatitis C virus-related hepatocellular carcinoma by postoperative interferon therapy. *Br J Surg* 2002; **89**: 418-422

14 **Nishiguchi S**, Tamori A, Kubo S. Effect of long-term postoperative interferon therapy on intrahepatic recurrence and survival rate after resection of hepatitis C virus-related hepatocellular carcinoma. *Intervirology* 2005; **48**: 71-75

15 **Sakaguchi Y**, Kudo M, Fukunaga T, Minami Y, Chung H, Kawasaki T. Low-dose, long-term, intermittent interferon-alpha-2b therapy after radical treatment by radiofrequency ablation delays clinical recurrence in patients with hepatitis C virus-related hepatocellular carcinoma. *Intervirology* 2005; **48**: 64-70

16 **Suou T**, Mitsuda A, Koda M, Matsuda H, Maruyama S, Tanaka H, Kishimoto Y, Kohno M, Hirooka Y, Kawasaki H. Interferon alpha inhibits intrahepatic recurrence in hepatocellular carcinoma with chronic hepatitis C: a pilot study. *Hepatol Res* 2001; **20**: 301-311

17 **Shiratori Y**, Shiina S, Teratani T, Imamura M, Obi S, Sato S, Koike Y, Yoshida H, Omata M. Interferon therapy after tumor ablation improves prognosis in patients with hepatocellular carcinoma associated with hepatitis C virus. *Ann Intern Med* 2003; **138**: 299-306

18 **The general rules for the clinical and pathological study of primary liver cancer**. Liver Cancer Study Group of Japan. *Jpn J Surg* 1989; **19**: 98-129

19 **Harada H**, Kitagawa M, Tanaka N, Yamamoto H, Harada K, Ishihara M, Taniguchi T. Anti-oncogenic and oncogenic potentials of interferon regulatory factors-1 and -2. *Science* 1993; **259**: 971-974

20 **Tanaka N**, Ishihara M, Kitagawa M, Harada H, Kimura T, Matsuyama T, Lamphier MS, Aizawa S, Mak TW, Taniguchi T. Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcription factor IRF-1. *Cell* 1994; **77**: 829-839

21 **Tanaka N**, Ishihara M, Lamphier MS, Nozawa H, Matsuyama T, Mak TW, Aizawa S, Tokino T, Oren M, Taniguchi T. Cooperation of the tumour suppressors IRF-1 and p53 in response to DNA damage. *Nature* 1996; **382**: 816-818

22 **Czaja MJ**, Weiner FR, Eghbali M, Giambrone MA, Eghbali M, Zern MA. Differential effects of gamma-interferon on collagen and fibronectin gene expression. *J Biol Chem* 1987; **262**: 13348-13351

23 **Capra F**, Casaril M, Gabrielli GB, Tognella P, Rizzi A, Dolci L, Colombari R, Mezzelani P, Corrocher R, De Sandre G. alpha-Interferon in the treatment of chronic viral hepatitis: effects on fibrogenesis serum markers. *J Hepatol* 1993; **18**: 112-118

24 **Ishibashi K**, Kashiwagi T, Ito A, Tanaka Y, Nagasawa M, Toyama T, Ozaki S, Naito M, Azuma M. Changes in serum fibrogenesis markers during interferon therapy for chronic hepatitis type C. *Hepatology* 1996; **24**: 27-31

25 **Hannigan GE**, Williams BR. Signal transduction by interferon-alpha through arachidonic acid metabolism. *Science* 1991; **251**: 204-207

26 **Swaminathan N**, Lai CM, Beilharz MW, Boyer SJ, Klinken SP. Biological activities of recombinant murine interferons alpha 1 and alpha 4: large difference in antiproliferative effect. *Antiviral Res* 1992; **19**: 149-159

27 **Chen LK**, Tourvieille B, Burns GF, Bach FH, Mathieu-Mahul D, Sasportes M, Bensussan A. Interferon: a cytotoxic T lymphocyte differentiation signal. *Eur J Immunol* 1986; **16**: 767-770

28 **Tabata Y**, Uno K, Yamaoka T, Ikada Y, Muramatsu S. Effects of recombinant alpha-interferon-gelatin conjugate on in vivo

murine tumor cell growth. *Cancer Res* 1991; **51**: 5532-5538
29 **Lai CL**, Lau JY, Wu PC, Ngan H, Chung HT, Mitchell SJ, Corbett TJ, Chow AW, Lin HJ. Recombinant interferon-alpha in inoperable hepatocellular carcinoma: a randomized controlled trial. *Hepatology* 1993; **17**: 389-394
30 **Koike K**. Molecular basis of hepatitis C virus-associated hepatocarcinogenesis: lessons from animal model studies. *Clin Gastroenterol Hepatol* 2005; **3**: S132-S135
31 **Sato Y**, Kato J, Takimoto R, Takada K, Kawano Y, Miyanishi K, Kobune M, Sato Y, Takayama T, Matunaga T, Niitsu Y. Hepatitis C virus core protein promotes proliferation of human hepatoma cells through enhancement of transforming growth factor alpha expression via activation of nuclear factor-kappaB. *Gut* 2006; **55**: 1801-1808
32 **Poynard T**, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, Bain V, Heathcote J, Zeuzem S, Trepo C, Albrecht J. Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet* 1998; **352**: 1426-1432
33 **McHutchison JG**, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; **339**: 1485-1492
34 **Fried MW**, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales FL Jr, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982
35 **Bailon P**, Palleroni A, Schaffer CA, Spence CL, Fung WJ, Porter JE, Ehrlich GK, Pan W, Xu ZX, Modi MW, Farid A, Berthold W, Graves M. Rational design of a potent, long-lasting form of interferon: a 40 kDa branched polyethylene glycol-conjugated interferon alpha-2a for the treatment of hepatitis C. *Bioconjug Chem* 2001; **12**: 195-202
36 **Harris JM**, Martin NE, Modi M. Pegylation: a novel process for modifying pharmacokinetics. *Clin Pharmacokinet* 2001; **40**: 539-551
37 **Glue P**, Fang JW, Rouzier-Panis R, Raffanel C, Sabo R, Gupta SK, Salfi M, Jacobs S. Pegylated interferon-alpha2b: pharmacokinetics, pharmacodynamics, safety, and preliminary efficacy data. Hepatitis C Intervention Therapy Group. *Clin Pharmacol Ther* 2000; **68**: 556-567
38 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965
39 **Lindsay KL**, Trepo C, Heintges T, Shiffman ML, Gordon SC, Hoefs JC, Schiff ER, Goodman ZD, Laughlin M, Yao R, Albrecht JK. 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
40 **Zeuzem S**, Feinman SV, Rasenack J, Heathcote EJ, Lai MY, Gane E, O'Grady J, Reichen J, Diago M, Lin A, Hoffman J, Brunda MJ. Peginterferon alfa-2a in patients with chronic hepatitis C. *N Engl J Med* 2000; **343**: 1666-1672
41 **Reddy KR**, Wright TL, Pockros PJ, Shiffman M, Everson G, Reindollar R, Fried MW, Purdum PP 3rd, Jensen D, Smith C, Lee WM, Boyer TD, Lin A, Pedder S, DePamphilis J. 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**: 433-438

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VIRAL HEPATITIS

Low-dose intermittent interferon-alpha therapy for HCV-related liver cirrhosis after curative treatment of hepatocellular carcinoma

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Abstract

AIM: To assess the efficacy of low-dose intermittent interferon (IFN) therapy in patients with hepatitis C virus (HCV)-related compensated cirrhosis who had received curative treatment for primary hepatocellular carcinoma (HCC).

METHODS: We performed a prospective case controlled study. Sixteen patients received 3 MIU of natural IFN-alpha intramuscularly 3 times weekly for at least 48 wk (IFN group). They were compared with 16 matched historical controls (non-IFN group).

RESULTS: The cumulative rate of first recurrence of HCC was not significantly different between the IFN group and the non-IFN group (0% *vs* 6.7% and 68.6% *vs* 80% at 1- and 3-year, $P = 0.157$, respectively). The cumulative rate of second recurrence was not also significantly different between the IFN group and the non-IFN group (0% *vs* 6.7% and 35.9% *vs* 67% at 1- and 3-year, $P = 0.056$, respectively). Although the difference in the Child-Pugh classification score between the groups at initial treatment of HCC was not significant, the score was significantly worse at the time of data analysis in the non-IFN group than IFN group (7.19 ± 1.42 *vs* 5.81 ± 0.75, $P = 0.0008$). The cumulative rate of deviation from objects of any treatment for recurrent HCC was also higher in the non-IFN group than IFN group (6.7% and 27% *vs* 0 and 0% at 1- and 3-year, $P = 0.048$, respectively).

CONCLUSION: Low-dose intermittent IFN-alpha therapy for patients with HCV-related compensated cirrhosis after curative HCC treatment was effective by making patients tolerant to medical or surgical treatment for recurrent HCC in the later period of observation.

Key words: Hepatitis C virus; Hepatocellular carcinoma; Interferon therapy; Liver cirrhosis; Liver function; Recurrence; Survival

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms worldwide. Approximately 80% of Japanese patients with HCC have a history of hepatitis C virus (HCV) infection, and most such patients have liver cirrhosis[1-3]. Although recent advances in imaging techniques and treatment of HCC have improved prognosis of patients with HCV-related HCC, the outcome is still unsatisfactory; the 5-year survival rate is only 50% to 70% even after curative treatment such as hepatic resection and local ablation[4]. The reasons for this unfavorable prognosis is considered to include high intrahepatic tumor recurrence rates and biochemical deterioration by sustained hepatic damage, both resulting from persistent HCV infection[5]. Even after curative hepatic resection for HCV-related HCC, the rate of intrahepatic tumor recurrence within 1 year is 20% to 40%, rising to about

80% by 5 years[4,6-8]. Intrahepatic recurrences of HCC may result from intrahepatic metastasis originating from the primary HCC or from ongoing multicentric carcinogenesis related to chronic HCV infection. In addition, sustained underlying HCV-related hepatic damage may compromise hepatic functional reserve, worsening clinical outcome. Thus, prevention of HCC recurrence and preservation of liver function are both highly important priorities in improving prognosis of patients with HCV-related HCC.

Interferon (IFN) therapy for patients with HCV infection is effective as evident by reduction of serum alanine transaminase (ALT) activity and eradication of HCV. Accordingly, IFN is valuable in minimizing hepatic necrosis, inflammation, and fibrosis, as well as reducing the likelihood of hepatocarcinogenesis[9-16]. The primary goal of treatment of patients with HCV infection is elimination of the virus. Several studies have reported recently that IFN therapy provided after curative treatment for HCV-related HCC prevents HCC recurrences and improves survival[17-23]. Such improvement of prognosis is more predominant when IFN therapy results in elimination of HCV RNA[24]. However, most patients with HCV-related HCC also have liver cirrhosis. Many centers do not advocate IFN therapy of patients with compensated cirrhosis, mainly because of the disappointing sustained virological response (SVR) rates in such patients[25]. Several studies indicated that the response of cirrhotic patients to antiviral therapy is low[26-28]. The reasons for the low SVR rate in such patients include inability to administer IFN at recommended doses due to adverse effects and dose-limiting cytopenia. On the other hand, several investigators suggested that the use of low-dose IFN therapy for viral elimination was as effective in the treatment of cirrhotic patients with HCV as it is in non-cirrhotic patients[29,30]. Furthermore, they indicated that the same therapy could improve the underlying liver histology. There is evidence to suggest that low-dose IFN therapy might be beneficial in HCV-related cirrhosis, not only because it prevents the progression of liver disease, but also because it reduces the risk of hepatocarcinogenesis[31,32]. In this regard, low-dose IFN therapy seems to be tolerable without significant life-threatening adverse effects than the standard dose of IFN.

However, it is not known whether low-dose IFN after curative treatment of primary HCC could slow disease progression or reduce the rate of clinical decompensation in cirrhotic patients, in addition to prevention of HCC recurrence. Several studies used the standard dose of IFN after HCC treatment[17,23,33], and studies using low-dose IFN therapy for HCV-related cirrhosis after HCC treatment also reported that such regimen may reduce late recurrence of HCC[34].

In this prospective case controlled trial, we assessed the efficacy of low-dose intermittent IFN therapy on HCV-related liver cirrhosis after curative treatment of primary HCC in terms of overall survival, HCC recurrence, and liver function.

MATERIALS AND METHODS

Patients

A total of 176 consecutive patients received their initial treatment for HCV-related primary HCC at Hiroshima University Hospital between August 2001 and December 2006. Of these, 67 patients with HCC underwent first medical or surgical therapeutic intervention with curative intent (defined as complete tumor eradication with no visible residual tumor in computed tomographic images, or resection of all evident tumor tissue). Medical treatments included percutaneous radiofrequency (RF) ablation and ethanol injection, while surgical procedures included hepatic resection and RF ablation under laparotomy. Among these 67 patients, 52 patients with liver cirrhosis (LC), which was diagnosed clinically and pathologically, were considered for this prospective study. Figure 1 shows our study flow. Among these 52 patients with HCV-related LC, we assessed 42 patients who met the following inclusion criteria: (1) the presence of up to three tumors with none exceeding 30 mm in diameter or a solitary tumor less than 50 mm in diameter; (2) tumor-node-metastasis (TNM) stage of I , II or III; (3) detectable serum HCV RNA; (4) all seronegativity for hepatitis B marker including hepatitis B surface antigen, hepatitis B anti-core antibody and hepatitis B surface antibody ; (5) compensated cirrhosis with a Child-Pugh class A or B; (6) platelet count j " 40000/μL; and (7) absence of local recurrence during the follow-up period and of any ectopic intrahepatic recurrence within 12 wk after treatment for primary HCC. We used the TNM classification system

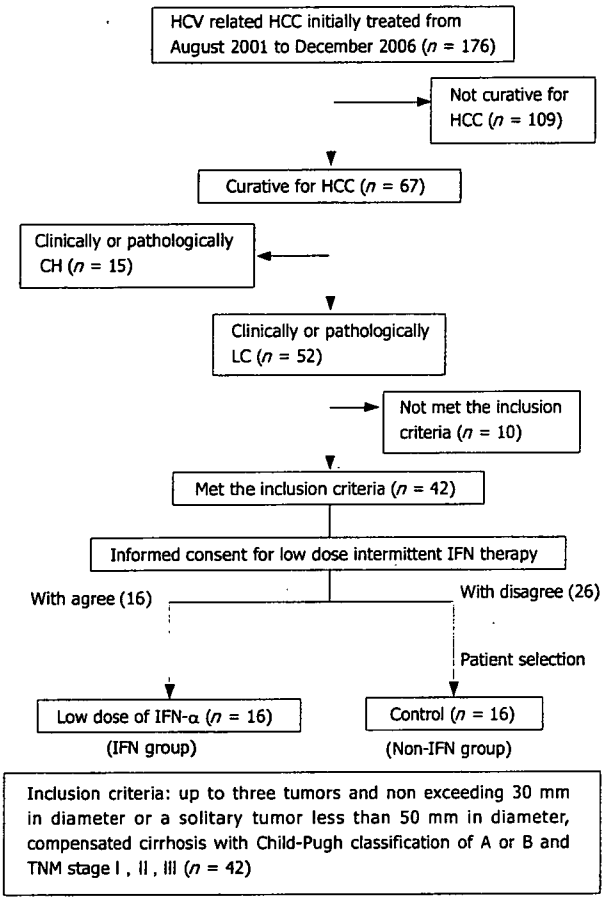

Figure 1 Schematic flow chart of enrolled patients.

Table 1 Characteristics of participating patients

	Interferon group	Non-interferon group	*P* value
No. of patients	16	16	
Age in years (range)	68.5[1] (53-73)	67.5[1] (58-75)	NS
Gender (Male/Female)	10/6	11/5	NS
Albumin (g/dL)	3.7[1] (3.0-4.8)	3.7[1] (3.0-4.5)	NS
Platelet count (× 10^4/L)	8.0[1] (4.5-14.2)	8.4[1] (4.6-14.3)	NS
ICG R-15 (%)	17.3[1] (6.1-40.8)	18.2[1] (5-45)	NS
Alanine aminotransferase (IU/L)	59[1] (35-99)	58[1] (21-143)	NS
Alpha fetoprotein (ng/mL)	54[1] (5.3-293.6)	38[1] (5.0-1217)	NS
Child-Pugh score (A/B)	13/3	13/3	NS
Main tumor size (mm)	15[1] (10-50)	18[1] (10-40)	NS
No. of HCC tumors (single/multiple)	9/7	10/6	NS
Stage (I / II /III)	8/3/5	7/5/4	NS
Treatment (medical/surgical)	8/8	9/7	NS
HCV genotype (1/2)	12/4	14/2	NS
Viral loads (low/high)	6/10	5/11	NS

ICG-R15: Indocyanine green retention at 15 min; Low viral loads: HCV RNA < 100 KIU/mL, high viral loads: HCV RNA j "100 KIU/mL. [1]median.

of the Liver Cancer Study Group of Japan as a staging system for HCC[35]. The underlying liver condition leading to LC was identified by histopathological examination of resected tissue samples. When this was not available, laboratory tests were performed including serum albumin, platelet, prothrombin time and indocyanine green retention at 15 min (ICG-R15), and radiological examination such as ultrasonography and computed tomography.

Of the 42 patients with LC who met the above eligibility criteria, 16 patients received low-dose IFN therapy after signing a written informed consent (IFN group). Of the remaining 26 patients who rejected IFN therapy, we selected 16 patients as the control (non-IFN group). These 16 patients, who met the eligibility criteria mentioned above, were matched by age, gender, tumor size, number of tumors, TNM stage of HCC, serum albumin level, platelet counts, ICG-R15 and Child-Pugh class with patients of the IFN group. Thus, a total of 32 patients (16 in the IFN group and 16 in the non-IFN group) were enrolled in this study. All agreed to participate in the research protocol, which was approved by the hospital research ethics board. Table 1 shows the baseline characteristics of patients of the two groups. The data indicates no significant differences between the groups for age, gender, liver function, tumor characteristics, and therapeutic methods used against primary HCC.

IFN therapy

In the IFN group, patients received 3 MIU of natural IFN-alpha (human lymphoblastoid IFN; Sumiferon, Dainippon Sumitomo Pharmaceuticals, Osaka, Japan) intramuscularly three times weekly for at least 48 wk as long as possible. IFN therapy commenced within 12 wk after initial treatment for HCC. Patients received post-treatment IFN therapy up to the detection of HCC recurrence, and then patients who could have curative treatment for recurrent HCC restarted IFN therapy when possible. However, patients who had advanced liver dysfunction or untreatable progressive HCC did not receive IFN therapy. In the control group, none of the patients received IFN therapy after curative treatment of HCC; instead, they were on ursodeoxycholic acid (UDCA) and stronger neo minophagen C (SNMC).

Follow-up

After curative treatment for primary HCC, all patients underwent liver function tests, serum tumor marker assays such as alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist (PIVKA)-II, every month, abdominal ultrasonography every 3 mo, and dynamic computed tomography (CT) every 6 mo. If recurrences of HCC were suspected, additional examinations including CT during arteriography or tumor biopsy were performed. Recurrence of HCC was defined as any new nodules appearing as hyperattenuation by CT during hepatic arteriography or as hypoattenuation in CT performed during arterioportography. Hypovascular HCC was confirmed histopathologically by fine-needle aspiration biopsy. Patients with recurrent HCC were treated medically or surgically, with curative intent if possible. Patients without curative treatment of recurrent HCC then received transcatheter chemoembolization. After repeated transcatheter chemoembolization, patients were finally unable to receive any treatment for recurrent HCC.

End points

We analyzed the outcome of this prospective study in December 2006. We compared the rate of HCC recurrence and the survival rate between IFN group and control group. We assessed whether low-dose of IFN therapy was effective in inhibiting recurrence of HCC, preserving liver function and prolonging survival. In addition, we also assessed the cumulative rate of deviation from objective of any treatment against recurrent HCC due to progression of HCC and/or underlying liver dysfunction.

Statistical analysis

The Chi-square and Fisher exact tests were used for categorical variables, while Student's *t*-test and the Mann-Whitney *U* test were used for continuous and ordinal variables, as appropriate. The Kaplan-Meier method used to assess cumulative survival and recurrence rates calculated from the date of diagnosis to the date of

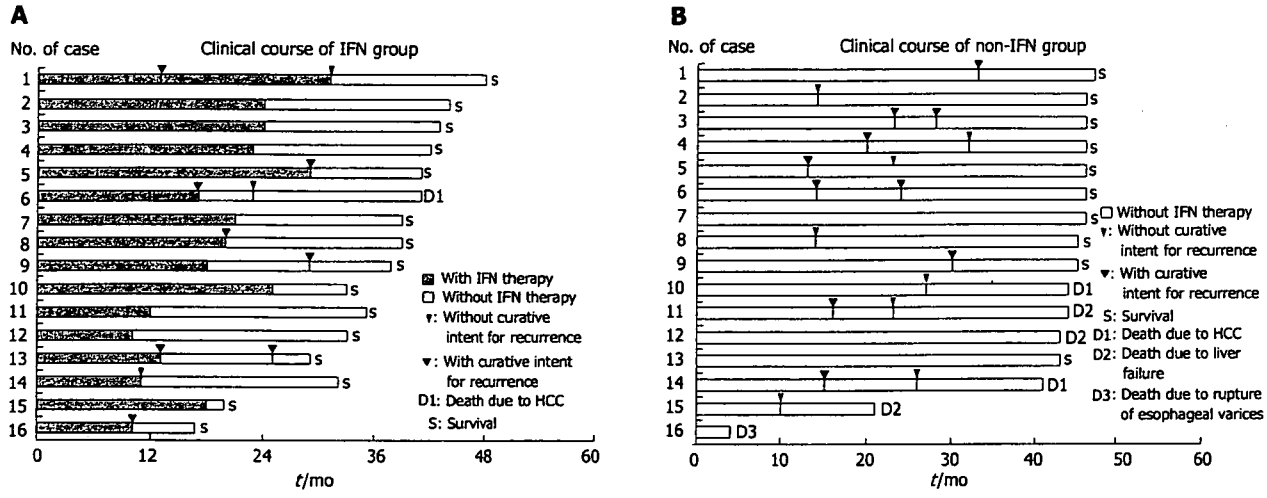

Figure 2 A: Clinical course of the interferon group. Patients who had a curative treatment for primary HCC received 3 MIU of natural interferon-alpha three times weekly for at least 48 wk as long as possible except Cases 12, 14 and 16. Recurrent HCCs were treated with or without curative treatment; B: Clinical course of the non-interferon group. Patients who had a curative treatment of primary HCC did not receive IFN therapy. Recurrent HCCs were also treated with or without curative treatment.

disease recurrence or death. Surviving patients and patients who died of causes unrelated to the liver were defined as censored cases, while patients who died of causes related to the liver were defined as noncensored cases. The log-rank test was used to compare survival and recurrence curves. *P* values below 0.05 were considered to indicate statistical significance. The JMP version 5.1 statistical software package (SAS Institute, Cary, NC) was used for analysis of data.

RESULTS

Clinical course of IFN group

Figure 2A shows the clinical course of 16 patients of the IFN group from the initial treatment of primary HCC to the date of data analysis. The duration of low-dose IFN therapy ranged from a minimum of 10 mo to a maximum of 25 mo (median 16 mo). Although 8 patients did not have HCC recurrence, HCC recurred in 8 patients after initial treatment of HCC during a median follow-up period of 37 mo. Of the recurred patients, 7 developed HCC recurrence during IFN therapy (Cases 1, 5, 6, 8, 13, 14 and 16) except 1 patient (Case 9) who had HCC recurrence after discontinuation of IFN therapy. Of the 8 patients with HCC recurrence, 4 were treated with surgical resection therapy (Cases 5, 9, 13 and 16), 3 patients with percutaneous RF ablation therapy (Cases 1, 6 and 8) and 1 patient transcatheter chemoembolization (Case 14). Of these patients, a patient with transcatheter chemoembolization (Case 14) could not have curative treatment and repeated transcatheter chemoembolization. He was excluded from the study concerning the next recurrence. Of the 7 patients with curative treatment for HCC recurrence, 2 restarted IFN therapy, one continued IFN therapy until next recurrence (Case 1), which was not curative, and the other continued until intolerant generalized fatigue (Case 8). The remaining 5 patients (Cases 5, 6, 13, 14 and 16) were followed without IFN therapy because of rejection of IFN therapy. Although one of these 5 patients was not curative for first recurrence (Case 14), he was tolerant to repeated transcatheter chemoembolization and was still alive at the date of data analysis. Two patients without curative treatment at the second recurrence (Cases 1 and 6) were also relatively tolerant to the repeated medical treatment such as transcatheter chemoembolization. Of these patients, one died of progression of HCC in spite of repeated transcatheter chemoembolization and hepatic arterial infusion (Case 6), another was alive at the date of data analysis (Case 1). Of 3 patients without curative treatment of HCC, two survivors' status of HCC were not progressive (stage II and stage III) and underlying liver function could be tolerant to the treatment such as transcatheter chemoembolization because of relatively preserved function (Cases 1 and 14).

The 16 patients who received IFN therapy included 2 patients with virological response (Cases 2 and 3) and 14 patients who did not get SVR [3 transient responders (Cases 8, 9 and 11), and 11 non-responders (Cases 1, 4, 5, 6, 7, 10, 12, 13, 14, 15 and 16)]. Among the 14 patients who did not show SVR, 8 were biochemical responders with normalized ALT (Cases 1, 4, 5, 7, 9, 10, 13 and 16), including 4 transient responders and 4 non-responders. Two sustained virological responders who received IFN therapy for 96 wk have viral characteristics of genotype 1 and low viral load. Among the patients who did not show SVR, 7 discontinued IFN treatment because of recurrence of HCC, while 2 patients restarted IFN therapy after the curative treatment of recurrent HCC. None of the patients who received IFN therapy developed life-threatening side effects.

Clinical course of non-IFN group

Among the non-IFN group, the first recurrence of HCC occurred in 13 patients during a median follow-up period of 45 mo (Figure 2B). HCC recurred in 6 of the 7 non-IFN patients who had a sustained normalized ALT. Of the 13 patients with recurrent HCC among the non-IFN group, 4 were treated with hepatic resection (Cases 1, 4, 9 and 11),

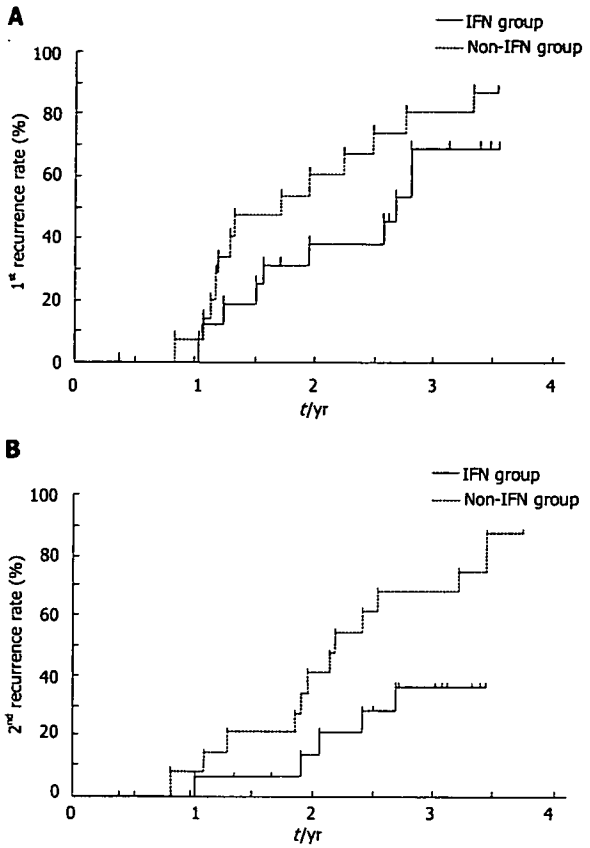

Figure 3 A: Cumulative rate of first recurrence. Rates of first recurrence for the IFN and non-IFN groups. The rate of first recurrence of HCC in the IFN group was not significantly different from that of the non-IFN group ($P = 0.157$); B: Cumulative rate of second recurrence. Rates of second recurrence for the IFN and non-IFN group. The rate of second recurrence of HCC in the IFN group was not significantly different from that of the non-IFN group ($P = 0.056$).

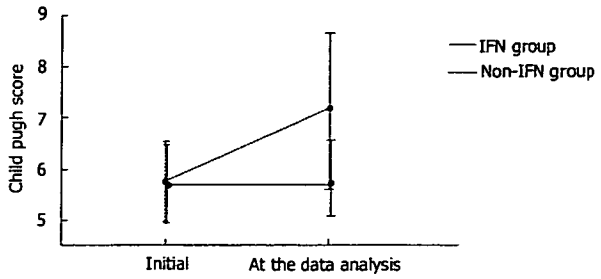

Figure 4 Effect of IFN therapy after curative treatment of HCC on Child-Pugh scores. IFN-treated patients were less likely to show deterioration of hepatic function. The average scores of Child-Pugh of the IFN group were significantly better preserved than the non-IFN group ($P = 0.0008$).

6 with local ablation including percutaneous RF ablation or ethanol injection (Cases 3, 5, 6, 7, 10 and 14) and 3 with transcatheter chemoembolization (Cases 2, 8 and 15). Of the 13 recurrent patients, 5 patients (2 received ethanol injection and 3 transcatheter chemoembolization) could not be treated curatively and was excluded from the study concerning the next recurrence. These 5 patients were treated repeatedly with transarterial chemoembolization after first recurrence. Among the remaining 8 patients who were treated curatively for first recurrence, 7 developed a second recurrence (Cases 3, 4, 5, 6, 9, 11 and 14). Among these 7 patients with second recurrence, 2 were treated curatively for HCC [1 with RF ablation (Case 3) and 1 with hepatic resection (Case 6)], while the remaining 5 patients were not (4 patients due to uncontrolled multiple HCC and one patient due to underlying liver dysfunction). The latter group of 5 patients received transarterial chemoembolization repeatedly after second recurrence.

Comparison of the first and second recurrence rates of HCC

We compared the overall cumulative rates for first and second recurrence between IFN and non-IFN groups (Figure 3). The 1-, 2- and 3- year rates of first recurrence of HCC in the IFN and non-IFN group were not different (0% *vs* 6.7%, 38.1% *vs* 60% and 68.6% *vs* 80%, respectively, Figure 3A, $P = 0.156$). The 1-, 2- and 3-year rates of second recurrence in the IFN and non-IFN groups were 0% *vs* 6.7%, 13.5% *vs* 33.3% and 35.9% *vs* 67%, respectively (Figure 3B, $P = 0.056$).

Liver function

Patients of the IFN group were less likely to develop worsening of hepatic dysfunction compared with the non-IFN group. We compared the average score determined for Child-Pugh classification at initial treatment of HCC with that at the time of data analysis (Figure 4). Although the difference in the Child-Pugh classification score between the two groups at initial treatment of HCC was not significant, the score was significantly worse at the time of data analysis in the non-IFN group than IFN group ($P = 0.0008$).

Deviation from objects of any treatments for recurrent HCC

At the date of data analysis, patients who developed recurrent HCC were treated repeatedly, as possible, for the purpose of curative treatment including surgical resection and ablative therapy such as RF ablation and ethanol injection. Patients who were difficult to treat with curative intent received transcatheter chemoembolization or hepatic arterial infusion. Although patients with recurrent HCC received repeated treatments, some patients finally could not be treated because of excessive progression of HCC or liver dysfunction. Figure 5 shows that the cumulative rate of deviation from objects of any treatment for recurrent HCC between the IFN group and non-IFN group. In the IFN group, one patient could not receive treatment due to progressively advanced HCC in later period. On the other hand, 8 patients in the non-IFN group could not receive treatment because of underlying liver dysfunction ($n = 2$) and progressively advanced HCC ($n = 6$). The 1-, 2- and 3- year rates of deviation from objects of any treatment for recurrent HCC in the IFN and non-IFN group were 0% *vs* 6.7%, 0% *vs* 20% and 0% *vs* 27%, respectively ($P = 0.048$). Thus, the IFN group tended to be treatable for recurrent HCC compared with the non-IFN group.

Survival of patients

At the date of data analysis, 1 patient among the IFN

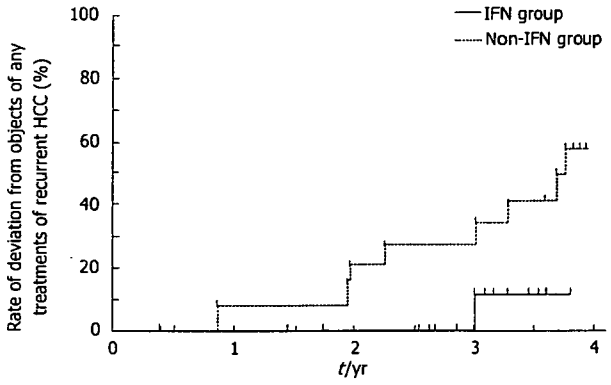

Figure 5 Cumulative rate of deviation from objects of any treatment of recurrent HCC. Recurrent HCC tended to be treatable later in the IFN group than non-IFN group ($P = 0.048$).

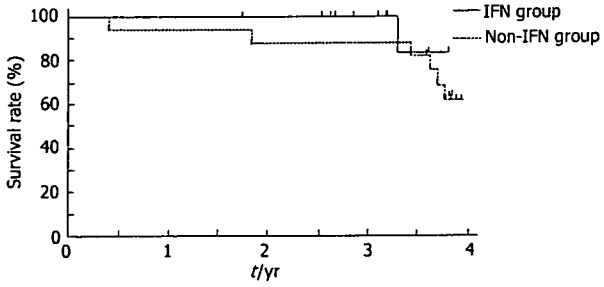

Figure 6 Cumulative survival rate. Comparison of the cumulative survival rates of the IFN and non-IFN groups. The cumulative survival rate was not significantly different between the two groups ($P = 0.45$).

group and 6 patients among the non-IFN group had died of liver disease. Of the 8 recurrence patients among the IFN group, 1 died of advanced multiple HCC and none died of liver failure. On the other hand, of the 13 recurrence patients among the non-IFN group, 2 died of advanced HCC and 2 died of liver failure in spite of the relatively early stage of HCC. Among the 3 patients without recurrent HCC of the non-IFN group, 1 died of liver dysfunction and 1 died of ruptured esophageal varices.

With regard to the cumulative survival rates of the IFN and non-IFN groups (Figure 6), the respective rates of survival were 100% *vs* 93.7% at 1 year, 100% *vs* 87.5% at 2 years, 100% *vs* 87.5% at 3 years and 83.3% *vs* 61.4% at 4 years. Thus, the cumulative survival rate was not significantly different between the two groups for first 4 years after curative treatment of HCC ($P = 0.45$). The median survival time following the first treatment of HCC was 37 mo (range, 17 to 45) for the IFN group and 45 mo (range, 4 to 47) for the non-IFN group.

DISCUSSION

HCC recurrence is still a risk even if HCV-related HCC is treated with curative intent. Most of such patients with HCC have underlying liver cirrhosis, and deterioration of underlying hepatic function may be a hindrance to treatment of recurrent HCC and be associated with prognosis. The present prospective case controlled study of cirrhotic patients shows that low-dose intermittent IFN therapy after curative treatment of HCC could preserve liver function and increase the chance of treatment for recurrent tumor.

Previous studies indicated that IFN therapy after curative treatment of HCC was effective in inhibiting or delaying the development of recurrent HCC[17-23,34,36]. Although several recent studies have reported the efficacy of chemoprevention with IFN therapy after treatment of HCV-related HCC, the basis of the benefit was not clear. Shiratori *et al*[23,33] and Ikeda *et al*[17] reported that IFN therapy in cirrhotic patients reduced recurrence of HCC and improved prognosis. Although they used standard IFN dosage per time, there are no other reports on the effect of low-dose IFN therapy after curative treatment of primary HCC in cirrhotic patients. Sakaguchi *et al*[21] reported that low-dose, long-term, intermittent IFN therapy in patients who had curative HCV-related HCC suppressed recurrence of HCC and improved survival, though it was not clear whether their patients had underlying liver cirrhosis or not. On the other hand, Mazzaferro *et al*[34] indicated that low-dose intermittent IFN therapy seemed to reduce late recurrence in patients with HCV-related cirrhosis after resection of HCC. Considered together, these results suggest that low-dose IFN therapy is potentially useful for cirrhotic patients when used as long as possible. However, our results of low-dose intermittent IFN therapy showed no significant difference in recurrence between those who received IFN therapy and those who did not. Unfortunately, since the difference in treatment outcome between the above three studies might be due to the use of different IFN regimens (e.g. dosage and frequency), and background characteristics of cirrhotic patients (e.g., performance status), the results varied and no standard IFN regimen to pursue after curative treatment of HCV-related HCC could be advocated.

The design of the present study was not randomized controlled type, and differed in details of the IFN protocol and characters of patients from the other studies. Although there was no significant difference in the recurrence rate between the IFN and non-IFN groups, the recurrence rate in the later period of observation including second recurrence appeared to be lower in patients with IFN therapy. Furthermore, the recurrent HCC in patients on IFN therapy did not seem to be aggressive compared with that in patients without IFN therapy, probably because they could be treated with curative intent during the observation period. Thus, low-dose intermittent IFN therapy seemed to have delayed or reduced the chance of development of recurrent HCC in the later period of observation, although IFN did not completely inhibit HCC recurrence in our cirrhotic patients.

Most cirrhotic patients cannot receive a standard full-dose IFN regimen due to underlying liver dysfunction and unfavorable complication such as cytopenia. Hence, it could be difficult to achieve SVR in most cirrhotic patients on low-dose intermittent IFN therapy. Valla *et al*[37] performed a randomized, controlled trial of IFN-alpha 2b but the results showed a lack of any benefits in terms of sustained biochemical response, liver function test

results, histology, occurrence of decompensation or HCC, or prolongation of survival. On the other hand, Everson and coworkers[29,30] suggested that the use of low-dose IFN therapy for viral elimination was as effective in the treatment of cirrhotic patients with HCV as it is in non-cirrhotic patients. Several recent studies have reported that IFN therapy following HCC treatment improved liver function of patients with HCV-related HCC, although it is not clear which specific IFN action is important for these benefits. We also demonstrated that preservation of liver function was significantly better in the IFN group than in the non-IFN group even when HCV was not completely eradicated. Thus, hepatic functional preservation increases the chance of treatment for recurrent. Therefore, the cumulative rate of deviation from objects of any treatment for recurrent HCC might be lower in patients with IFN therapy than in patients without IFN therapy as we showed that low-dose IFN resulted in less advanced recurrence and hepatic functional preservation. Although the survival rates were not significantly different between the two groups in our observation period, we need a longer observation to determine differences in survival rates. Although we also assessed the correlation between the observed beneficial effects of the low-dose intermittent IFN therapy and HCV genotype, we could not reach the clear conclusion due to small sample size. In the future, the study with large sample size may be needed to conclude.

In our study, only about 12.5% (2/16) of patients who received IFN therapy had sustained viral elimination. And there were no significant difference in population of patients with normalized ALT between the IFN and non-IFN group (n = 10, n = 7, respectively). In spite of these results, patients treated with low-dose intermittent IFN therapy have a hepatic functional preservation greater than IFN untreated patients who received continuous medication with UDCA or SNMC after curative treatment of HCC. Although the mechanism of this reason is not well known, we suggested that the anti-inflammatory activity by low-dose intermittent IFN therapy may be stronger than medication with UDCA or SNMC and induce regression or retardation of underlying hepatic fibrosis, and finally, inhibits the progression of hepatic dysfunction.

Adverse effects such as reduction in blood counts by low-dose of IFN-alpha were not observed in our study, although neutropenia and/or thrombocytopenia were identified before IFN therapy. Furthermore, none of the patients required dose reduction in our study. Although 4 patients discontinued IFN therapy because of generalized fatigue, 2 of these patients restarted IFN therapy after that. Therefore, low-dose intermittent IFN-alpha therapy can be used relatively safely for cirrhotic patients with thrombocytopenia. However, patients who can not receive even low-doses of IFN also exist due to severe cytopenia or advanced liver cirrhosis. Medication with UDCA or SNMC or phlebotomy may be useful in decreasing ALT level for those patients.

Most cirrhotic patients who had received curative treatment for primary HCC have a limited hepatic reserve or thrombocytopenia. Therefore, low-dose intermittent IFN therapy might be effective for better prognosis. However, further studies of larger samples followed-up for longer periods should be conducted to establish a definite conclusion about the effect of low-dose IFN therapy for the prevention of progressive liver disease and effect of treatment for recurrent HCC.

REFERENCES

1 **Tsukuma H**, Hiyama T, Tanaka S, Nakao M, Yabuuchi T, Kitamura T, Nakanishi K, Fujimoto I, Inoue A, Yamazaki H. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N Engl J Med* 1993; **328**: 1797-1801

2 **Takano S**, Yokosuka O, Imazeki F, Tagawa M, Omata M. Incidence of hepatocellular carcinoma in chronic hepatitis B and C: a prospective study of 251 patients. *Hepatology* 1995; **21**: 650-655

3 **Shiratori Y**, Shiina S, Imamura M, Kato N, Kanai F, Okudaira T, Teratani T, Tohgo G, Toda N, Ohashi M. Characteristic difference of hepatocellular carcinoma between hepatitis B- and C- viral infection in Japan. *Hepatology* 1995; **22**: 1027-1033

4 **Ikeda K**, Saitoh S, Tsubota A, Arase Y, Chayama K, Kumada H, Watanabe G, Tsurumaru M. Risk factors for tumor recurrence and prognosis after curative resection of hepatocellular carcinoma. *Cancer* 1993; **71**: 19-25

5 **Kubo S**, Nishiguchi S, Shuto T, Tanaka H, Tsukamoto T, Hirohashi K, Ikebe T, Wakasa K, Kuroki T, Kinoshita H. Effects of continuous hepatitis with persistent hepatitis C viremia on outcome after resection of hepatocellular carcinoma. *Jpn J Cancer Res* 1999; **90**: 162-170

6 **Kumada T**, Nakano S, Takeda I, Sugiyama K, Osada T, Kiriyama S, Sone Y, Toyoda H, Shimada S, Takahashi M, Sassa T. Patterns of recurrence after initial treatment in patients with small hepatocellular carcinoma. *Hepatology* 1997; **25**: 87-92

7 **Shimada M**, Takenaka K, Gion T, Fujiwara Y, Kajiyama K, Maeda T, Shirabe K, Nishizaki T, Yanaga K, Sugimachi K. Prognosis of recurrent hepatocellular carcinoma: a 10-year surgical experience in Japan. *Gastroenterology* 1996; **111**: 720-726

8 **Nagasue N**, Uchida M, Makino Y, Takemoto Y, Yamanoi A, Hayashi T, Chang YC, Kohno H, Nakamura T, Yukaya H. Incidence and factors associated with intrahepatic recurrence following resection of hepatocellular carcinoma. *Gastroenterology* 1993; **105**: 488-494

9 **Chayama K**, Saitoh S, Arase Y, Ikeda K, Matsumoto T, Sakai Y, Kobayashi M, Unakami M, Morinaga T, Kumada H. Effect of interferon administration on serum hepatitis C virus RNA in patients with chronic hepatitis C. *Hepatology* 1991; **13**: 1040-1043

10 **Hagiwara H**, Hayashi N, Mita E, Ueda K, Takehara T, Kasahara A, Fusamoto H, Kamada T. Detection of hepatitis C virus RNA in serum of patients with chronic hepatitis C treated with interferon-alpha. *Hepatology* 1992; **15**: 37-41

11 **Shindo M**, Di Bisceglie AM, Hoofnagle JH. Long-term follow-up of patients with chronic hepatitis C treated with alpha-interferon. *Hepatology* 1992; **15**: 1013-1016

12 **McHutchison J**, Blatt L, Sedghi-Vaziri A, Russell J, Schmid P, Conrad A. Is there an optimal time to measure quantitative HCV RNA to predict non-response following interferon treatment for chronic HCV infection? *J Hepatol* 1998; **29**: 362-368

13 **Castilla A**, Prieto J, Fausto N. Transforming growth factors beta 1 and alpha in chronic liver disease. Effects of interferon alfa therapy. *N Engl J Med* 1991; **324**: 933-940

14 **Manabe N**, Chevallier M, Chossegros P, Causse X, Guerret S, Trepo C, Grimaud JA. Interferon-alpha 2b therapy reduces liver fibrosis in chronic non-A, non-B hepatitis: a quantitative histological evaluation. *Hepatology* 1993; **18**: 1344-1349

15 **Jimenez SA**, Freundlich B, Rosenbloom J. Selective inhibition of human diploid fibroblast collagen synthesis by interferons. *J Clin Invest* 1984; **74**: 1112-1116

16 **Baron S**, Tyring SK, Fleischmann WR Jr, Coppenhaver DH, Niesel DW, Klimpel GR, Stanton GJ, Hughes TK. The interferons. Mechanisms of action and clinical applications.

JAMA 1991; **266**: 1375-1383
17 **Ikeda K**, Arase Y, Saitoh S, Kobayashi M, Suzuki Y, Suzuki F, Tsubota A, Chayama K, Murashima N, Kumada H. Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumor-A prospective randomized study of hepatitis C virus-related liver cancer. *Hepatology* 2000; **32**: 228-232
18 **Kubo S**, Nishiguchi S, Hirohashi K, Tanaka H, Shuto T, Yamazaki O, Shiomi S, Tamori A, Oka H, Igawa S, Kuroki T, Kinoshita H. Effects of long-term postoperative interferon-alpha therapy on intrahepatic recurrence after resection of hepatitis C virus-related hepatocellular carcinoma. A randomized, controlled trial. *Ann Intern Med* 2001; **134**: 963-967
19 **Kubo S**, Nishiguchi S, Hirohashi K, Tanaka H, Shuto T, Kinoshita H. Randomized clinical trial of long-term outcome after resection of hepatitis C virus-related hepatocellular carcinoma by postoperative interferon therapy. *Br J Surg* 2002; **89**: 418-422
20 **Nishiguchi S**, Tamori A, Kubo S. Effect of long-term postoperative interferon therapy on intrahepatic recurrence and survival rate after resection of hepatitis C virus-related hepatocellular carcinoma. *Intervirology* 2005; **48**: 71-75
21 **Sakaguchi Y**, Kudo M, Fukunaga T, Minami Y, Chung H, Kawasaki T. Low-dose, long-term, intermittent interferon-alpha-2b therapy after radical treatment by radiofrequency ablation delays clinical recurrence in patients with hepatitis C virus-related hepatocellular carcinoma. *Intervirology* 2005; **48**: 64-70
22 **Suou T**, Mitsuda A, Koda M, Matsuda H, Maruyama S, Tanaka H, Kishimoto Y, Kohno M, Hirooka Y, Kawasaki H. Interferon alpha inhibits intrahepatic recurrence in hepatocellular carcinoma with chronic hepatitis C: a pilot study. *Hepatol Res* 2001; **20**: 301-311
23 **Shiratori Y**, Shiina S, Teratani T, Imamura M, Obi S, Sato S, Koike Y, Yoshida H, Omata M. Interferon therapy after tumor ablation improves prognosis in patients with hepatocellular carcinoma associated with hepatitis C virus. *Ann Intern Med* 2003; **138**: 299-306
24 **Akamatsu M**, Yoshida H, Shiina S, Teratani T, Obi S, Tateishi R, Mine N, Kondo Y, Kawabe T, Omata M. Sustained viral response prolonged survival of patients with C-viral hepatocellular carcinoma. *Liver Int* 2006; **26**: 536-542
25 **Schalm SW**, Fattovich G, Brouwer JT. Therapy of hepatitis C: patients with cirrhosis. *Hepatology* 1997; **26**: 128S-132S
26 **Hadziyannis SJ**, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H Jr, Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **140**: 346-355
27 **Zeuzem S**, Feinman SV, Rasenack J, Heathcote EJ, Lai MY, Gane E, O'Grady J, Reichen J, Diago M, Lin A, Hoffman J, Brunda MJ. Peginterferon alfa-2a in patients with chronic hepatitis C. *N Engl J Med* 2000; **343**: 1666-1672
28 **Poynard T**, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, Bain V, Heathcote J, Zeuzem S, Trepo C, Albrecht J. Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet* 1998; **352**: 1426-1432
29 **Everson GT**, Jensen DM, Craig JR, van Leeuwen DJ, Bain VG, Ehrinpreis MN, Albert D, Joh T, Witt K. Efficacy of interferon treatment for patients with chronic hepatitis C: comparison of response in cirrhotics, fibrotics, or nonfibrotics. *Hepatology* 1999; **30**: 271-276
30 **Everson GT**. Management of cirrhosis due to chronic hepatitis C. *J Hepatol* 2005; **42** Suppl: S65-S74
31 **Mazzella G**, Accogli E, Sottili S, Festi D, Orsini M, Salzetta A, Novelli V, Cipolla A, Fabbri C, Pezzoli A, Roda E. Alpha interferon treatment may prevent hepatocellular carcinoma in HCV-related liver cirrhosis. *J Hepatol* 1996; **24**: 141-147
32 **Serfaty L**, Aumaitre H, Chazouilleres O, Bonnand AM, Rosmorduc O, Poupon RE, Poupon R. Determinants of outcome of compensated hepatitis C virus-related cirrhosis. *Hepatology* 1998; **27**: 1435-1440
33 **Shiratori Y**, Imazeki F, Moriyama M, Yano M, Arakawa Y, Yokosuka O, Kuroki T, Nishiguchi S, Sata M, Yamada G, Fujiyama S, Yoshida H, Omata M. Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. *Ann Intern Med* 2000; **132**: 517-524
34 **Mazzaferro V**, Romito R, Schiavo M, Mariani L, Camerini T, Bhoori S, Capussotti L, Calise F, Pellicci R, Belli G, Tagger A, Colombo M, Bonino F, Majno P, Llovet JM. Prevention of hepatocellular carcinoma recurrence with alpha-interferon after liver resection in HCV cirrhosis. *Hepatology* 2006; **44**: 1543-1554
35 **Liver Cancer Study Group of Japan**. The general rules for the clinical and pathological study of primary liver cancer. Liver Cancer Study Group of Japan. *Jpn J Surg* 1989; **19**: 98-129
36 **Lin SM**, Lin CJ, Hsu CW, Tai DI, Sheen IS, Lin DY, Liaw YF. Prospective randomized controlled study of interferon-alpha in preventing hepatocellular carcinoma recurrence after medical ablation therapy for primary tumors. *Cancer* 2004; **100**: 376-382
37 **Valla DC**, Chevallier M, Marcellin P, Payen JL, Trepo C, Fonck M, Bourliere M, Boucher E, Miguet JP, Parlier D, Lemonnier C, Opolon P. Treatment of hepatitis C virus-related cirrhosis: a randomized, controlled trial of interferon alfa-2b versus no treatment. *Hepatology* 1999; **29**: 1870-1875

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Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon

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Abstract **We developed a reverse genetics system of hepatitis C virus (HCV) genotypes 1a and 2a using infectious clones and human hepatocyte chimeric mice. We inoculated cell culture-produced genotype 2a (JFH-1) HCV intravenously. We also injected genotype 1a CV-H77C clone RNA intrahepatically. Mice inoculated with HCV by both procedures developed measurable and transmissible viremia. Interferon (IFN) alpha treatment resulted in greater reduction of genotype 2a HCV levels than genotype 1a, as seen in clinical practice. Genetically engineered HCV infection system should be useful for analysis of the mechanisms of resistance of HCV to IFN and other drugs.**

Keywords: Human hepatocyte chimeric mouse; Human serum albumin; HCV RNA; Interferon

1. Introduction

The hepatitis C virus (HCV) infects an estimated 170 million people worldwide [1]. HCV causes persistent infection in adults leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [2,3]. The most effective therapy for viral clearance is a 48-week combination therapy of pegylated interferon (IFN)-alpha and ribavirin. However, the success rate of this combination therapy is only about 50% [4]. Development of new anti-HCV drug had been severely restricted by the absence of a cell culture system that supports the efficient replication of HCV, as well as the lack of a small animal model. A cell culture system has been developed recently using a unique genotype 2a HCV gnome (JFH-1), which does not require adaptive mutations for efficient replication [5–7]. Chimpanzee was the only useful animal for the study of HCV until recently, although the availability of this model is severely restricted [8]. Recently, HCV-infected mice have been developed by inoculating HCV-infected human serum into chimeric urokinase-type plasminogen activator (uPA)-severe combined immunodeficiency (SCID) mice with engrafted human hepatocytes [9]. This HCV-infected mouse model has been reported to be useful for evaluating anti-HCV drugs such as IFN-alpha and anti-NS3 protease [10]. We have generated a human hepatocyte chimeric mouse where mouse hepatocytes were extensively replaced by human hepatocytes [11], and established a genetically engineered hepatitis B virus (HBV) system [12]. Using this mouse, we show in this paper the development of reverse genetics system of genotypes 1a and 2a after intrahepatic injection of transcribed RNA and intravenous injection of cell culture-produced virus, respectively. We also show here that HCV in these mice can be transmitted to naïve mice. Interferon treatment of these mice resulted in a greater reduction of HCV titer in genotype 2a clone infected mice than in genotype 1a infected mice. As these results are consistent with our clinical experience, we consider this model suitable for the study of resistance of HCV against IFN and other drugs.

2. Materials and methods

2.1. Generation of human hepatocyte chimeric mice and quantification of human serum albumin

Generation of the uPA$^{+/+}$/SCID$^{+/+}$ mice and transplantation of human hepatocytes were performed as described recently by our group [11,12]. All mice used in this study were transplanted with frozen

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Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator

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human hepatocytes obtained from one donor. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentrations of human serum albumin (HSA) correlate with the repopulation index [11], and were measured as described previously [12]. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Graduate School of Biomedical Sciences, Hiroshima University.

2.2. HCV RNA transcription and inoculation into chimeric mice

A plasmid containing the full-length genotype 1a HCV cDNA clone, pCV-H77C, was kindly provided by Dr. Robert H. Purcell (National Institutes of Health). Ten micrograms of plasmid DNA, linearized by *Xba*I (Promega, Madison, WI) digestion, was transcribed in a 100-μl reaction volume with T7 RNA polymerase (Promega) at 37 °C for 2 h [13], and analyzed by agarose gel electrophoresis. Each transcription mixture was diluted with 400 μl of phosphate-buffered saline (PBS) and injected into the liver of chimeric mice. Transcripts of plasmid pJFH-1 containing the full-length HCV genotype 2a were transfected into Huh7 cells as described previously [6]. Seventy-two hours after transfection, 200 μl of the culture medium was injected intravenously into the chimeric mice. IFN-treatment was also performed by intramuscular injection of diluted IFN solutions. IFN-alpha was a kind gift from Hayashibara Biochemical Labs, Inc. (Okayama, Japan). Serum samples collected every 2 weeks after inoculation were frozen at −80 °C until further analysis.

2.3. Human serum samples

For control infection experiments, human serum containing a high titer of genotype 1b HCV (2.2×10^6 copies/ml) was obtained from a patient with chronic hepatitis after obtaining a written informed consent. The individual serum samples were divided into small aliquots and separately stored in liquid nitrogen until use.

2.4. RNA extraction and amplification

RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), dissolved in 8.8 μl RNase-free H_2O, and reverse transcribed by using a random primer (Takara Bio, Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in a 20 μl reaction mixture according to the instructions provided by the manufacturer. One microliter of cDNA solution was amplified by Light Cycler (Roche Diagnostic, Japan, Tokyo) for quantitation of HCV. The primers used for amplification were 5′-TTTATCCAAGAAAGGACCC-3′ and 5′-TTCACGCAGAAAGCGTCTAGC-3′. The amplification conditions included initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 5 s, and extension at 72 °C for 6 s. The lower detection limit of this assay is 10^3 copies/ml. Nested PCR was used with the outer primers NC1 (5′-CAACACTACTCGGCTAGCAGT-3′) and NC2 (5′-CCTGTGAGGAACTACTGTC-3′) and inner primers cc6 (5′-TTTATCCAAGAAAGGACCC-3′) and cc7 (5′-TTCACGCAGAAAGCGTCTAGCttc-3′). The amplification condition included 35 cycles of 94 °C for 30 s, 58 °C for 1 min 30 s, and 72 °C for 1 min after 5 min of initial denaturation at 94 °C followed by 7 min of final extension using Gene Taq (Wako Pure Chemicals, Tokyo) with anti-Taq high according to the instructions provided by the manufacturer (TOYOBO).

2.5. Histochemical analysis of mouse liver

Histopathological analysis and immunohistochemical staining using an antibody against HSA (Bethyl Laboratories Inc.) were performed as described previously [12].

3. Results

3.1. High serum HCV RNA titer in human hepatocyte chimeric mice after inoculation of serum samples obtained from HCV-infected patient

We inoculated 50 μl of genotype 1b serum samples into five chimeric mice intravenously to test their susceptibility to HCV infection. All mice became positive for HCV RNA by nested PCR at 2 weeks after inoculation (Fig. 1). The viremia reached a plateau level at 6–8 weeks after infection, and persisted for more than 12 weeks.

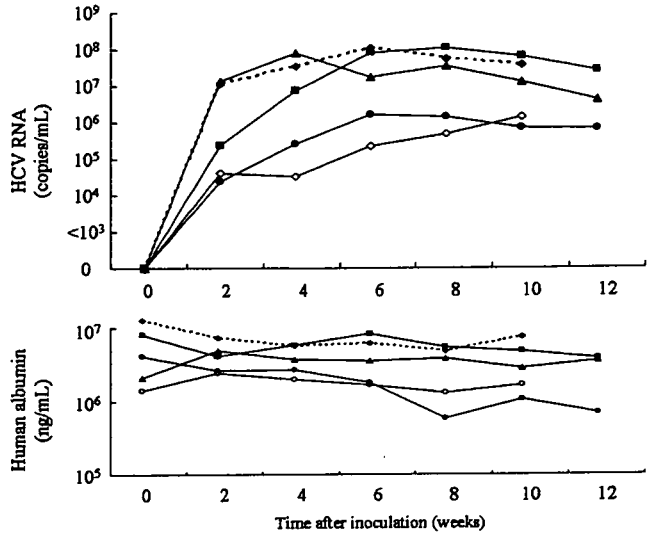

Fig. 1. Serial changes in HCV RNA and human serum albumin in sera of mice inoculated with human serum samples positive for genotype 1b HCV. Fifty microliter serum samples were injected intravenously into each mouse. Mice serum samples were obtained every 2 weeks after injection, and HCV RNA titer was analyzed.

3.2. Infection with in vitro-transcribed genotype 1a HCV RNA and cell culture generated genotype 2a HCV

In the next step, we tried to establish infection of cloned HCV using infectious genotype 1a and genotype 2a clones. In these experiments, we used two different strategies to establish infection using these two clones because genotype 1a has not been confirmed to replicate in cell culture system. We used genotype 1a HCV RNA (CV-H77C), which has been reported to be infectious to chimpanzee [13]. In vitro-transcribed HCV RNA was directly injected intrahepatically in three chimeric mice. We also infected three chimeric mice by intravenous injection of Huh7 cell-produced genotype 2a HCV after transfection of in vitro transcribed RNA from an infectious clone JFH-1. This clone has been shown to be infectious to a chimpanzee [6] and a chimeric mouse [7]. All mice developed measurable viremia 2 weeks after inoculation. At 6 weeks after inoculation, HCV RNA titer was 2.4×10^7 copies/ml (range: 8.8×10^6–2.9×10^7 copies/ml) in genotype 1a HCV-infected mice, and 2.5×10^5 copies/ml (range: 1.4×10^5–3.7×10^5 copies/ml) in genotype 2a HCV-infected mice (Fig. 2).

3.3. Passage experiment of HCV to naïve chimeric mice

We then performed passage experiments using naïve mice. Each of three mice was inoculated intravenously with 10 μl serum samples obtained from the above genotype 1a and genotype 2a HCV-infected mice at week 6. Two weeks after injection, all mice developed measurable viremia, and the titer was 8.5×10^6 copies/ml (range: 1.4×10^6–2.4×10^7 copies/ml) in genotype 1a, and 1.7×10^5 copies/ml (range: 1.5×10^5–2.5×10^5 copies/ml) in genotype 2a HCV-infected mice (Fig. 3).