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Randomized, Phase II Study Comparing Interferon Combined with Hepatic Arterial Infusion of Fluorouracil plus Cisplatin and Fluorouracil Alone in Patients with Advanced Hepatocellular Carcinoma

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Key Words
Hepatic arterial infusion · Hepatocellular carcinoma · 5-Fluorouracil · Cisplatin · Interferon

Abstract
Objective: This randomized phase II trial compared the response rates to treatment with interferon (IFN) combined with hepatic arterial infusion of fluorouracil (FU) plus cisplatin (CDDP) or FU alone in patients with advanced hepatocellular carcinoma (HCC). ***Methods:*** A total of 114 patients with measurable advanced HCC were enrolled and randomized into 2 groups. FU (300 mg/m^2, days 1–5, days 8–12) with or without CDDP (20 mg/m^2, days 1 and 8) was administered via the hepatic artery. IFNα-2b was administered 3 times per week for 4 weeks. ***Results:*** The response rates were 45.6% for the IFN/FU + CDDP group and 24.6% for the IFN/FU group. The response rate was significantly higher in the IFN/FU + CDDP group ($p = 0.030$). The median overall survival period was 17.6 months in the IFN/FU + CDDP group versus 10.5 months in the IFN/FU group ($p = 0.522$). The median progression-free survival period was 6.5 months in the IFN/FU + CDDP group versus 3.3 months in the IFN/FU group ($p = 0.0048$). Hematological toxicity was common, but no toxicity-related deaths were observed. ***Conclusion:*** These results show the clinical efficacy of adding CDDP to the hepatic arterial infusion of FU in combined chemotherapy regimens with IFN.

Introduction

Hepatocellular carcinoma (HCC) is the 6th most frequent type of cancer in the world and ranks third among the various causes of cancer death. In recent years, the incidence of HCC has been increasing in Western and Asian countries [1–3].

Clinical practice guidelines for HCC are currently available in Japan, and the number of early cases with an early single tumor with a major diameter of 2 cm or less detected by regular screening is generally increasing [4]. The treatment of early cases, including the use of hepatectomy and local therapy such as radiofrequency abla-

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tion and percutaneous ethanol injection therapy, has progressed markedly, achieving a 5-year survival rate of 60–70% [5]. Most patients with HCC experience the repeated recurrence of tumors after treatment, and the disease may eventually reach an advanced stage. Furthermore, it is still not uncommon to find patients with symptomatic advanced HCC who have not participated in regular screening.

The efficacy of hepatectomy, local ablation therapy and transarterial chemoembolization (TACE) is limited for advanced HCC, and the prognosis of such cases is poor. Under these circumstances, systemic therapy with the molecular targeting drug sorafenib has shown a statistically significant survival benefit compared with placebo treatment in two large-scale phase III clinical trials [6, 7]. Based on these findings, this drug is now recommended as a standard treatment for advanced HCC. These trials did not compare sorafenib with other conventional treatments of advanced HCC but with best supportive care as the placebo treatment. Although a significant difference in the survival time was noted, the response rate was as low as 2–3.3%, with no significant difference from the results in the placebo arm (1–1.3%) [6, 7].

As another optional treatment for advanced HCC, hepatic arterial infusion chemotherapy (HAIC) has been employed mainly in Japan and other Asian countries. HAIC has been used not only for unresectable HCC accompanied by vascular invasion but also uncontrollable cases of repeated recurrences within a short period of time despite a number of sessions of TACE.

In recent years, fluorouracil (FU) and cisplatin (CDDP) have been reported as the most commonly used anticancer drugs for HAIC [8–17]. Favorable results with an HAIC protocol using low-dose CDDP and FU have also been reported [8, 14, 16, 17]. Similarly, combination of interferon (IFN) with FU has demonstrated relatively good results in HAIC [11, 13, 18].

With this background in mind and with the aim of establishing the most effective HAIC protocol for advanced HCC, we planned a phase II randomized clinical comparative study to examine whether or not IFN combined with HAIC consisting of FU and CDDP might be associated with a higher response rate. Patients with advanced HCC were randomly allocated to two treatment arms, i.e. IFN combined with hepatic arterial infusion of FU with CDDP or IFN combined with hepatic arterial infusion of FU alone without CDDP. The results were then compared with regard to the efficacy, safety and prognosis.

Patients and Methods

Patients

Patients who had histologically or clinically diagnosed HCC were included in this study. A clinical diagnosis of HCC was made based on underlying chronic liver disease, radiologic findings and elevation of tumor markers.

With regard to the tumor stage, the following patients were included: patients who had (1) severe vascular invasion (i.e. vascular invasion found in the main trunk to the secondary branches of the portal vein, or invasion in the right, middle or left hepatic vein) and (2) intrahepatic multiple lesions (i.e. 5 or more nodules in the left and/or right lobes as confirmed by radiology).

Patients were eligible when they were 20 years old or older, had an Eastern Clinical Oncology Group performance status of 2 or less and had appropriate bone marrow, liver, kidney and cardiac functions as determined by the following measurements obtained within 1 week before enrollment: hemoglobin ≥8.0 g/dl; white blood cell count ≥2,000/mm^3; platelet count ≥30,000/mm^3; blood urea nitrogen ≤30 mg/dl; serum creatinine ≤2.0 mg/dl; percentage of prothrombin time ≥30%, and total bilirubin ≤5 mg/dl or less (excluding elevations caused by biliary tract obstruction as a result of HCC).

Assignment

The present study was an open randomized single-center study consisting of a two-group comparison. All the patients who satisfied the inclusion criteria were randomized to either of the two treatments. The treatment protocol was approved by the ethical committee of Kanazawa University (approval number 5169). Patients were given full information regarding the details of the clinical study and provided their written consent prior to participation in the study. This clinical study adhered to the Declaration of Helsinki and good clinical practice.

Treatment Schedule

A reservoir for hepatic arterial infusion was implanted prior to HAIC. A catheter with a side hole was inserted from the right femoral artery using an image-guided procedure, and the tip of the catheter was placed in the gastroduodenal artery or splenic artery. When more than one hepatic artery was present, the hepatic arteries were unified to the original proper hepatic artery alone. When blood flow into the gastrointestinal tract was confirmed by catheter angiography, the route was embolized to prevent complications. The reservoir was placed beneath the skin in the lower right abdomen. Medication was started at least 3 days after implantation.

In the IFN/FU treatment group, patients underwent continuous hepatic arterial infusion of FU (5-FU®, Kyowa Hakko, Tokyo, Japan) at a dose of 300 mg/m^2/day for 5 days in the 1st and 2nd weeks (for 120 h) using an infuser pump (Baxter Infusor SV1®, Tokyo, Japan) in the same manner as described in previous reports [18]. The maximum amount of FU infused over 5 days was 2,500 mg. IFNα-2b (Intron A®, Schering-Plough, Osaka, Japan) at a dose of 3,000,000 units was injected intramuscularly 3 times a week for 4 weeks. In the IFN/FU + CDDP treatment group, CDDP (Randa®, Nippon Kayaku, Tokyo, Japan) at a dose of 20 mg/m^2 was given by hepatic arterial infusion over 1.5 h on days 1 and 8 prior to the administration of FU and after appropriate hy-

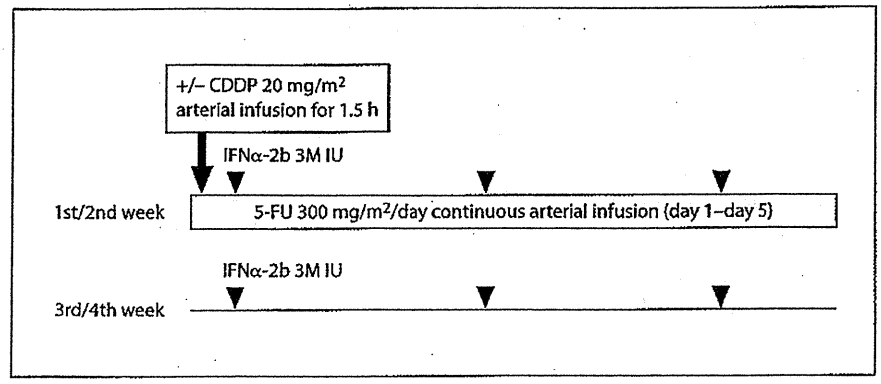

Fig. 1. Treatment protocol. 3M = 3 million.

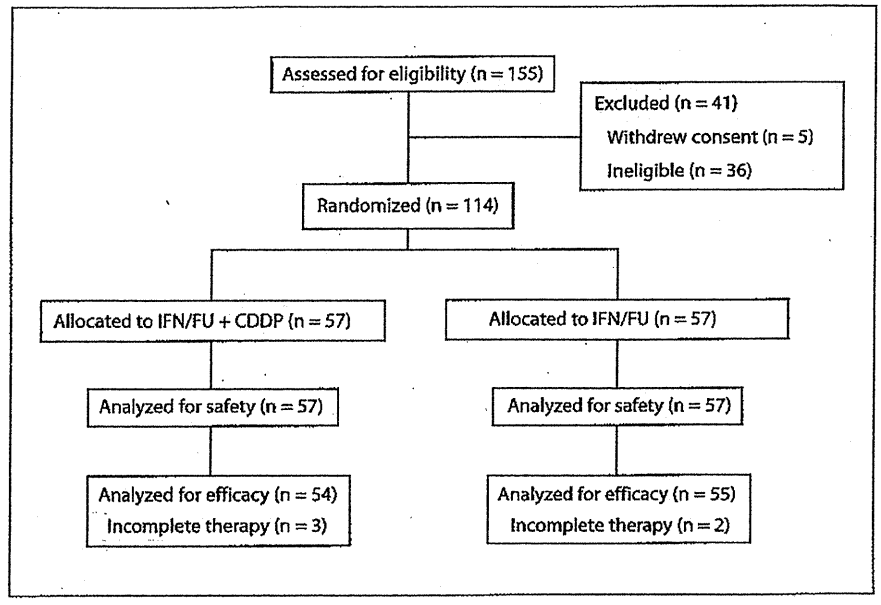

Fig. 2. Consolidated Standards of Reporting Trials flow diagram.

duration and antiemetic medication. A treatment cycle comprised 4 weeks of drug administration including IFN administration and a subsequent 2-week rest period (fig. 1).

Sample Size

Based on previous reports in the literature [9, 19] and the results of our studies of HAIC for the treatment of HCC using single-drug regimens, it was assumed that the response rate in the IFN/FU treatment group would be 20% and that in the IFN/FU + CDDP treatment group would be 50%. Based on the assumption that the ratio of the numbers of patients was 1:1, the α error was 0.05, the β error was 0.1, and 52 patients were necessary for each treatment group. Therefore, the number of patients to be included was 114, allowing a 10% dropout rate, which would result in a total of 104 patients for the two groups.

Response Assessment

The primary endpoint was the response rate, as determined using dynamic computed tomography or magnetic resonance imaging performed at the end of each treatment cycle according to the Response Evaluation Criteria in Solid Tumors, version 1.0 [20].

Secondary endpoints were the overall survival time, progression-free survival time and adverse events. The overall survival time was defined as the period from the time of randomization until death, and the progression-free survival time was defined as the period from the beginning of treatment until confirmation of progression or death. Adverse events were evaluated according to the Common Toxicity Criteria for Adverse Events, version 3.0.

Statistical Analyses

The two treatment groups were compared using the Fisher direct method and the Wilcoxon rank sum test. Response factors were analyzed using logistic regression analysis. The cumulative survival and prognostic factors were analyzed using the Kaplan-Meier method, log-rank test and the Cox proportional hazard regression model.

Table 1. Patient demographics and baseline characteristics

	IFN/FU + CDDP (n = 57)	IFN/FU (n = 57)	p value
Gender (male/female)	49/8	46/11	0.62[a]
Median age, years (range)	65 (40–82)	68 (40–82)	0.27[b]
ECOG PS (0/1/2)	36/19/2	34/21/2	0.92[b]
Primary/recurrence	20/37	23/34	0.70[a]
Prior TACE (+/–)	32/25	33/24	1.00[a]
Prior chemotherapy (+/–)	4/53	3/54	1.00[a]
HCV-Ab (positive/negative)	32/25	35/22	0.70[a]
HBsAg (positive/negative)	16/41	18/39	0.83[a]
Liver cirrhosis (+/–)	46/11	47/10	1.00[a]
Child-Pugh class (A/B/C)	33/23/1	32/22/3	0.74[b]
LCSGJ TNM stage (II/III/IVA/IVB)	7/26/17/7	7/20/25/5	0.53[b]
UICC TNM stage (II/III/IV)	6/43/8	12/38/7	0.30[b]
BCLC stage (B/C/D)	33/23/1	23/31/3	0.13[b]
Median diameter of tumor, mm (range)	37 (10–250)	40 (11–200)	0.71[b]
Major portal vein invasion (+/–)	12/45	19/38	0.21[a]
Lymph node metastasis (+/–)	2/55	4/53	0.68[a]
Distant metastasis (+/–)	7/50	5/52	0.76[a]
Treatment cycles, n	3.2 ± 2.6	2.9 ± 2.4	0.37[b]
Albumin, g/dl	3.36 ± 0.6	3.49 ± 0.5	0.22[b]
Total bilirubin, mg/dl	1.10 ± 0.7	1.44 ± 0.88	0.07[b]
Active prothrombin, %	78.6 ± 18.9	74.9 ± 13.8	0.22[b]
Platelet count, $\times 10^4/\mu l$	12.3 ± 6.4	11.0 ± 5.4	0.26[b]
AST, IU/l	83.1 ± 74.4	82.5 ± 51.8	0.47[b]
ALT, IU/l	64.2 ± 53.6	68.5 ± 88.6	1.00[b]
DCP (<100/≥100 mAU/ml)	33/24	37/20	0.56[a]
AFP (<400/≥400 ng/ml)	24/33	28/29	0.57[a]
AFP-L3 (<30/≥30%)	22/35	27/30	0.45[a]

Values represent numbers of patients or mean ± SD, except where indicated otherwise. ECOG = Eastern Cooperative Oncology Group; PS = performance status; HBsAg = hepatitis B surface antigen; LCSGJ = Liver Cancer Study Group of Japan; UICC = Union for International Cancer Control; BCLC = Barcelona Clinic Liver Cancer; Major portal vein invasion = tumor invasion in main trunk or 1st branches of portal vein; ALT = alanine aminotransferase; DCP = des-gamma-carboxy prothrombin; AFP = α-fetoprotein.
[a] Fisher's exact test. [b] Wilcoxon rank sum test.

Results

Patients

A total of 155 patients with advanced HCC were treated at our hospital between October 2003 and September 2007. Eventually, 114 patients were allocated to the IFN/FU + CDDP treatment group or the IFN/FU treatment group. Three patients in the IFN/FU + CDDP group and 2 in the IFN/FU group dropped out before the end of the first cycle; therefore, a total of 109 patients, comprising 54 patients from the former group and 55 from the latter, were included in the efficacy evaluation (fig. 2).

The baseline clinical features of the 114 patients are shown in table 1. No significant differences in the clinical features and test results were observed between the two groups, with the exception of a slightly higher bilirubin level in the IFN/FU group. The patients classified as having Barcelona Clinic Liver Cancer stage B had 5 or more nodules in the left and/or right lobes and were considered to have disease that was difficult to control by TACE after repeated TACE (68%) or multiple lesions that showed an inadequate response to TACE.

Response to Treatment

Among the 57 patients in the IFN/FU + CDDP treatment group, the best study response was a complete response (CR) in 1 (1.7%); partial response (PR) was observed in 25 (43.9%) patients, stable disease was observed

Table 2. Comparison of best study response between treatment arms

Best study response	IFN/FU + CDDP (n = 57)	IFN/FU (n = 57)	p value
CR	1 (1.7)	3 (5.3)	
PR	25 (43.9)	11 (19.3)	
SD	15 (26.3)	19 (33.3)	
PD	13 (22.8)	22 (38.6)	
NE	3 (5.3)	2 (3.5)	
RR (CR + PR)	26 (45.6)	14 (24.6)	0.030
TCR (CR + PR + SD)	41 (71.9)	33 (57.9)	0.169

Values represent n (%). Between-group p values were determined using Fisher's exact test. SD = Stable disease; PD = progressive disease; NE = not evaluable; RR = response rate; TCR = tumor control rate.

in 15 (26.3%), and progressive disease was observed in 13 (22.8%). Among the 57 patients in the IFN/FU treatment group, the response was CR in 3 (5.3%), PR in 11 (19.3%), stable disease in 19 (33.3%) and progressive disease in 22 (38.6%). The response rate (CR + PR) was 45.6% in the IFN/FU + CDDP group and 24.6% in the IFN/FU group; the figure was significantly higher in the former group (p = 0.030; table 2).

The only factor that improved the response to treatment as indicated by a multivariate analysis was the addition of CDDP to the treatment [odds ratio 2.5, 95% confidence interval (CI) 1.1–6.0; table 3].

Safety

Table 4 shows the major adverse events. Grade 3 or 4 adverse events were found in 75 of the 114 patients (65.8%). Bone marrow suppression of any grade was found in 65–90% of the patients. Leukopenia and neutropenia were noted in about 70% of the patients, and no significant difference was found between the IFN/FU + CDDP group and the IFN/FU group. An overall reduction in hemoglobin was observed more frequently in the IFN/FU + CDDP group than in the IFN/FU group (91.2 vs. 75.4%; p = 0.021), although the difference was not significant for hemoglobin reductions of grade 3 or 4. No significant difference in all-grade thrombocytopenia was observed between the two groups, but thrombocytopenia of grade 3 or 4 was found significantly more frequently in the IFN/FU + CDDP group (45.6 vs. 22.8%; p = 0.017). However, no serious complications secondary to a reduction in platelets occurred.

Nonhematologic toxicities including general malaise, nausea, vomiting, stomatitis and elevation of serum creatinine were significantly more common in the IFN/FU + CDDP group, but no intergroup difference was found for grade 3 or grade 4 toxicities.

Peptic ulcer arising from the leakage of arterially infused anticancer drugs into the gastrointestinal tract, a complication characteristic of HAIC, was found in 6 patients (10.5%) in the IFN/FU + CDDP group and 1 patient (1.8%) in the IFN/FU group; the incidence was higher, but not significantly, in the IFN/FU + CDDP group (p = 0.06), and no grade 3 or grade 4 cases occurred.

Survival

The median overall survival period of the 114 patients who underwent HAIC was 12.0 months (95% CI 11.6–12.4). In the IFN/FU + CDDP group, the median survival time (MST) was 17.6 months (95% CI 9.9–25.3). In the IFN/FU group, the MST was 10.5 months (95% CI 5.6–15.4). Although the survival period tended to be longer in the group given FU combined with CDDP, no statistically significant differences were observed between the two groups (p = 0.522, log-rank test; hazard ratio 0.88, 95% CI 0.60–1.30; fig. 3a).

In the subgroup with the presence of major vascular invasion, the MST was 5.8 months (95% CI 3.3–8.3) in the IFN/FU + CDDP group and 4.7 months (95% CI –7.6 to 31.6) in the IFN/FU group. In contrast, in the subgroup with absence of major vascular invasion, the MST was 20.0 months (95% CI 13.6–26.6) in the IFN/FU + CDDP group and 12.0 months (95% CI 4.4–19.6) in the IFN/FU group. Subanalysis according to the presence or absence of major vascular invasion showed no significant difference between the two treatment groups (p = 0.571 in the presence of major vascular invasion, p = 0.399 in its absence). In the subgroup with tumor stage II and III, the MST was 22.6 months (95% CI 0.4–44.7) in the IFN/FU + CDDP group and 12.0 months (95% CI 5.5–18.5) in the IFN/FU group. In the subgroup with tumor stage IVA and IVB, the MST was 7.5 months (95% CI 5.7–9.3) in the IFN/FU + CDDP group and 7.5 months (95% CI 0.4–14.5) in the IFN/FU group. Subanalysis according to tumor stage (stage II and III or stage IVA and IVB) also showed no difference between the two treatment groups (p = 0.625 for stage II and III, p = 0.906 for stage IVA and IVB).

The median overall progression-free survival period of the 114 patients was 4.5 months (95% CI 3.5–5.5). In the IFN/FU + CDDP group, the median progression-free survival time was 6.5 months (95% CI 2.6–10.4). In the IFN/FU group, the median progression-free survival

Table 3. Factorial analysis of predictors of response

	Response rate %	Univariate analysis, p value[a]	Multivariate analysis, p value[b]	Odds ratio (95% CI)
IFN/FU + CDDP/IFN/FU	45.6/25.6	0.0302	0.0268	2.5 (1.1–6.0)
Gender (male/female)	35.8/31.6	0.7979		
Age (<65/≥65 years)	31.1/39.6	0.4318		
Primary/recurrence	32.6/36.6	0.6909		
Prior TACE (+/–)	35.4/36.7	1.00		
Prior chemotherapy (+/–)	57.1/33.6	0.2382		
HCV-Ab (positive/negative)	40.3/27.7	0.2315		
HBsAg (positive/negative)	38.2/33.7	0.6722		
Liver cirrhosis (+/–)	37.6/23.8	0.3134		
Child-Pugh class (A/B, C)	41.5/26.5	0.115		
LCSGJ TNM stage (II, III/IVA, IVB)	46.7/22.2	0.0102	0.2877	1.3 (0.4–4.0)
Diameter of tumor (<50/≥50 mm)	44.0/17.9	0.008	0.1817	2.2 (0.7–7.0)
Major portal vein invasion (+/–)	16.1/42.2	0.0143	0.1266	1.8 (0.5–6.8)
Lymph node metastasis (+/–)	33.3/35.2	1.00		
Distant metastasis (+/–)	16.7/37.3	0.2098		
Albumin (<3.5/≥3.5 g/dl)	27.6/42.9	0.1165		
Total bilirubin (<1.5/≥1.5 mg/dl)	39.2/25.7	0.2038		
Active prothrombin (<70/≥70%)	26.8/39.7	0.2203		
Platelets (<10/≥10 × 10^4/μl)	33.3/36.7	0.8444		
AST (<80/≥80 IU/l)	40.8/25.6	0.1096		
ALT (<80/≥80 IU/l)	33.7/40.0	0.6372		
DCP (<100/≥100 mAU/ml)	42.5/57.5	0.5511		
AFP (<400/≥400 ng/ml)	38.7/30.8	0.4334		
AFP-L3 (<30/≥30%)	46.6/22.4	0.0094	0.2898	1.7 (0.7–4.4)

HBsAg = Hepatitis B surface antigen; LCSGJ = Liver Cancer Study Group of Japan; Major portal vein invasion = tumor invasion in main trunk or 1st branches of portal vein; ALT = alanine aminotransferase; DCP = des-gamma-carboxy prothrombin; AFP = α-fetoprotein. [a] Fisher's exact test. [b] Logistic procedure model.

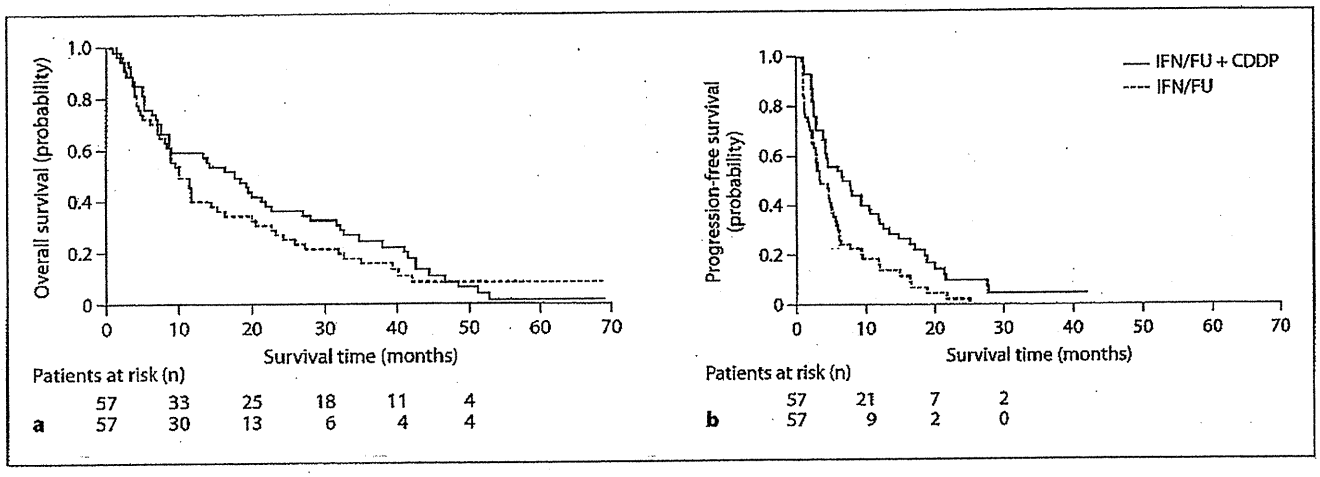

Fig. 3. Kaplan-Meier analysis of overall survival (**a**) and progression-free survival (**b**) according to the different chemotherapeutic regimens.

Table 4. Most common adverse events

Adverse event	IFN/FU + CDDP (n = 57)		IFN/FU (n = 57)	
	any grade	CTC grade 3–4	any grade	CTC grade 3–4
Neutropenia	44 (77.2)	17 (29.8)	37 (64.9)	19 (33.3)
Leukopenia	43 (75.4)	12 (21.1)	38 (66.7)	18 (31.6)
Reduced hemoglobin	52 (91.2)[a]	4 (7.0)	43 (75.4)[a]	2 (3.5)
Thrombocytopenia	50 (89.5)	26 (45.6)[b]	48 (84.2)	13 (22.8)[b]
Prothrombin time	30 (52.6)	3 (5.3)	32 (56.1)	1 (1.8)
Asthenia	34 (59.6)[a]	1 (1.8)	21 (36.8)[a]	3 (5.3)
Fever	41 (71.9)	1 (1.8)	37 (64.9)	0
Nausea	32 (56.1)[a]	10 (17.5)	22 (38.6)[a]	3 (5.3)
Vomiting	15 (26.3)[a]	4 (7.0)	4 (7.0)[a]	1 (1.8)
Mucositis	22 (38.6)[a]	3 (5.3)	9 (15.8)[a]	1 (1.8)
Liver function	42 (73.7)	4 (7.0)	43 (75.4)	10 (17.5)
Creatinine elevation	10 (17.5)[a]	0	2 (3.5)[a]	0
Peptic ulcer	6 (10.5)	0	1 (1.8)	0

Values represent numbers of events with percentages in parentheses. [a] $p < 0.05$ (Wilcoxon rank sum test); [b] $p < 0.05$ (Fisher's exact test). CTC = Common Toxicity Criteria.

time was 3.3 months (95% CI −0.6 to 7.2). The progression-free survival period was significantly longer in the IFN/FU + CDDP group than in the IFN/FU group ($p = 0.0048$, log-rank test; hazard ratio 0.57, 95% CI 0.38–0.85; fig. 3b).

As predictors for survival, a multivariate analysis showed that positivity for hepatitis C virus antibody (HCV-Ab), an albumin level of 3.5 g/dl or more and an aspartate aminotransferase (AST) value of lower than 80 IU/l were associated with improved survival (table 5).

Discussion

The present study showed that the addition of CDDP to IFN combined with HAIC using FU significantly enhanced the antitumor effect from 24.6 to 45.6%. The response rates obtained in previous studies of HAIC involving at least 30 patients varied from 14 to 71% [8–17]. Regarding the use of IFN combined with HAIC using FU, Obi et al. [13] used this treatment in patients with advanced HCC and a tumor embolus in the main trunk or the first branch of the portal vein and achieved a response rate of 52.6%. Ota et al. [18] also used IFN combined with HAIC using FU for similar cases of advanced HCC and reported a response rate of 43.6%. We previously reported a response rate of 45% in 34 patients who underwent multidrug HAIC using FU and CDDP in combination with IFN treatment [11]. Uka et al. [21] used IFN in combination with HAIC using FU in 55 patients who had a tumor embolus of the portal vein and reported a response rate of 29%. The response rates obtained in the present study were similar to those obtained in the report by Uka et al. [21] and lower than those obtained in the other two reports. This discrepancy may be explained by the different criteria used to evaluate antitumor efficacy, as Uka et al. [21] suggested in their discussion. Obi et al. [13] and Ota et al. [18] used the Eastern Clinical Oncology Group criteria, whereas Uka et al. [21] and the present study used the Response Evaluation Criteria in Solid Tumors.

The combined use of FU and IFN is reportedly beneficial because IFN serves as a modulator to enhance the antitumor effect of FU. More specifically, IFN induces p53, which enhances apoptosis by FU, and influences the cell cycle via $p27^{Kip1}$ or apoptosis via Bcl-xL [22, 23]. From a clinical perspective, Takaki-Hamade et al. [24] and Eun et al. [25] concluded that combined IFN treatment did not have an incremental effect. Thus, the benefit of adding IFN to HAIC with FU has not been proven clinically. However, experimental data suggest that IFN should enhance the antitumor effect of FU [22, 26], and this supports the current use of IFN-combined HAIC in clinical practice.

On the other hand, with regard to the effect of CDDP combined with FU in a clinical setting, Ando et al. [8] used HAIC with FU combined with low-dose CDDP for the treatment of patients with advanced HCC and a portal

Table 5. Factorial analysis of predictors of survival

	MST, months	Univariate analysis, p value[a]	Multivariate analysis, p value[b]	Hazard ratio (95% CI)
IFN/FU + CDDP/IFN/FU	17.6/10.5	0.522		
Gender (male/female)	12.0/12.0	0.236		
Age (<65/≥65 years)	9.9/19.5	0.115		
Primary/recurrence	7.7/16.5	0.394		
Prior TACE (+/–)	14.4/12.0	0.491		
Prior chemotherapy (+/–)	18.6/12.0	0.936		
HCV-Ab (positive/negative)	19.5/7.6	0.0049	0.0219	0.60 (0.39–0.93)
HBsAg (positive/negative)	7.6/15.4	0.1145		
Liver cirrhosis (+/–)	13.7/9.0	0.5063		
Child-Pugh class (A/B, C)	18.6/9.2	0.0636		
LCSGJ TNM stage (II, III/IVA, IVB)	19.4/7.5	0.0019	0.6326	0.87 (0.49–1.54)
Diameter of tumor (<50/≥50 mm)	19.4/5.8	0.0014	0.1068	0.64 (0.37–1.10)
Major portal vein invasion (+/–)	5.1/18.6	0.0005	0.3203	0.73 (0.40–1.35)
Lymph node metastasis (+/–)	4.5/12.0	0.0789		
Distant metastasis (+/–)	4.5/14.0	0.0037	0.1806	0.60 (0.29–1.27)
Albumin (<3.5/≥3.5 g/dl)	9.3/16.5	0.0200	0.0017	0.50 (0.32–0.77)
Total bilirubin (<1.5/≥1.5 mg/dl)	15.4/9.5	0.2774		
Active prothrombin (<70/≥70%)	9.3/14.5	0.9470		
Platelets (<10/≥10 × 10^4/μl)	16.5/10.5	0.6273		
AST (<80/≥80 IU/l)	19.4/7.4	0.0056	0.0356	0.62 (0.39–0.97)
ALT (<80/≥80 IU/l)	13.7/9.5	0.8973		
DCP (<100/≥100 mAU/ml)	20.0/9.4	0.2294		
AFP (<400/≥400 ng/ml)	21.5/6.6	0.0002	0.1588	0.69 (0.41–1.16)
AFP-L3 (<30/≥30%)	20.8/7.5	0.0002	0.0730	0.61 (0.35–1.05)

HBsAg = Hepatitis B surface antigen; LCSGJ = Liver Cancer Study Group of Japan; Major portal vein invasion = tumor invasion in main trunk or 1st branches of portal vein; ALT = alanine aminotransferase; DCP = des-gamma-carboxy prothrombin; AFP = α-fetoprotein. [a] Log-rank test. [b] Cox proportional hazard model.

tumor embolus and reported a response rate of 48%. Following their report, several other reports on HAIC with FU combined with low-dose CDDP were published, with reported response rates ranging from 38.5 to 71% [14, 16, 17, 27]. Experimental studies have shown that low-dose CDDP blocks methionine transport into the cell, causing a decrease in intracellular methionine and an increase in reduced folic acid, thus serving as a modulator of FU to enhance its antitumor efficacy [28]. It has also been reported that low-dose CDDP is involved in the inhibition of p53-mediated apoptosis and drug resistance [29]. The present study used two agents, IFN and CDDP, in combination with FU. Although IFN and CDDP seem to enhance the antitumor effect of FU through these pathways, a large amount of basic experimental research on FU combined with these two agents remains to be performed.

Our present study showed that the antitumor effect was significantly greater and the progression-free survival time was significantly longer in the IFN/FU + CDDP group. However, there was no statistically significant difference in the overall survival time. Subgroup analysis also did not show survival benefit in the IFN + CDDP group. Since there were no limitations on treatment after the end of the protocol treatment, 88 of the 114 patients (77.2%) underwent some treatment subsequently, and 34 patients (59.6%) in the IFN/FU group received HAIC (mainly IFN/FU + CDDP) eventually. This might have had some effects on the results for overall survival.

The factors that improved survival in this study included positivity for HCV-Ab, an albumin level of 3.5 g/dl or more and an AST value of lower than 80 IU/l. Previous reports have documented the presence of response to chemotherapy, the Cancer of the Liver Italian Program score, the Okuda stage, the Child-Pugh score and α-fetoprotein as prognostic factors for HAIC in advanced HCC [30, 31]. Obi et al. [13] also reported that positivity for HCV-Ab was a predictor of a CR to IFN combined with HAIC using FU. Uka et al. [21] reported that positivity

for HCV-Ab was a factor associated with the early antitumor effect, progression-free time and overall survival after IFN combined with HAIC. Thus, positivity for HCV-Ab was determined to be a factor that improved prognosis. A possible explanation for this discrepancy may be that viral differences between hepatitis B virus and HCV may be involved in the heterogeneity of the anticancer drug sensitivity of HCC, or differences in the cytokine patterns of hepatitis B virus and HCV infections may influence the effect of IFN [32–35]. However, the true explanation remains unclear. In connection with an AST value of lower than 80 IU/l, Cheong et al. [36] also reported that low levels of AST and alkaline phosphatase were associated with long-term survival exceeding 8 months in a study examining chemotherapy including HAIC for the treatment of patients with advanced HCC. The basis of their findings requires further investigation.

Most patients with HCC have concomitant hepatic cirrhosis and thus have pancytopenia. Therefore, with regard to adverse events, we expected to see enhanced blood toxicity when IFN and CDDP were added to FU. Thus, this study showed a significantly higher frequency of cytopenia in the IFN/FU + CDDP group. However, as far as severe hematologic toxicities of grade 3 or 4 were concerned, thrombocytopenia alone was significantly more frequent in the IFN/FU + CDDP group, but no complications secondary to thrombocytopenia occurred. Although some nonhematologic toxicities were significantly more frequent in the IFN/FU + CDDP group, these adverse events were controllable. Thus, IFN combined with HAIC using FU and CDDP seems to be tolerable with regard to the occurrence of adverse events. The frequency of grade 3 or 4 toxicity with IFN-combined HAIC in our study was higher than that with sorafenib therapy reported previously [6, 7]. We enrolled 45 patients (39.5%) in Child-Pugh class B, and the pretreatment blood cell count in patients of Child-Pugh class B was generally lower than that of those patients in Child-Pugh class A. In addition, IFN has the effect of decreasing the blood cell count, especially neutrophils and platelets. However, these toxicities were controllable and there were no toxicity-related deaths.

In conclusion, the results of this phase II randomized clinical study on the effect of adding CDDP to IFN in combination with HAIC using FU for the treatment of advanced HCC show that the addition of CDDP significantly increases the antitumor effect of the treatment and results in a significant improvement in the progression-free survival time. Although there was no significant difference in the overall survival time of the two treatment groups, the survival benefit of IFN combined with HAIC using CDDP should be examined in comparison with systemic therapy using sorafenib, the current standard treatment for advanced HCC. In this regard, a multicenter study of hepatic arterial infusion of FU versus sorafenib therapy is now under way in Japan, and the results are awaited.

Disclosure Statement

There are no financial disclosures from any authors. There are no conflicts of interest for any authors.

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Research Article

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The *let-7* family of microRNAs inhibits Bcl-xL expression and potentiates sorafenib-induced apoptosis in human hepatocellular carcinoma

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Background & Aims: Bcl-xL, an anti-apoptotic member of the Bcl-2 family, is over-expressed in human hepatocellular carcinoma, conferring a survival advantage to tumour cells. The mechanisms underlying its dysregulation have not been clarified. In the present study, we explored the involvement of microRNAs that act as endogenous sequence-specific suppressors of gene expression.

Methods: The expression profiles of microRNAs in Huh7 hepatoma cells and primary human hepatocytes were compared by microarray analysis. The effect of *let-7* on Bcl-xL expression was examined by Western blot and a reporter assay. The involvement of *let-7* microRNAs in human tissues was analysed by western blot and reverse transcription-PCR.

Results: Microarray analysis, followed by *in silico* target prediction, identified *let-7* microRNAs as being downregulated in Huh7 hepatoma cells in comparison with primary human hepatocytes, as well as possessing a putative target site in the *bcl-xl* mRNA. Over-expression of *let-7c* or *let-7g* led to a clear decrease of Bcl-xL expression in Huh7 and HepG2 cell lines. Reporter assays revealed direct post-transcriptional regulation involving *let-7c* or *let-7g* and the 3′-untranslated region of *bcl-xl* mRNA. Human hepatocellular carcinoma tissues with low expression of *let-7c* displayed higher expression of Bcl-xL protein than those with high expression of *let-7c*, suggesting that low *let-7* microRNA expression contributes to Bcl-xL over-expression. Finally, expression of *let-7c* enhanced apoptosis of hepatoma cells upon exposure to sorafenib, which downregulates expression of another anti-apoptotic Bcl-2 protein, Mcl-1.

Conclusions: *let-7* microRNAs negatively regulate Bcl-xL expression in human hepatocellular carcinomas and induce apoptosis in cooperation with an anti-cancer drug targeting Mcl-1.

Cancer

Introduction

MicroRNAs (miRNAs), a novel class of non-coding, small RNAs, repress gene expression by binding to the 3′-untranslated region (3′UTR) of target messenger RNAs (mRNAs) [1]. More than 500 miRNAs have been identified in humans. Each miRNA is capable of modulating the expression of many mRNAs to which it binds by imperfect sequence complementarity, although only a limited number of targeted genes has been identified. Through its activity of gene silencing, miRNA functions in a variety of cellular processes, such as development, organ homeostasis, and cancer development and progression [2]. In the context of cancer development and progression, miRNAs targeting oncogenes function as tumour suppressors, whereas those targeting tumour suppressor genes serve as oncogenes [3]. Accumulating evidence has revealed the aberrant expression of miRNAs in human hepatocellular carcinoma (HCC) [4–6]. *miR-122a* has been reported to be downregulated in HCC, in turn, leading to upregulation of cyclin G1 [7]. On the other hand, recent reports have demonstrated that *miR-21* [8], *miR-221* [9], and *miR-224* [10] are upregulated in HCC, leading to downregulation of PTEN, CDK inhibitors, and API-5, respectively. Furthermore, the miRNA expression signature was reported to be related to the clinical outcome of patients with HCC [11,12]. Thus, miRNAs may play an important role in HCC development and progression by modulating a variety of gene expression and cellular processes.

Apoptosis resistance is an important characteristic of tumour cells, in addition to dysregulated proliferation and aberrant differentiation. Apoptosis is regulated by a fine balance of Bcl-2 family proteins, such as anti-apoptotic Bcl-xL and Mcl-1 and pro-apoptotic Bak and Bax. We previously demonstrated that Bcl-xL

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Abbreviations: miRNA, microRNA; 3′UTR, 3′-untranslated region; mRNA, messenger RNA; HCC, hepatocellular carcinoma; CDK, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle medium; RT, reverse transcription; PCR, polymerase chain reaction; 7-AAD, 7-amino-actinomycin D; DMSO, dimethyl sulfoxide.

is over-expressed in one-third of human HCC and confers resistance to hepatoma cells toward a variety of apoptotic insults generated by serum starvation and *p53* activation [13]. Patients with Bcl-xL-overexpressing HCC were shown to have significantly shorter disease-free survival after surgery [14]. Recently, it was proposed that autophagy defect is another mechanism of the malignant phenotype of Bcl-xL-overexpressing HCC through interaction between Bcl-xL and Beclin1 [15]. The underlying mechanisms of Bcl-xL over-expression in HCC are not clearly understood. Several reports show that transcription factors such as NF-κB [16] and STAT3 [17] could upregulate Bcl-xL expression in hepatoma cells. In addition, hepatitis C virus-related proteins, such as core [18] and NS5A [19], could upregulate Bcl-xL at a transcriptional level. However, we noticed that Bcl-xL-overexpressing hepatocarcinoma tissues do not always display upregulation of *bcl-xl* mRNA [13]. This observation led us to examine the possibility that post-transcriptional regulation by miRNAs may be involved in Bcl-xL expression in human HCC.

In the present study, we demonstrate that *let-7* family miRNAs, a prototype of human miRNAs [20], negatively regulate Bcl-xL expression in human HCC. *let-7* miRNAs are downregulated in human hepatoma cells and tissues in association with enhanced expression of Bcl-xL. Over-expression of *let-7* miRNAs in hepatoma cells downregulates Bcl-xL in a *bcl-xl* 3′UTR sequence-specific manner and enhances apoptosis induced by sorafenib, a recently approved anti-cancer drug for HCC [21]. The present study demonstrates for the first time that *let-7* miRNAs directly target Bcl-xL and induce apoptosis in cooperation with an anti-cancer drug targeting Mcl-1 in HCC.

Materials and methods

miRNA target predictions

The algorithms miRanda (http://www.microrna.org/), Pictar (http://pictar.mdc-berlin.de/), and TargetScan (http://www.targetscan.org/) were used to predict miRNAs that could potentially bind to *bcl-xl* mRNA.

Cell lines and tissues

Primary human hepatocytes were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured with the provided medium. Human hepatoma cell lines, Huh7 and HepG2, were cultured with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (Sigma, St. Louis, MO). HCCs and adjacent non-tumour counterparts were obtained at the time of surgical resection. Written informed consent was obtained from each patient. All tissues were stored at −80 °C until the time of use.

RNA extractions

Total RNA including the miRNA fraction was isolated from cell lines and tissue samples using the miRNeasy Mini Kit (QIAGEN, Valencia, CA). After extraction, the quality of each RNA sample was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

miRNA microarray analysis

RNA labelling and hybridisation were performed using a human miRNA microarray kit and a miRNA complete labelling and hybridisation kit (Agilent Technologies). After washing with Gene Expression Wash Buffer, the slides were scanned with an Agilent Microarray Scanner and analysed by GeneSpring GX software.

Western blot

Cells or tissues were lysed and Western blotted as previously described [22]. For immunodetection, the following antibodies were used: anti-Bcl-xL polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Mcl-1 polyclonal antibody (Santa Cruz Biotechnology), anti-Bak polyclonal antibody (Millipore, Billerica, MA), anti-Bax polyclonal antibody (Cell Signaling Technology, Danvers, MA). Optical densities of bands in each blot were analysed using ImageJ 1.40 g (NIH, Bethesda, MD).

Real time reverse transcription (RT)-PCR assays for mature miRNAs

To quantify the expression of mature miRNA, we synthesised cDNA from 10 ng of RNA sample using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed with TaqMan MicroRNA Assays (Applied Biosystems) specific for *let-7c* (P/N 4373167) and *let-7g* (P/N 4395393). To normalise the expression levels of miRNAs, we used TaqMan MicroRNA Assays specific for RNU6B (P/N 4373381) as the endogenous control.

Real time RT-PCR assays for bcl-xl mRNA

We reverse-transcribed RNA with High Capacity RNA-to-cDNA Master Mix (Applied Biosystems), and *bcl-xl* mRNA expression was measured using TaqMan Gene Expression Assays (Applied Biosystems, Assay ID: Hs99999146_m1). We also quantified *β-actin* mRNA as an endogenous control (Assay ID: Hs99999903_m1).

Transfections with miRNAs

Huh7 and HepG2 cells were transfected with 50 nM Pre-miR miRNA precursor molecules (Ambion, Austin, TX) of either *let-7c* or *let-7g* using RNAiMAX (Invitrogen, Carlsbad, CA) in six-well plates according to the manufacturer's instructions. Pre-miR negative control (Ambion) was also used as a control.

Luciferase assay

To generate the pMIR-Bcl-xL-3′UTR construct that contains the putative binding site of *bcl-xl* 3′UTR downstream of the firefly luciferase gene, we synthesised oligonucleotides to mimic the target sequence and inserted them into the SpeI–HindIII site of pMIR-REPORT Luciferase vector (Ambion). We also generated a pMIR-Bcl-xL-3′UTR mutant that has a point mutation in the putative binding site, using the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

Each of these constructs was transfected into Huh7 cells together with 50 nM Pre-miR miRNA precursor molecules and pMIR-REPORT β-Gal vector (Ambion), which contains the β-galactosidase gene for normalisation of transfection efficiency. Transfection was performed using Lipofectamine 2000 (Invitrogen). We measured firefly luciferase activity 24 h after transfection using the Luciferase Assay System (Promega, Madison, WI) and normalised it to the β-galactosidase expression level.

In vitro staurosporine or sorafenib treatment

Huh7 cells were transfected with 50 nM Pre-miR miRNA precursor molecules as described above, and 48 h after transfection, the medium was changed to DMEM containing staurosporine (Calbiochem, Gibbstown, NJ) or sorafenib. Sorafenib was kindly provided by Bayer HealthCare Pharmaceuticals Inc. (Wayne, NJ). Cells were additionally cultured and assayed for apoptosis by monitoring the activity of caspase-3/7 using a luminescent substrate assay for caspase-3 and caspase-7 (Caspase-Glo assay, Promega, Madison, WI), or by flow cytometry using the Annexin V-PE Apoptosis Detection Kit I (BD Biosciences, San Jose, CA). We defined apoptotic cells as Annexin V-PE-positive and 7-amino-actinomycin D (7-AAD)-negative cells. Cell viability was determined by the WST assay using cell count reagent SF (Nacalai Tesque, Kyoto, Japan).

Statistical analysis

Data are presented as mean ± SD. Comparisons between two groups were performed by the unpaired *t*-test. Multiple comparisons were performed by ANOVA with the Scheffe post hoc test. $p < 0.05$ was considered statistically significant.

Results

let-7 miRNAs were downregulated in hepatoma cells with upregulated expression of Bcl-xL

As observed in human HCC tissues, Bcl-xL was over-expressed, according to Western blot analysis, in Huh7 and HepG2 human hepatoma cell lines compared to normal hepatocytes (Fig. 1A). Previous research established that 30 and 32 kDa species are original and post-translationally modified Bcl-xL, respectively [23]. Mcl-1 was also over-expressed in human hepatoma cells, but the levels of expression of Bak and Bax did not differ between hepatoma cells and normal hepatocytes. We reasoned that miRNA regulating Bcl-xL expression would be downregulated in those hepatoma cell lines. To search for the candidate miRNA, microarray analysis was performed. More specifically, miRNA expression in Huh7 cells and normal hepatocytes was compared. When levels of expression less than 50% were considered significant, 26 miRNAs were identified as being downregulated in Huh7 cells: *let-7b*, *let-7g*, *let-7i*, *miR-127-3p*, *miR-214*, *miR-376a*, *miR-381*, *miR-409-3p*, *miR-376c*, *miR-493**, *miR-432*, *miR-487b*, *let-7d*, *let-7a*, *let-7f*, *let-7c*, *miR-200a*, *let-7e*, *miR-134*, *miR-503*, *miR-34a*, *miR-638*, *miR-150**, *miR-1225-5p*, *miR-21**, and *miR-223*. Among them, *in silico* analysis revealed that only the *let-7* family is capable of potentially targeting the 3′UTR of the *bcl-xl* mRNA. To confirm the results of the microarray analysis, quantitative real time RT-PCR was performed to evaluate the expression of *let-7c* and *let-7g* (Fig. 1B). After normalisation to endogenous RNU6B expression levels, the expression levels of both miRNAs were substantially lower in Huh7 cells than in normal hepatocytes. These results were consistent with the results of microarray analysis. Furthermore, the expression levels of both miRNAs were found to be downregulated in another human hepatoma cell line, HepG2, compared to normal hepatocytes.

let-7c and let-7g downregulate Bcl-xL expression by directly targeting the 3′UTR of bcl-xl mRNA

To examine whether *let-7* miRNAs are capable of suppressing translation of Bcl-xL, hepatoma cell lines were transfected with *let-7c* or *let-7g* or the negative control. Three days after transfection, Huh7 cells showed a decrease in Bcl-xL protein levels in both the *let-7c*-transfected group and the *let-7g*-transfected group in comparison with the negative control group (Fig. 2A). The transfection of *let-7c* and *let-7g* showed suppression of Bcl-xL protein levels in HepG2 cells as well (Fig. 2B). It did not affect expression of Bak and Bax, but increased Mcl-1 expression, which may be a secondary phenomenon of suppression of Bcl-xL. Normal hepatocytes were also transfected with *let-7c* or *let-7g* (Suppl. Fig. 1). The transfection led to a decrease in Bcl-xL expression in normal hepatocytes, but the decline was lesser than that observed in hepatoma cells. This finding may be explained by the observation that endogenous expression of *let-7c* and *let-7g* was extremely high in normal hepatocytes.

To examine whether the downregulation of Bcl-xL by *let-7c* or *let-7g* is caused by direct binding to a putative targeting site in the *bcl-xl* mRNA, we constructed the luciferase reporter plasmid pMIR-Bcl-xL-3′UTR containing the putative *let-7* binding site of *bcl-xl* 3′UTR downstream of the luciferase open reading frame (Fig 3A). The pMIR-Bcl-xL-3′UTR construct was cotransfected with the control pMIR-REPORT β-gal vector into Huh7 cells together with *let-7c* or *let-7g* or the negative control. When *let-7c* or *let-7g* Pre-miR was cotransfected with pMIR-Bcl-xL-3′UTR, the expression of firefly luciferase was significantly reduced compared to the negative control cotransfected group. There was no difference in firefly luciferase expression levels when pMIR-REPORT, which does not contain the putative *let-7* binding site, was cotransfected with *let-7c*, *let-7g* or the negative control (Fig. 3B). We also generated a pMIR-Bcl-xL-3′UTR mutant with a single base mutation in the seed region of the putative binding sequence to investigate whether the downregulation of firefly luciferase can be attributed to the insert (Fig. 3A). A single base mutation prevented the downregulation of firefly luciferase

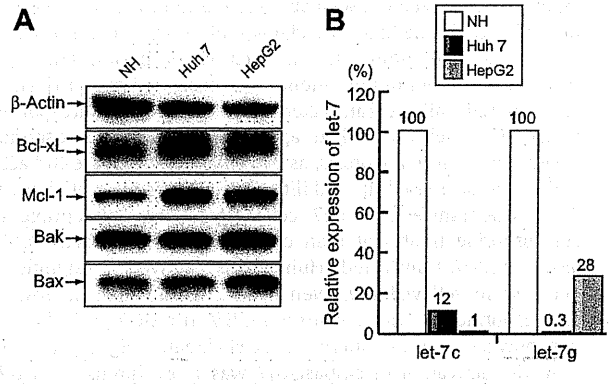

Fig. 1. Expression of Bcl-xL and *let-7* miRNAs in cultured human hepatocytes and hepatoma cells. Human hepatoma cell lines, Huh7 and HepG2, and normal hepatocytes (NH) were cultured and then lysed. (A) Western blot analysis for Bcl-2 family proteins. Bcl-xL migrates as a doublet band (see text). (B) Real time RT-PCR analysis for *let-7c* and *let-7g* expression. After normalisation to endogenous RNU6B expression, the expression of each miRNA in hepatoma cells was expressed in comparison to the levels observed in normal hepatocytes.

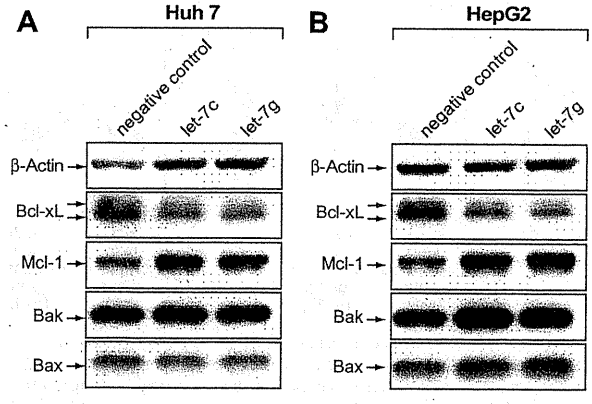

Fig. 2. Over-expression of *let-7* miRNAs downregulates Bcl-xL expression in hepatoma cells. Hepatoma cell lines Huh7 (A) and HepG2 (B) were transfected with *let-7c*, *let-7g*, or negative control miRNA at 50 nM and cultured for 3 days. Expression levels of Bcl-2 family proteins were determined by Western blot analysis.

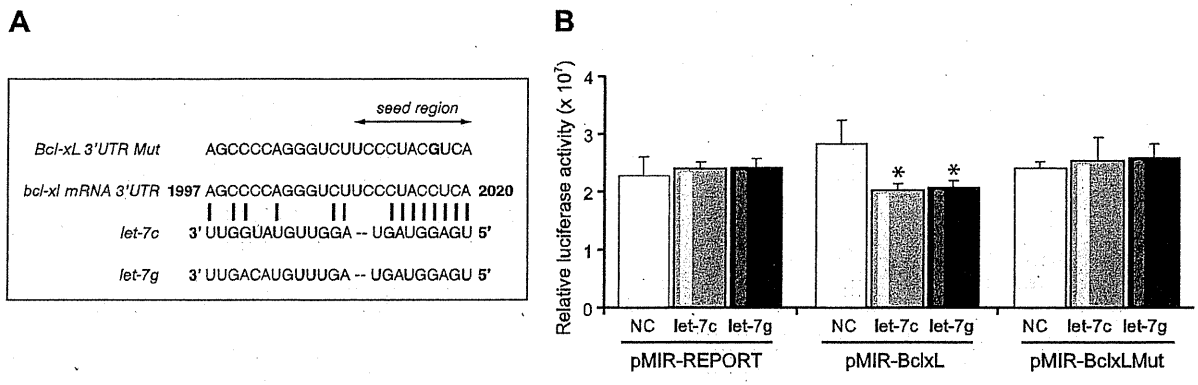

Fig. 3. Sequence-specific suppression of *bcl-xl* gene expression by *let-7c* or *let-7g* miRNAs. (A) The putative target site of *bcl-xl* mRNA 3′UTR determined by computational predictions. The target sequence was cloned into pMIR-REPORT vector (pMIR-Bcl-xL-3′UTR). pMIR-Bcl-xL-3′UTR mutant was also generated with a single mutation (indicated by a bold character) in the target site. (B) Each of these constructs was transfected into Huh7 cells together with *let-7c*, *let-7g* or negative control miRNA (NC). At 24 h after transfection, the activity of firefly luciferase was measured and normalised to β-galactosidase expression levels ($n = 3$). $^*p < 0.05$.

induced by *let-7c* or *let-7g*, which strongly suggests a direct inhibitory effect of *let-7* on Bcl-xL expression (Fig. 3B).

Downregulation of let-7c miRNA in human HCC tissues overexpressing Bcl-xL but not bcl-xl mRNA

To investigate the relationship between *let-7* expression levels and Bcl-xL protein levels in human HCC samples, we used 22 pairs of surgically resected human HCC tissue samples and adjacent non-tumour tissue samples with highly preserved RNA. Compared to the non-tumour counterparts, *bcl-xl* mRNA was found to be over-expressed in HCC tissue samples in only two cases; Bcl-xL was also over-expressed at the protein level in these cases. To assess the significance of *let-7* in post-transcriptional regulation of Bcl-xL *in vivo*, we selected 20 pairs of HCC tissue samples that did not over-express *bcl-xl* mRNA. When these samples were divided into two groups according to relative *let-7c* expression levels, the relative expression of Bcl-xL protein was significantly higher in the *let-7c* low expression group than in the *let-7c* high expression group (Fig. 4). By contrast, there was no significant difference in *bcl-xl* mRNA expression between the two groups. We also examined the relationship between relative *let-7g* expression and Bcl-xL expression. The *let-7g* low expression group tended to over-express Bcl-xL protein compared to the *let-7g* high expression group, although the difference did not reach statistical significance (data not shown). These results are consistent with the hypothesis that *let-7* miRNAs negatively regulate Bcl-xL expression independent of transcriptional regulation.

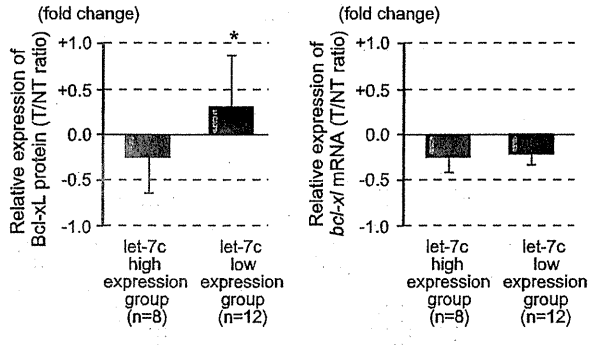

Fig. 4. Expression of Bcl-xL, *bcl-xl* mRNA and *let-7* miRNAs in human HCC tissue. Relationship between *let-7* and Bcl-xL expression in human HCC tissue samples. HCC tissue samples that did not show transcriptional upregulation of *bcl-xl* mRNA were divided into two groups according to relative *let-7c* expression levels with the threshold set at a 0.4-fold change in the tumour to non-tumour (T/NT) ratio. Relative expression of Bcl-xL protein and *bcl-xl* mRNA was calculated as the optical densities of the Bcl-xL blots normalised with the β-actin blots and those of real time RT-PCR assays, respectively, and are shown as the ratio of expression in the tumour to non-tumour expression in $\log_{10}$ scale. $^*p < 0.05$.

let-7c miRNA sensitises human Huh7 cells to sorafenib, which downregulates Mcl-1 expression

To investigate the effect of *let-7* in the resistance of hepatoma cells to apoptosis, we transfected Huh7 hepatoma cells with *let-7c* miRNAs and then subjected them to apoptosis analysis and a cell viability assay. There was no significant difference in caspase-3/7 activation or cell viability between *let-7c* miRNA-transfected Huh7 cells and control miRNA-transfected Huh7 cells (represented by the DMSO-treated group of Fig. 5A and B). These results are in agreement with our previous finding that anti-sense oligonucleotide-mediated knockdown of Bcl-xL sensitised hepatoma cells to apoptotic stimuli, such as serum starvation and *p53* activation, but did not induce apoptosis by itself [13]. Next, we exposed miRNA-transfected Huh7 cells to staurosporine, which is a well-established apoptosis inducer. Staurosporine treatment induced apoptosis, as determined by caspase-3/7 activation and decreased the viability of Huh7 cells by itself, but *let-7c* miRNA-transfected Huh7 cells were more susceptible to staurosporine treatment than control miRNA-transfected cells. *let-7c* miRNA-transfected Huh7 cells showed a significant decrease in cell viability, even upon exposure to low-dose of staurosporine at which control miRNA-transfected Huh7 did not show a significant difference in cell viability (Fig. 5B). In addition, the activation of caspase-3/7 was more intense in *let-7c* miRNA-transfected Huh7 cells than in control miRNA-transfected Huh7 cells (Fig. 5A). Thus, suppression of *let-7* expression leading to over-expression of Bcl-xL, may be a mechanism by which hepatoma cells resist apoptotic stimuli. While normal hepatocytes were more sensitive to staurosporine than hepatoma cells, transfection of *let-7* miRNA did not affect sensitivity to staurosporine

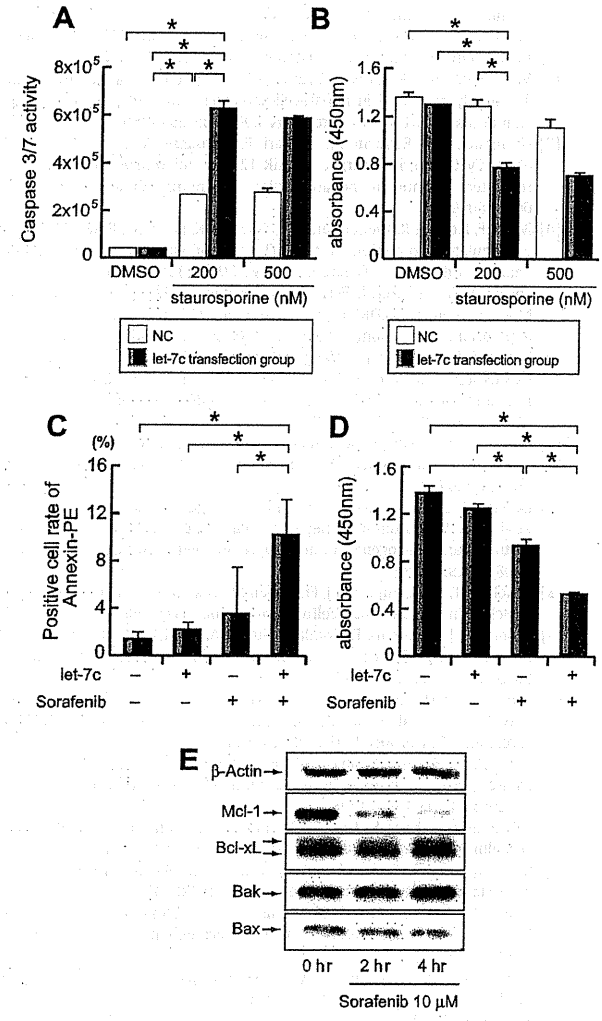

Fig. 5. Introduction of *let-7* miRNAs sensitises hepatoma cells to apoptotic stimuli. (A and B) Response to staurosporine treatment. Huh7 cells were transfected with *let-7c* (grey bars) or control miRNA (white bars) for 48 h and then further treated with staurosporine or DMSO alone for 12 h. The activities of caspase-3 and -7 were determined by luminescent substrate assays for caspase-3 and -7 ($n = 4$) (A). Cell viability was determined by the WST assay ($n = 4$) (B). *$p < 0.05$. (C and D) Response to sorafenib treatment. Huh7 cells were transfected with *let-7c* or control miRNA for 48 h and then further treated with sorafenib (5 μM) or DMSO alone for 48 h (C) or 72 h (D) 7-AAD negative cells were gated and the positive cell rate for annexin V-PE was determined ($n = 4$) (C). Cell viability was determined by the WST assay ($n = 4$) (D). *$p < 0.05$. E. Western blot analysis for Bcl-2 family proteins in lysates of Huh7 cells treated with sorafenib.

in normal hepatocytes (Suppl. Fig. 2), which is in agreement with the modest decline of Bcl-xL expression described earlier.

To examine the impact of *let-7* family miRNAs as a therapeutic tool, we investigated the effect of *let-7* miRNAs on apoptosis resistance to sorafenib, a recently approved anti-cancer drug for HCC. It has been reported that sorafenib was capable of downregulating Mcl-1 expression in tumour cells [24], and HCC has been reported to over-express Mcl-1, which is another anti-apoptotic Bcl-2 protein capable of conferring resistance to apoptosis [24–27]. In agreement with these findings, sorafenib treatment clearly downregulated Mcl-1 expression in hepatoma cells, but did not affect Bcl-xL expression (Fig. 5E). In contrast, sorafenib treatment did not affect Mcl-1 expression in normal hepatocytes (Suppl. Fig. 3). We hypothesised that *let-7* miRNA targeting Bcl-xL may induce apoptosis of hepatoma cells in cooperation with sorafenib. Apoptosis determined by Annexin V staining did not increase in *let-7c* miRNA-treated Huh7 cells compared to control miRNA-treated cells (represented by the DMSO-treated group in Fig. 5C). Sorafenib treatment of Huh7 cells led to a slight increase in the annexin V-positive cell rate, although the difference did not reach statistical significance levels under our experimental conditions (Fig. 5C). Of importance is the finding that sorafenib-induced apoptosis was markedly enhanced in *let-7c* miRNA-transfected cells. In addition, sorafenib treatment significantly reduced the viability of Huh7 cells and this decrease was markedly enhanced in cells transfected with *let-7c* miRNA (Fig. 5D). This finding implies that *let-7* miRNA transfection potentiates sorafenib-induced apoptosis and toxicity in hepatoma cells.

Discussion

Anti-apoptotic members of the Bcl-2 family, which consists of five members, Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and Bfl-1, are critically involved in the mitochondrial pathway of apoptosis [28]. Cancer cells frequently over-express one or more members of this family to acquire a survival advantage [29]. These proteins are over-expressed in a variety of ways, including genetic translocation, particularly in the case of Bcl-2, and transcriptional regulation. Unlike the case of the *bcl-2* gene, mutations or amplification of the *bcl-x* gene have not been demonstrated in tumour cells. With regard to miRNA regulation, previous research clearly demonstrated that Bcl-2 is a direct target of *miR-15* and *miR-16*. The expression levels of *miR-15* and *miR-16* inversely correlate with Bcl-2 expression in chronic lymphocytic leukaemia [30]. More recently, Mcl-1 was reported to be suppressed by *miR-29* [31]. Our present study is the first demonstration of miRNA-mediated regulation of Bcl-xL expression. Since Bcl-xL is over-expressed not only in HCC but also in other tumours, the present findings may shed light on the mechanisms of Bcl-xL over-expression in other malignancies.

While more than 500 human miRNAs have been identified, *let-7* is a prototype of human miRNA and was first identified in 2001 [32]. *let-7* miRNAs are downregulated in several malignancies. A highly characterised example is non-small cell lung cancer in which downregulation of *let-7* miRNAs is well correlated with poor prognosis in patients [33]. In HCC, some reports showed downregulation of *let-7*, while others did not [7]. In the present study, *let-7c* miRNA was under-expressed at less than 40% of the normal level in approximately half of the HCC tissues. Further study is needed to determine the clinical significance of *let-7* miRNA in HCC. Several target genes have been identified for *let-7* miRNA, including Ras [34], Myc [35], HMGA2 [36], CDC25A, and CDK6 [37]. The major function of this miRNA is to promote cell proliferation. Since these proteins could act as oncogenes in tumour cells, *let-7* miRNA is believed to serve as a tumour suppressor [38]. In the present study, we have demonstrated that *bcl-xl* is a direct target for *let-7* miRNA, implying that this well-known tumour suppressor miRNA directly regulates apoptosis, another important process in malignancy.

Sorafenib is a recent FDA-approved anti-cancer drug for HCC [21]. It functions as a multi-kinase inhibitor and can induce

apoptosis at least in part by downregulating Mcl-1 in tumour cells [24]. Like Bcl-xL, several reports have identified Mcl-1 as being over-expressed in some HCCs [25–27]. Since Bcl-xL and Mcl-1 share a similar structure and functions, we reasoned that downregulation of both proteins would efficiently kill hepatoma cells. To verify this hypothesis, we treated hepatoma cells with sorafenib and *let-7* miRNA. As expected, sorafenib treatment downregulated Mcl-1 expression as early as 2 h post-treatment; however, it did not efficiently induce apoptosis. Transfection of *let-7* miRNA itself was not capable of inducing apoptosis of hepatoma cells despite a clear reduction in Bcl-xL expression. Importantly, *let-7* miRNA substantially increased sensitivity to sorafenib. Since both *let-7* miRNA and sorafenib may have pleiotropic effects on gene expression and cellular processes, downregulation of Bcl-xL and Mcl-1 may not be a single mechanism for killing hepatoma cells. However, our study revealed that Bcl-xL-targeting miRNA, *let-7*, controls resistance of hepatoma cells to this novel class of anti-HCC drug.

In conclusion, we have demonstrated that *let-7* miRNA negatively regulates Bcl-xL expression in HCCs. Reconstitution of *let-7* miRNA may reduce apoptosis resistance to anti-cancer drugs targeting Mcl-1 in HCC. Further study is needed to examine the significance of *let-7* miRNA expression for predicting responses to sorafenib therapy in patients with HCC.

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Disclosures

All authors have nothing to disclose.

Conflicts of interest

All authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2009.12.024.

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Meeting Summary

Will There Be an HCV Meeting in 2020? Summary of the 17th International Meeting on Hepatitis C Virus and Related Viruses

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Hepatitis C virus (HCV), which was discovered in 1989, is a major etiologic agent in human liver disease. Approximately 130 million people, or 2% of the population, worldwide are infected. The 17th International Meeting on Hepatitis C Virus and Related Viruses was held September 10–14, 2010, in Yokohama, Japan. The meeting was attended by almost 700 scientists from all over the world who are interested in the fundamental aspects of the molecular virology, immunology, pathogenesis, prevention, and treatment of HCV infection. Two special opening lectures given by Masaaki Komatsu and Takashi Gojobori focused attention on the related research fields of autophagy and genome biology, respectively. In the subsequent sessions, the latest research, original studies, and controversies were presented in 9 keynote lectures, 82 oral presentations, and 329 poster presentations.

Viral Entry

The opening scientific session of this meeting focused on the viral host cell entry processes. Thomas Baumert presented the keynote lecture, which included an overview of the HCV cell entry process and recent advances at his laboratory. These included the finding that HCV variants that reinfect the liver after transplantation demonstrate more efficient cell entry and are less susceptible to neutralization by host antibodies. He also described the isolation of monoclonal antibodies against claudin-1 that do not inhibit either extracellular or direct cell-to-cell HCV transfer.

Alexander Ploss described the establishment of a mouse model for studying HCV cell entry. They utilized an HCV cell culture virus (HCVcc) expressing recombinase and transgenic mice bearing a recombinase-activatable fluorescent protein. Bioluminescent imaging indicated that only mice transduced with CD81 and occludin supported HCVcc entry. The presence of an intact immune system in these animals makes it particularly important for the testing of HCV vaccine candidates. Danyelle N. Martin described a role for transferrin receptor 1 (TfR1) in mediating HCV cell entry. The inhibition of HCV entry with TfR1 antibodies and silencing, suggest this factor should be added to the growing list of cellular proteins required for HCV cell entry. Joachim Lupberger presented results from a study showing an essential role for the epidermal growth factor receptor (EGFR) in HCV cell entry. He found that EGFR is required for both mediating the interactions between two other entry factors, CD81 and CLDN1, and catalyzing the fusion activity of viral glycoproteins.

Translation/Replication

Volker Lohmann began the session by describing what is known of the functions of viral nonstructural proteins and their associated host cellular factors in viral translation and replication. He included an overview of viral isolates and model systems currently used, and presented data addressing the mechanisms for efficient replication of the JFH-1 isolate.

Several reports have focused on the molecular basis of the architecture and composition of membrane-associated sites for HCV replication, which often induce membrane alterations, such as the so-called membranous web. Brenno Wolk demonstrated that NS4B is sufficient to direct all nonstructural proteins into the viral replication complex compartment, and that intragenotype-specific interactions are required for NS4B-dependent recruitment of NS5A. Ines Romero-Brey showed that the membranous web predominantly contains double-membrane vesicles with various diameters. These vesicle structures were connected to the endoplasmic reticulum (ER) through funnel-like structures.

Several DDX DEAD-box RNA helicases were identified as host factors associated with HCV replication. Yasuo Ariumi presented the cross-talk of HCV with DDX proteins and the role of distinct DDX proteins in viral replication. Tetsuro Shimakami and Selena M. Sagan reported the importance of miR-122 to not only enhance IRES-mediated translation, but stabilize positive-strand HCV RNA by binding to its 5′ extremity. Enzymatic activity of host phosphatidyl-inositol-4 kinase III alpha was shown to be critically involved in HCV replication and the activity is regulated by HCV NS5A (Simon Reiss). Nam-Joon Cho reconstituted a functionally active full-

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length HCV polymerase on a biomimetic membrane platform. Deborah Harrus found that guanosine triphosphate specifically stimulates the initial step of de novo initiation by stimulating transition of newly formed linker primer.

Assembly and Release

In the keynote lecture, Guangxiang G. Luo presented an overview of particle assembly and release, and the impact of apolipoprotein (Apo) E in the entry and assembly of HCV. He demonstrated the inhibition of HCVcc entry by treatment with anti-ApoE antibody and the direct interaction of ApoE with NS5A.

Ann L. Wozniak showed an important role for p7 in the production of infectious particles. Their data suggest that p7 stimulates virus production through the alkalization of intracellular vesicles. Ophelia Granio showed that both p7 and NS2 are required for the recruitment of core from lipid droplets (LDs) to ER. Costin-Ioan I. Popescu showed that NS2 accumulated in dotted structures in the ER in juxtaposition with Core and LDs. They concluded that cross-talk among Core, E1, E2, p7, and NS2 was essential for virion assembly. Vlastimil Jirasko demonstrated point mutations in the transmembrane regions of NS2 impaired the particle production and suggested that NS2 serves as a platform of viral and cellular proteins that coordinates HCV assembly. Qisheng Li identified the proviral function of IKKα by genome wide siRNA screening. IKKα regulates lipid metabolism and biogenesis of LDs and may enhance production of virus particles. The very low-density lipoproteins are secreted via a Golgi-dependent pathway. Bryan R. Bishe demonstrated the important role of phosphatidylinositol-4-phosphate and its interacting protein GOLPH3 in HCV secretion in the trans-Golgi network. Roland Remenyi showed 3-dimensional visualization of the HCV life cycle in cultured cells by electron tomography. They detected virus-like particles at various cytoplasmic locations. Viral particles in the proximity of LDs and within sponge-like inclusion were observed. These results provide ultrastructural visualization of putative assembly sites close to LDs.

Host Factors

In the invited lecture, Sara Cherry presented an overview of high-throughput screening toward the identification of host factors required for viral infection.

The contribution of autophagy to the HCV life cycle was also presented in this section, most notably, host factors linked with lipids. Tsubasa Munakata showed that the fatty acid synthase is required for efficient HCV replication. They also suggested the importance of palmitate for HCV replication. Samantha L. Blackham presented both the thioredoxin-interacting protein and the peroxisome proliferator activated receptor-α have significant effects on HCV replication. The host factors functioning on infectious HCV particle production were also reported. Takayuki Hishiki demonstrated the isoform dependent binding affinities of ApoE for low-density lipoprotein receptors and they affect infectivity of HCV. Laurent Chatel-Chaix found that Y-box binding protein interacted with HCV NS3 protein and viral RNA and was relocalized from nucleocytoplasmics site to the core-containing surface of LDs. Mohsan Saeed reported that the ER-associated degradation pathway was activated by HCV infection in a viral envelope protein-dependent manner. Po-Yuan Ke showed that HCV infection induces the unfolded protein response and activates the autophagic pathway. They proposed that autophagy contributes to the suppression of HCV in an autolysosome formation-dependent manner. Hiroto Kambara did not find any effects on HCV replication by inhibition of autophagosome formation in replicon cells. They proposed a role for autophagy induced by HCV infection to avoid the generation of vacuolation harmful to cell survival. Qisheng Li reported the network map of cellular pathways and machineries that are associated with HCV life cycle.

Very low-density lipoprotein is now considered to be one of a component of HCV particles. LDs are composed of fatty acid, triglyceride, and cholesterol, surrounded by several types of lipoproteins. In addition, Daniel J. Felmlee reported that chylomicron-associated viruses may be generated by virion association while in the vascular compartment. Francois Jean showed that the serine protease inhibitor protein Spn4A was modified to be directed to Site-1 protease specifically and was introduced into adenovirus vector to inhibit cholesterol and fatty acid syntheses for down-regulation of HCV propagation. The modified serpin could suppress Site-1 protease activity, reduce the LD, and block HCVcc infection. Nicolas Menzel tried to identify novel cellular factors involved in HCV assembly and release and found ERK inhibitor and cytosolic phospholipase A2 (cPLA2) inhibitor reduce viral production. cPLA2 inhibitor also reduced the amount of LD-associated core and supernatant ApoB/E. cPLA2 may be crucial for assembly of infectious HCV particles, possibly through participating in the formation of lipoproteins. Kohji Moriishi reported that the proteasome activator PA28γ participates in HCV propagation. PA28γ may participate in the propagation of HCV by regulating the degradation of Core in both ubiquitin-dependent and -independent manners. NS5A is regulated by phosphorylation of several host protein kinases. Takahiro Masaki identified 79 serine threonine protein kinases that were tightly associated with NS5A. Two of these may regulate the production of viral particles and/or viral replication.

Innate Immunity

The early phase of host defense against viral infection has largely been delineated based on recent advances in innate immunity. In the invited lecture, Manoj N. Krishnan introduced his comprehensive study on the Toll-like receptor 3-TRIF (TICAM-1) pathway. Using RNAi and polyI:C, he screened the genes specifically upregulated via the TRIF (TICAM-1) pathway. He expected that some viral infections are selectively blocked by the IPS-1 pathway, while others are blocked by the TRIF pathway.

Michael Gale, Jr., identified IFITM1 inhibits HCV infection. IFITM1 assembles with CD81 and tranlocates to the tight junction. This translocation of CD81 hampers the receptor function of CD81. They also discovered a novel pathway for ISGF3 activation. A non-receptor type tyrosine kinase-1 triggers activation of ISGF3 independent from the classical IFNAR pathway. IP-10 is a chemokine and is a negative predictor for pegylated interferon (IFN)/ribavirin therapy. Matthew L. Albert indicated that there is a 2-amino-acid-deleted form of IP-10 that serves as an antagonist for intact IP-10, and this form abrogates an early virologic response. As this IP-10 truncation is mediated by dipeptidylpeptidase IV, they believed that dipeptidylpeptidase IV is a novel therapeutic target for HCV patients during IFN therapy. Joo Chun Yoon suggested that activation of natural killer cells is inhibited by HCV-infected hepatocytes. They claimed that the early phases of HCV infection may be established through the failure of virus-inducible natural killer cell activation. Shin-ichiro Nakagawa reported that polyI:C induces both type I IFN and IFN-λ in human hepatocytes. The antiviral effect appears to parallel the induction of IFN-λ. This, together with the report by Emmanuel Thomas, suggests that the IFN-λ system is activated in HCV infected hepatocytes.

Adaptive Immunity

In a keynote lecture, Robert Thimme summarized the mechanisms of HCV-induced T-cell dysfunction. Multifaceted factors contribute to the hyporesponsiveness of T cells, including viral mutations, primary T-cell failure, lack of support from dendritic cells, expression of inhibitory molecules on T cells, and abundance of regulatory T cells (Tregs). Whether the ability of HCV-specific CTLs is comparable with that of CTLs having other specificities remains controversial. Bianca Seigel showed that HCV-specific CTLs are functionally impaired when compared with other CTLs, irrespective of their expression of inhibitory receptors or differentiation stages. CD161 is a C-type lectin that is expressed in HCV-specific $CD8^+$ T cells with tissue homing phenotype. Vicki M. Fleming found that $CD4^+CD161^+$ T cells produce large amounts of inflammatory cytokines and accumulate in the liver, where they are thought to exert pro-inflammatory roles. Naruyasu Kakita reported that certain adaptive Tregs, known as interleukin (IL)-10-producing type 1 Tregs, are increased in HCV-positive hepatocellular carcinoma patients, and their significance in hepatocellular carcinoma was greater than that of natural Tregs. Even in patients who have attained a sustained virologic response, trace amounts of HCV RNA are sporadically detectable in plasma. Barbara Rehermann reported the inoculation studies of such plasma. Residual HCV RNA in patients was able to infect chimpanzees and induced broad, HCV-specific T-cell responses. HCV RNA levels continued to be high when T-cell responses declined, suggesting that such HCV remains transmissible as hepatotropic pathogens.

Pathogenesis

In the invited lecture, Michael Diamond presented new mechanisms for West Nile virus immune evasion via 2'O methylation of viral RNA to subvert host innate immunity.

Genome-wide analysis of quantitative data (transcriptomics, proteomics, and metabolomics) facilitates systems biology analysis of HCV infection. Deborah L. Diamond analyzed the pathways involved in the progression of chronic hepatitis, namely, fibrosis and carcinogenesis, and found that molecules relating to cell metabolism including fatty acid oxidation enzymes and antioxidant systems may be master regulators of liver disease progression in HCV infection. HCV core protein has been shown to play a key role in the development of steatosis in HCV infected liver, especially in patients with genotype 3a HCV infection. Sophie Clement-Leboube showed that PTEN expression was down-regulated in the HCV infected liver. Analysis of lipo-viral-particle from hepatitis C patients by Olivier Diaz revealed that empty lipo-viral-particle lacking HCV RNA outnumbers those with RNA. The presence of virus-modified lipoproteins in HCV-infected patients may play a role in the pathogenesis of hepatitis C. Massimiliano Pagani used serum miRNA signatures to monitor liver disease in HCV infection and found miRNome candidates that are specific for HCV disease progression. Shuhei Taguwa showed that Con1 replicon induces incomplete autophagy through the dysfunction of autolysosomal acidification, which results in the secretion of immature cathepsin B in cells. Because the secretion of the protein is enhanced in many types of tumors, this observation may be associated with the pathogenesis of liver tumorigenesis in HCV infection.

The existence of extrahepatic manifestations is another issue of interest. Essential mixed cryoglobulinemia, membranoproliferative glomerulonephritis, and Sjögren syndrome are conditions that have been shown to correlate