Table 1 Demographic and baseline characteristics of patients ($n = 223$)

Variable, n (%)	
Age (years)[a]	64.3 ± 10.6
Male sex	176 (78.9)
ECOG performance status	
0	159 (71.3)
1	57 (25.6)
2	7 (3.1)
Viral infection	
HBsAg, positive	58 (26.0)
Anti HCVAb, positive	125 (56.1)
Both positive	4 (1.8)
Both negative	36 (16.1)
Child–Pugh classification	
Class A	166 (74.4)
Class B	57 (25.6)
Platelet count ($10^3/\mu l$)[b]	127 (34–840)
BCLC stage	
B	22 (9.9)
C	201 (90.1)
Viable intrahepatic lesion, present	213 (95.5)
Macroscopic vascular invasion, present[c]	103 (46.2)
Portal vein	73
Hepatic vein or vena cava	51
Maximum tumor size (cm)[b]	5.2 (1.0–20.0)
AFP >100 ng/mL	143 (64.1)
AFP-L3 >15.0 %[d]	147 (66.2)
DCP >100 mAU/mL[e]	152 (68.8)
Extrahepatic metastasis, present[c]	166 (74.4)
Lung	91
Lymph node	52
Bone	33
Adrenal gland	11
Dissemination	20
Others	5
Previous therapy[c]	
None	13 (5.8)
Surgical resection	78 (35.0)
Percutaneous ablation	95 (42.6)
Transarterial chemoembolization	150 (67.3)
Radiotherapy	32 (14.3)
Transarterial chemotherapy	65 (29.1)
Systemic chemotherapy	46 (20.6)
Cycles of systemic 5-FU + IFN therapy[b]	2 (1–13)

ECOG Eastern Cooperative Oncology Group, *HBsAg* hepatitis B surface antigen, *HCVAb* hepatitis C virus antibody , *BCLC* Barcelona-Clinic Liver Cancer, *AFP* alpha fetoprotein, *DCP* des-gamma-carboxy prothrombin, *5-FU* 5-fluorouracil, *IFN* interferon

[a] Mean ± SD

[b] Median (range)

[c] Including overlap

[d] Missing in one case

[e] Missing in two cases

Table 2 Summary of efficacy measures ($n = 223$)

Level of response, n (%)	
Complete response	6 (2.7)
Partial response	15 (6.7)
Stable disease	52 (23.3)
Progressive disease	132 (59.2)
Not assessable	18 (8.1)
Response rate (%)	9.4
Disease-control rate (%)	32.7
Time to progression (months)	
Median	2.0
95 % confidence interval (CI)	2.0–3.1
Overall survival (months)	
Median	6.5
95 % CI	5.13–9.13
1-year survival rate (%)	31.2
2-year survival rate (%)	12.7
3-year survival rate (%)	7.1

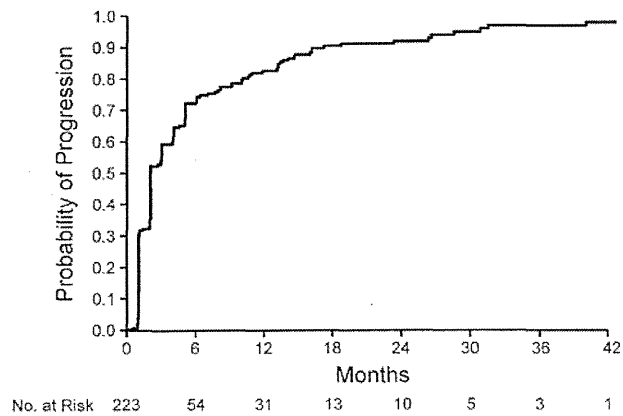

Fig. 1 Kaplan–Meier analysis of time to progression

132 (59.2 %) had PD. Treatment response was not assessable in the remaining 18 (8.1 %) patients due to symptomatic PD or their being lost to follow up before evaluation. The response rate was 9.4 % and the disease-control rate was 32.7 % (Table 2). The median TTP was 2.0 months (Fig. 1). There was no statistically significant difference in TTP between Child–Pugh class A and class B patients (median 3.0 vs. 2.0 months, $P = 0.19$).

Survival

The overall MST was 6.5 months (Fig. 2a). The survival rates at 1, 2, and 3 years were 31.2, 12.7, and 7.1 %, respectively (Table 2). MST was significantly longer in Child–Pugh class A as compared with class B patients (9.2 vs. 2.8 months, $P < 0.001$) (Fig. 2b). The MSTs of patients

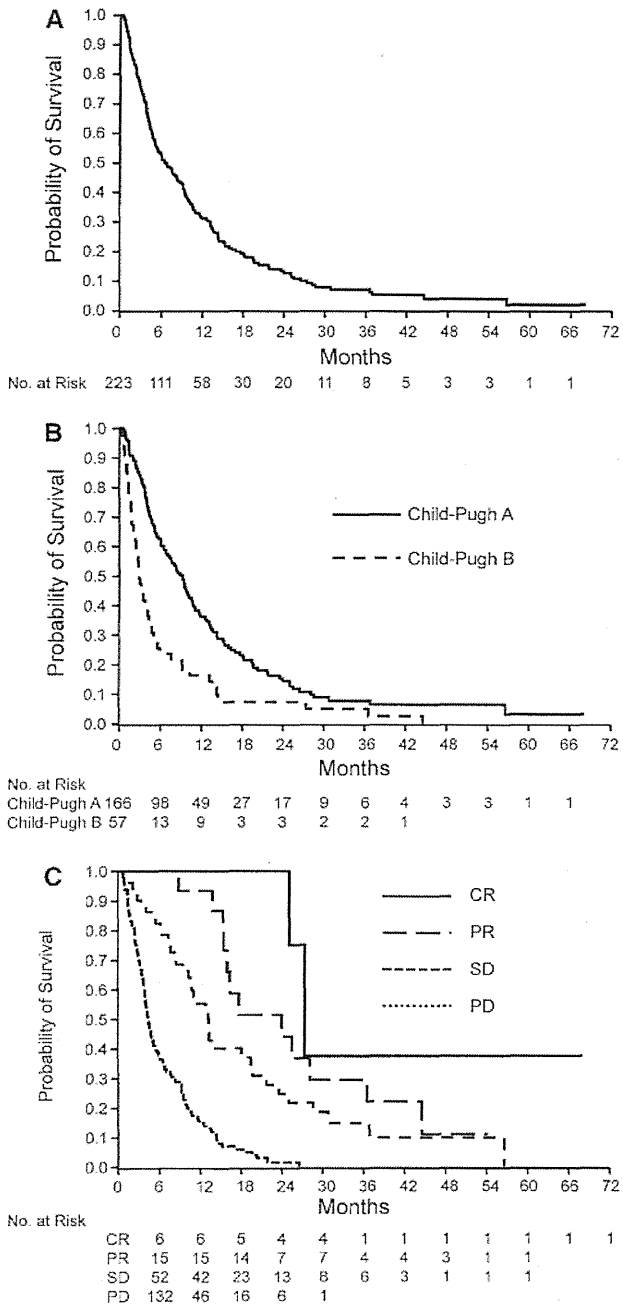

Fig. 2 Kaplan–Meier analysis of overall survival (**a**); stratified based on Child–Pugh classification (**b**) and response to treatment (**c**). *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease

with CR, PR, SD, and PD were 27.4, 24.0, 13.2, and 4.4 months, respectively (Fig. 2c, $P < 0.001$). Based on a univariate analysis, the following factors were significantly associated with shorter survival time: ECOG performance status >0, Child–Pugh class B, and presence of macroscopic vascular invasion (Table 3). A multivariate analysis showed that all of these factors were also independent prognostic factors (Table 4).

Table 3 Predictors of overall survival: univariate analysis ($n = 223$)

Variable	Hazard ratio (95 % CI)	*P*
Age (years) >65	1.01 (0.76–1.35)	0.94
Male sex	1.03 (0.73–1.45)	0.87
ECOG performance status >0	1.73 (1.25–2.39)	<0.001
HBsAg, positive	0.87 (0.63–1.20)	0.38
Anti HCVAb, positive	1.06 (0.80–1.42)	0.68
Child–Pugh class B versus A	2.12 (1.54–2.92)	<0.001
Platelet count >127,000/μL	1.25 (0.94–1.67)	0.13
BCLC stage C	1.46 (0.89–2.41)	0.14
Viable intrahepatic lesion, present	1.85 (0.76–4.49)	0.17
Macroscopic vascular invasion, present	1.37 (1.03–1.83)	0.03
Extrahepatic metastasis, present	1.35 (0.97–1.87)	0.08
Previous chemotherapy, present	1.16 (0.86–1.55)	0.34

Table 4 Predictors of overall survival: multivariate analysis ($n = 223$)

Variable	Hazard ratio (95 % CI)	*P*
ECOG performance status >0	1.46 (1.04–2.05)	0.03
Child–Pugh class B	1.83 (1.31–2.55)	<0.001
Macroscopic vascular invasion, present	1.39 (1.03–1.88)	0.03
Extrahepatic metastasis, present	1.35 (0.96–1.92)	0.09

Table 5 Safety profile

	Grade 1–2, *n* (%)	Grade 3–4, *n* (%)
Leukopenia	25 (11.2)	31 (13.9)
Anemia	0 (0)	1 (0.4)
Thrombocytopenia	20 (9.0)	13 (5.8)
Stomatitis	11 (4.9)	3 (1.3)
Anorexia	2 (0.9)	1 (0.4)
Diarrhea	2 (0.9)	0 (0)
Skin rash	2 (0.9)	1 (0.4)

Safety

Adverse events graded as 3 or 4 were observed in 28 (12.6 %) patients. The incidence of major adverse events is presented in Table 5. The major grade 3–4 adverse events were leucopenia (13.9 %) and thrombocytopenia (5.8 %). A common non-hematological toxicity was stomatitis (6.2 %, any grade). Fever, which was mostly low-grade, occurred in about 90 % of the patients, usually after the first administration of peginterferon, and was gradually

attenuated during subsequent administrations. Elevations in bilirubin, AST, and ALT levels from baseline occurred in 7.6 % of patients, although most cases of such elevation occurred due to progression of the intrahepatic lesion, and not due to the treatment itself. There were no catheter-related problems, including infection or occlusion. No treatment-related deaths occurred.

Discussion

Wadler et al. first reported combination therapy with intravenous 5-FU and subcutaneous interferon for a malignant neoplasm. They treated 30 patients with advanced colorectal cancer using this protocol [19]. However, the following phase III trial failed to establish the efficacy of the treatment [20]. Subsequently, Patt et al. [21] reported systemic combination therapy for HCC patients, reporting that the treatment induced a decrease of more than 50 % in the size of each measurable lesion in 18 % of the treated patients. Since then, several studies have demonstrated the efficacy of combination therapy of intraarterial 5-FU and subcutaneous interferon for patients with advanced HCC with portal venous invasion, reporting response rates of 44–63 % [13, 22, 23]. Furthermore, other studies have revealed the mechanism underlying the antitumor effects of this combination therapy [24–31]. However, only a case series of a small number of patients has reported on this systemic combination therapy in HCC patients [32]. The present study is the first report of this therapy in a large number of patients ($n = 223$).

In the past, systemic chemotherapy for advanced HCC using various cytotoxic agents, such as doxorubicin, 5-FU, cisplatin, and etoposide, has been investigated. However, few agents showed response rates above 20 %, and the number of patients included in those studies was small. Furthermore, no regimens demonstrated convincing survival benefits in phase III trials [33, 34]. Single-agent 5-FU [35–37] and related drugs such as eniluracil/5-FU [38, 39] and uracil/tegafur [40, 41] showed low response rates. An impressive result came from phase II and phase III studies of PIAF (combination of cisplatin, interferon alfa, doxorubicin, and 5-FU). The response rates of these studies were 26 and 20.9 %, respectively [42, 43], which were actually better than that of the present study, although the number of patients was small and the characteristics of the patients differed from those in our study.

At present, sorafenib is the standard treatment for advanced HCC with extrahepatic metastasis or vascular invasion. Before the availability of sorafenib, we treated such patients with a combination of systemic intravenous 5-FU and subcutaneous interferon. The MSTs in the SHARP study and the Asian-Pacific study of sorafenib (both randomized controlled trials) were 10.7 and 6.5 months, respectively, whereas the MST in the present study was 6.5 months. However, both these trials of sorafenib consisted only of Child–Pugh class A patients, and the MSTs in these two studies were comparable to the MST of the Child–Pugh class A patients in our study (9.2 months). The disease-control rate in our study was 32.7 %, which was comparable to that of sorafenib (43 % in the SHARP study; 35.3 % in the Asian-Pacific study). Moreover, there were no complete responders in either of these randomized controlled trials, and the response rates were also low (2 % in the SHARP study; 3.3 % in the Asian-Pacific study). On the other hand, in the present study, six (2.7 %) patients achieved a complete response, and the response rate of 9.4 % was higher than that in these two studies. Thus, the combination of intravenous 5-FU and subcutaneous interferon is worth consideration as a choice of treatment for advanced HCC.

The response rate of 52.6 % that we observed in our previous study where we treated HCC patients with portal venous invasion with a combination of intraarterial 5-FU and subcutaneous interferon [13] was much better than that observed here. This may be partly because the local concentration of 5-FU in the liver is higher after intraarterial infusion than after systemic administration. However, systemic rather than intraarterial administration is appropriate for patients with extrahepatic metastases because intraarterially administered 5-FU is substantially removed by the liver in the first pass [44, 45].

In our previous study [13], we combined interferon alfa, not pegylated, with the intraarterial administration of 5-FU. Here, we combined pegylated interferon alfa with the systemic administration of 5-FU mainly because of the convenience in an outpatient setting. Whereas non-pegylated interferon needs to be administered three times a week, pegylated interferon requires only once-a-week administration.

Cirrhotic patients have lower clearance rates of 5-FU than non-cirrhotic patients [46]. Thus, such patients with poor liver function may have more severe adverse events. However, there were few serious adverse events in the present study, although as many as 25.6 % of the patients were Child–Pugh class B. Although grade 3 or 4 leucopenia and thrombocytopenia were observed, the baseline white blood cell and platelet counts in the patients with these events were almost always low because of background cirrhosis, and they were able to continue to receive treatment. In addition, we did not observe any serious adverse events in relation to infection.

According to our data, ECOG performance status, Child–Pugh classification, and the presence of vascular invasion were independent prognostic factors. This is consistent with our previous study findings on the prognosis of patients with

extrahepatic metastasis of HCC [47]. In the present study, we also analyzed prognosis as stratified by treatment response, and better treatment response resulted in better prognosis. This point is to be confirmed in future prospective studies.

The combination therapy described in the present study was performed before the advent of sorafenib. It will now be important to evaluate the efficacy of this combination therapy in cases of sorafenib failure. It is also necessary to assess the efficacy and safety of this treatment, as well as that of sorafenib, for patients with poor liver function [48, 49].

In conclusion, the combination of continuous intravenous infusion of 5-FU and subcutaneous peginterferon alfa-2a was well tolerated and showed promising efficacy in a subset of patients with advanced HCC. Further studies; for example validating the efficacy of this treatment in patients with sorafenib failure and conducting a randomized controlled trial comparing this treatment with sorafenib, are needed to definitively establish the usefulness of this treatment.

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References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin. 2005;55(2):74–108.
2. Matsuda T, Marugame T, Kamo K, Katanoda K, Ajiki W, Sobue T. Cancer incidence and incidence rates in Japan in 2003: based on data from 13 population-based cancer registries in the Monitoring of Cancer Incidence in Japan (MCIJ) Project. Jpn J Clin Oncol. 2009;39(12):850–8.
3. Shiratori Y, Shiina S, Imamura M, Kato N, Kanai F, Okudaira T, et al. Characteristic difference of hepatocellular carcinoma between hepatitis B- and C-viral infection in Japan. Hepatology. 1995;22(4 Pt 1):1027–33.
4. Okuda K, Ohtsuki T, Obata H, Tomimatsu M, Okazaki N, Hasegawa H, et al. Natural history of hepatocellular carcinoma and prognosis in relation to treatment. Study of 850 patients. Cancer. 1985;56(4):918–28.
5. Arii S, Yamaoka Y, Futagawa S, Inoue K, Kobayashi K, Kojiro M, et al. Results of surgical and nonsurgical treatment for small-sized hepatocellular carcinomas: a retrospective and nationwide survey in Japan. The Liver Cancer Study Group of Japan. Hepatology. 2000;32(6):1224–9.
6. Shiina S, Tagawa K, Niwa Y, Unuma T, Komatsu Y, Yoshiura K, et al. Percutaneous ethanol injection therapy for hepatocellular carcinoma: results in 146 patients. AJR Am J Roentgenol. 1993; 160(5):1023–8.
7. Llovet JM, Real MI, Montana X, Planas R, Coll S, Aponte J, et al. Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. Lancet. 2002;359(9319):1734–9.
8. Shiina S, Teratani T, Obi S, Sato S, Tateishi R, Fujishima T, et al. A randomized controlled trial of radiofrequency ablation with ethanol injection for small hepatocellular carcinoma. Gastroenterology. 2005;129(1):122–30.
9. Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, et al. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. N Engl J Med. 1996;334(11):693–9.
10. Shiina S, Tateishi R, Arano T, Uchino K, Enooku K, Nakagawa H, et al. Radiofrequency ablation for hepatocellular carcinoma: 10-year outcome and prognostic factors. Am J Gastroenterol 2011 [Epub ahead of print].
11. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med. 2008;359(4):378–90.
12. Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, et al. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol. 2009;10(1):25–34.
13. Obi S, Yoshida H, Toune R, Unuma T, Kanda M, Sato S, et al. Combination therapy of intraarterial 5-fluorouracil and systemic interferon-alpha for advanced hepatocellular carcinoma with portal venous invasion. Cancer. 2006;106(9):1990–7.
14. Oken MM, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. Am J Clin Oncol. 1982;5(6):649–55.
15. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst. 2000;92(3):205–16.
16. Torzilli G, Minagawa M, Takayama T, Inoue K, Hui AM, Kubota K, et al. Accurate preoperative evaluation of liver mass lesions without fine-needle biopsy. Hepatology. 1999;30(4):889–93.
17. Bruix J, Sherman M. Management of hepatocellular carcinoma. Hepatology. 2005;42(5):1208–36.
18. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet. 2003;362(9399):1907–17.
19. Wadler S, Schwartz EL, Goldman M, Lyver A, Rader M, Zimmerman M, et al. Fluorouracil and recombinant alfa-2a-interferon: an active regimen against advanced colorectal carcinoma. J Clin Oncol. 1989;7(12):1769–75.
20. Greco FA, Figlin R, York M, Einhorn L, Schilsky R, Marshall EM, et al. Phase III randomized study to compare interferon alfa-2a in combination with fluorouracil versus fluorouracil alone in patients with advanced colorectal cancer. J Clin Oncol. 1996; 14(10):2674–81.
21. Patt YZ, Yoffe B, Charnsangavej C, Pazdur R, Fischer H, Cleary K, et al. Low serum alpha-fetoprotein level in patients with hepatocellular carcinoma as a predictor of response to 5-FU and interferon-alpha-2b. Cancer. 1993;72(9):2574–82.
22. Sakon M, Nagano H, Dono K, Nakamori S, Umeshita K, Yamada A, et al. Combined intraarterial 5-fluorouracil and subcutaneous interferon-alpha therapy for advanced hepatocellular carcinoma with tumor thrombi in the major portal branches. Cancer. 2002;94(2):435–42.
23. Ota H, Nagano H, Sakon M, Eguchi H, Kondo M, Yamamoto T, et al. Treatment of hepatocellular carcinoma with major portal vein thrombosis by combined therapy with subcutaneous interferon-alpha and intra-arterial 5-fluorouracil; role of type 1 interferon receptor expression. Br J Cancer. 2005;93(5):557–64.
24. Damdinsuren B, Nagano H, Sakon M, Kondo M, Yamamoto T, Umeshita K, et al. Interferon-beta is more potent than interferon-alpha in inhibition of human hepatocellular carcinoma cell

growth when used alone and in combination with anticancer drugs. Ann Surg Oncol. 2003;10(10):1184–90.
25. Eguchi H, Nagano H, Yamamoto H, Miyamoto A, Kondo M, Dono K, et al. Augmentation of antitumor activity of 5-fluorouracil by interferon alpha is associated with up-regulation of p27Kip1 in human hepatocellular carcinoma cells. Clin Cancer Res. 2000;6(7):2881–90.
26. Moriyama M, Hoshida Y, Kato N, Otsuka M, Yoshida H, Kawabe T, et al. Genes associated with human hepatocellular carcinoma cell chemosensitivity to 5-fluorouracil plus interferon-alpha combination chemotherapy. Int J Oncol. 2004;25(5):1279–87.
27. Kondo M, Nagano H, Wada H, Damdinsuren B, Yamamoto H, Hiraoka N, et al. Combination of IFN-alpha and 5-fluorouracil induces apoptosis through IFN-alpha/beta receptor in human hepatocellular carcinoma cells. Clin Cancer Res. 2005;11(3):1277–86.
28. Takaoka A, Hayakawa S, Yanai H, Stoiber D, Negishi H, Kikuchi H, et al. Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. Nature. 2003;424(6948):516–23.
29. Nagano H, Miyamoto A, Wada H, Ota H, Marubashi S, Takeda Y, et al. Interferon-alpha and 5-fluorouracil combination therapy after palliative hepatic resection in patients with advanced hepatocellular carcinoma, portal venous tumor thrombus in the major trunk, and multiple nodules. Cancer. 2007;110(11):2493–501.
30. Yamamoto T, Nagano H, Sakon M, Wada H, Eguchi H, Kondo M, et al. Partial contribution of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/TRAIL receptor pathway to antitumor effects of interferon-alpha/5-fluorouracil against hepatocellular carcinoma. Clin Cancer Res. 2004;10(23):7884–95.
31. Nakamura M, Nagano H, Sakon M, Yamamoto T, Ota H, Wada H, et al. Role of the Fas/FasL pathway in combination therapy with interferon-alpha and fluorouracil against hepatocellular carcinoma in vitro. J Hepatol. 2007;46(1):77–88.
32. Patt YZ, Hassan MM, Lozano RD, Brown TD, Vauthey JN, Curley SA, et al. Phase II trial of systemic continuous fluorouracil and subcutaneous recombinant interferon alfa-2b for treatment of hepatocellular carcinoma. J Clin Oncol. 2003;21(3):421–7.
33. Burroughs A, Hochhauser D, Meyer T. Systemic treatment and liver transplantation for hepatocellular carcinoma: two ends of the therapeutic spectrum. Lancet Oncol. 2004;5(7):409–18.
34. Nowak AK, Chow PK, Findlay M. Systemic therapy for advanced hepatocellular carcinoma: a review. Eur J Cancer. 2004;40(10):1474–84.
35. Link JS, Bateman JR, Paroly WS, Durkin WJ, Peters RL. 5-Flourouracil in hepatocellular carcinoma: report of twenty-one cases. Cancer. 1977;39(5):1936–9.
36. Zaniboni A, Simoncini E, Marpicati P, Marini G. Phase II study of 5-fluorouracil (5-FU) and high dose folinic acid (HDFA) in hepatocellular carcinoma. Br J Cancer. 1988;57(3):319.
37. Tetef M, Doroshow J, Akman S, Coluzzi P, Leong L, Margolin K, et al. 5-Fluorouracil and high-dose calcium leucovorin for hepatocellular carcinoma: a phase II trial. Cancer Invest. 1995;13(5):460–3.
38. Llovet JM, Ruff P, Tassopoulos N, Castells L, Bruix J, El-Hariry I, et al. A phase II trial of oral eniluracil/5-fluorouracil in patients with inoperable hepatocellular carcinoma. Eur J Cancer. 2001;37(11):1352–8.
39. Benson AB 3rd, Mitchell E, Abramson N, Klencke B, Ritch P, Burnhan JP, et al. Oral eniluracil/5-fluorouracil in patients with inoperable hepatocellular carcinoma. Ann Oncol. 2002;13(4):576–81.
40. Mani S, Schiano T, Garcia JC, Ansari RH, Samuels B, Sciortino DF, et al. Phase II trial of uracil/tegafur (UFT) plus leucovorin in patients with advanced hepatocellular carcinoma. Invest New Drugs. 1998;16(3):279–83.
41. Ishikawa T, Ichida T, Sugitani S, Tsuboi Y, Genda T, Sugahara S, et al. Improved survival with oral administration of enteric-coated tegafur/uracil for advanced stage IV-A hepatocellular carcinoma. J Gastroenterol Hepatol. 2001;16(4):452–9.
42. Leung TW, Patt YZ, Lau WY, Ho SK, Yu SC, Chan AT, et al. Complete pathological remission is possible with systemic combination chemotherapy for inoperable hepatocellular carcinoma. Clin Cancer Res. 1999;5(7):1676–81.
43. Yeo W, Mok TS, Zee B, Leung TW, Lai PB, Lau WY, et al. A randomized phase III study of doxorubicin versus cisplatin/interferon alpha-2b/doxorubicin/fluorouracil (PIAF) combination chemotherapy for unresectable hepatocellular carcinoma. J Natl Cancer Inst. 2005;97(20):1532–8.
44. Ensminger WD, Rosowsky A, Raso V, Levin DC, Glode M, Come S, et al. A clinical-pharmacological evaluation of hepatic arterial infusions of 5-fluoro-2′-deoxyuridine and 5-fluorouracil. Cancer Res. 1978;38(11 Pt 1):3784–92.
45. Goldberg JA, Kerr DJ, Watson DG, Willmott N, Bates CD, McKillop JH, et al. The pharmacokinetics of 5-fluorouracil administered by arterial infusion in advanced colorectal hepatic metastases. Br J Cancer. 1990;61(6):913–5.
46. Ueno H, Okada S, Okusaka T, Ikeda M, Kuriyama H. Phase I and pharmacokinetic study of 5-fluorouracil administered by 5-day continuous infusion in patients with hepatocellular carcinoma. Cancer Chemother Pharmacol. 2002;49(2):155–60.
47. Uchino K, Tateishi R, Shiina S, Kanda M, Masuzaki R, Kondo Y, et al. Hepatocellular carcinoma with extrahepatic metastasis: clinical features and prognostic factors. Cancer. 2011;117(19):4475–83.
48. Worns MA, Weinmann A, Pfingst K, Schulte-Sasse C, Messow CM, Schulze-Bergkamen H, et al. Safety and efficacy of sorafenib in patients with advanced hepatocellular carcinoma in consideration of concomitant stage of liver cirrhosis. J Clin Gastroenterol. 2009;43(5):489–95.
49. Pinter M, Sieghart W, Graziadei I, Vogel W, Maieron A, Konigsberg R, et al. Sorafenib in unresectable hepatocellular carcinoma from mild to advanced stage liver cirrhosis. Oncologist. 2009;14(1):70–6.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Mortality and morbidity of hepatectomy, radiofrequency ablation, and embolization for hepatocellular carcinoma: a national survey of 54,145 patients

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Abstract
Background Reported mortalities and morbidities of therapeutic procedures for liver tumors vary between studies, because of different designs and small sample sizes. We investigated the mortalities and complication rates for hepatectomy, radiofrequency ablation (RFA), and trans-catheter arterial embolization (TAE) for hepatocellular carcinoma (HCC) in a large sample, using a nationwide Japanese database (the Diagnosis Procedure Combination database).
Methods Data from the Diagnosis Procedure Combination database were analyzed for July 1 to December 31, 2007 and the same period in 2008. We identified 54,145 patients with HCC who underwent hepatectomy ($n = 5{,}270$), RFA ($n = 11{,}688$), or TAE ($n = 37{,}187$). In-hospital mortality and morbidity were analyzed for each procedure. The relationships between mortality and factors including patient characteristics and procedural backgrounds were assessed.
Results In-hospital mortalities associated with hepatectomy, RFA, and TAE were 2.6 % [95 % confidence interval (CI) 2.2–3.1], 0.3 % (0.2–0.4), and 1.0 % (0.9–1.1), and post-procedural complication rates were 14.5 % (13.5–15.5), 4.5 % (4.2–4.9), and 4.5 % (4.3–4.7), respectively. Increased mortality following hepatectomy was significantly associated with older age, extended lobectomy (vs. partial hepatectomy; odds ratio [OR] 3.80, $p < 0.001$), lower hospital volume (OR 2.74, $p < 0.001$), and renal comorbidity (OR 3.01, $p = 0.02$). Older age and cardiac comorbidity (OR 5.14, $p = 0.001$) were significantly associated with RFA-related mortality, and lower hospital volume was significantly associated with TAE-related mortality (OR 1.60, $p < 0.001$).
Conclusions Mortalities and morbidities associated with therapeutic procedures for liver tumors were acceptably low in Japan, but were affected by patient and institutional characteristics.

Keywords Liver tumor · Hospital volume · Nationwide database

Abbreviations

RFA	Radiofrequency ablation
TAE	Trans-catheter arterial embolization
DPC	Diagnosis Procedure Combination
ICD-10	International Classification of Diseases and Related Health Problems, Tenth Revision

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Introduction

The liver is one of the commonest sites of primary and metastatic tumors [1, 2]. Hepatectomy has been considered as a treatment of choice in patients with liver tumors, and can offer survival for 5 years in 50–70 % of patients with early hepatocellular carcinoma (HCC) [3–7], and in

50–60 % with liver metastases of colorectal carcinoma [9–10]. However, image-guided minimally invasive techniques have been widely used for the treatment of HCC in the past decade, and radiofrequency ablation (RFA) has yielded promising clinical results, with survival rates comparable with those of hepatectomy. During the past decade, there has been growing interest in the use of RFA in patients with liver tumors considered to be unresectable because of impaired hepatic function [11–14]. Despite the fact that surveillance programs have reduced the proportion of HCC detected at an advanced stage in certain populations [15, 16], the majority of patients with HCC are still not eligible for curative treatments, such as hepatectomy and RFA, because of advanced features, and trans-catheter arterial embolization (TAE) has been widely used as a palliative treatment in such patients. A recent meta-analysis showed improved overall survival in patients with well-preserved liver function who were treated with TAE [17].

Before the 1980s, hepatectomy-related mortality was reported to be as high as 10 %. In recent years, however, this has decreased to less than 5 % at some surgical centers, and several recent studies have reported large series of successful hepatectomies with no mortality [18, 19]. On the other hand, although the safety of image-guided therapies has been generally accepted, various complications have been reported. The reported complication rates vary substantially, primarily because of the small sample sizes used in most studies. Published studies may also be liable to publication bias, i.e., authors may be less enthusiastic about reporting studies with higher complication rates.

The Diagnosis Procedure Combination (DPC) database is a discharge abstract and administrative claims database of inpatient admissions to secondary and tertiary care hospitals in Japan [20–22], representing approximately 40 % of inpatient admissions to such hospitals. This database represents a large number of samples, and can thus be used to investigate the mortalities and morbidities associated with different treatment modalities, on an objective basis. The aim of this study was to investigate the mortalities and complication rates associated with hepatectomy, RFA, and TAE for HCC in a large patient sample, using the DPC database.

Subjects, materials, and methods

Data source

The DPC database includes the following information: location of hospital; patient demographics; diagnosis, comorbidities at admission and complications after admission recorded with text in Japanese and the International Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10) codes; therapeutic procedures coded by the Japanese original K-code; length of stay; and discharge status, including in-hospital death. The survey of the DPC hospitals is conducted by the DPC Research Group between July 1 and December 31 each year, and is funded by the Ministry of Health, Labour and Welfare, Japan. All 82 university teaching hospitals are obliged to adopt the DPC system, but adoption by community hospitals is voluntary. The survey started in 2003 with 82 teaching hospitals; 926 hospitals participated in 2007, and 855 in 2008. Data for 2.99 and 2.86 million patients were included in 2007 and 2008, respectively. The number in 2008 represented approximately 40 % of all the inpatient admissions to secondary and tertiary care hospitals in Japan.

The requirement for informed consent was waived in this study, because of the anonymous nature of the data. Study approval was obtained from the institutional review board of the University of Occupational and Environmental Health, Fukuoka.

Samples

The data used in this survey were derived from the DPC database for July 1 to December 31, 2007 and the same period in 2008. Patients with a diagnosis of HCC (ICD-10 code C220) were identified. We then selected patients who underwent hepatectomy (DPC procedure code K695), RFA (K697-3), or TAE (K615). Hepatectomy (K695) patients were divided into five sub-categories (K695-1, partial hepatectomy; K695-2, hepatic segmentectomy; K695-3, hepatic lobectomy; K695-4, extended hepatic lobectomy; K695-5, extended hepatic lobectomy with revascularization procedure). Finally, patients who underwent two or more types of the above procedures, and those who underwent TAE for controlling tumor bleeding during emergency hospitalization were excluded.

Endpoints

The primary endpoint was the in-hospital mortality after each procedure. The secondary endpoint was the occurrence of post-procedural complications during hospitalization, including hemorrhage, pneumothorax, liver abscess, gastrointestinal perforation, peritonitis, and hepatic infarction.

Statistical analysis

Patient characteristics were analyzed in terms of sex, age, and comorbidities. The characteristics of patients who underwent hepatectomy were analyzed in terms of each operative method. Hospital volume for each therapeutic procedure during the survey period was determined using the unique identifier for each hospital, and categorized into

three (low-, intermediate-, and high-volume) groups, such that the numbers of patients in each group were almost equal. We assessed the relationships between mortality and various factors, including patient characteristics and hospital volume. The univariate association between each factor and in-hospital mortality was evaluated using the χ^2 test or analysis of variance, as appropriate. Stepwise logistic regression analysis was used to model the concurrent effects of procedures and other factors on in-hospital mortality. Statistical analyses were performed using PASW version 18.0 (SPSS, Chicago, IL, USA). The threshold of reported p values for significance was accepted as <0.05.

Results

Patient characteristics

Of 118,524 patients with HCC, 54,145 eligible patients at 808 hospitals were finally enrolled. Among these, 5,270, 11,688, and 37,187 patients underwent hepatectomy, RFA, and TAE, respectively. Approximately 70 % were male and the mean ages ranged from 67.7 to 71.2 years, according to the mode of treatment. Patients who underwent hepatectomy were younger, and had a higher probability of having diabetes or cardiac disease (Table 1).

Procedural outcomes

The numbers of in-hospital deaths among patients who underwent hepatectomy, RFA, and TAE were 137 (2.60 %), 29 (0.25 %), and 383 (1.03 %), respectively.

Table 2 shows the in-hospital mortality associated with each procedure, and the univariate association between patient characteristics and procedural backgrounds. The in-hospital mortality associated with hepatectomy was higher in older patients ($p = 0.002$), those treated with more invasive procedures ($p < 0.001$), those in hospitals with lower procedure volumes ($p < 0.001$), and patients with chronic renal disease ($p = 0.02$) (Table 2). The results of multivariate logistic regression analysis of in-hospital mortality for hepatectomy, RFA, and TAE are shown in Fig. 1. Multivariate logistic regression analysis revealed that lobectomy and partial lobectomy were significantly associated with higher mortalities. Segmentectomy was associated with higher in-hospital mortality than partial hepatectomy, but the difference was not statistically significant [adjusted odds ratio (OR) 1.21, $p = 0.38$] (Fig. 1a).

The in-hospital mortality associated with RFA was significantly higher in older patients ($p = 0.001$) and those with cardiac diseases ($p < 0.001$) (Table 2). Both of these features were identified as significant factors associated with increased mortality (Fig. 1b).

The in-hospital mortality associated with TAE was 1.03 % (Table 2). Univariate comparison showed that in-hospital mortality was significantly higher in hospitals with lower procedure volumes ($p < 0.001$) and in younger patients ($p = 0.04$). Multivariate logistic regression analysis identified higher procedure volume as a significant factor associated with lower mortality (OR 0.62, $p < 0.001$) (Fig. 1c).

The mean intervals between the date of procedure and death in fatal cases were 43.1 days (range 0–167 days) for hepatectomy, 42.1 days (range 0–178 days) for RFA, and 40.5 days (range 0–204 days) for TAE. The mortalities within the 30 days following the procedure were 1.08 % for hepatectomy, 0.14 % for RFA, and 0.45 % for TAE.

Table 1 HCC patient characteristics and details of procedures

	Total (n = 54,145)	Hepatectomy (n = 5,270)	RFA (n = 11,688)	TAE (n = 37,187)	p value
Sex, n (%)					<0.001
Female	15,266 (28.2)	1,250 (29.3)	3,831 (32.8)	10,185 (27.4)	
Male	38,879 (71.8)	4,020 (70.7)	7,857 (67.2)	27,002 (72.6)	
Age (years), mean ± SD	70.7 ± 8.8	67.7 ± 9.5	70.7 ± 8.6	71.2 ± 8.6	
Age (years), n (%)					<0.001
≤59	5,498 (10.2)	883 (16.8)	1,197 (10.2)	3,418 (9.2)	
60–69	14,662 (27.1)	1,703 (32.3)	3,162 (27.1)	9,797 (26.4)	
70–79	25,247 (46.6)	2,261 (42.9)	5,471 (46.8)	17,515 (47.1)	
≥80	8,738 (16.1)	423 (8.0)	1,858 (15.9)	6,457 (17.3)	
Comorbidities, n (%)					
Diabetes mellitus	9,878 (18.2)	1,162 (22.0)	1,945 (16.6)	6,771 (18.2)	<0.001
Cardiac diseases	2,138 (3.9)	317 (6.0)	365 (3.1)	1,456 (3.9)	<0.001
Chronic renal diseases	771 (1.4)	72 (1.4)	161 (1.4)	538 (1.5)	0.8

RFA radiofrequency ablation, *TAE* trans-catheter arterial embolization, *HCC* hepatocellular carcinoma

Table 2 In-hospital mortality of hepatectomy, RFA, and TAE

	Hepatectomy			RFA			TAE		
	n/N	% (95 % CI)	*P*	*n/N*	% (95 % CI)	*P*	*n/N*	% (95 % CI)	*P*
Overall	137/5,270	2.60 (2.19–3.10)		29/11,688	0.25 (0.17–0.36)		383/37,187	1.03 (0.93–1.14)	
Sex			0.26			0.08			0.17
Female	38/1,250	3.04 (2.16–4.15)		14/3,831	0.37 (0.20–0.61)		93/10,185	0.91 (0.73–1.12)	
Male	99/4,020	2.46 (2.01–3.00)		15/7,857	0.19 (0.11–0.31)		290/27,002	1.07 (0.95–1.20)	
Age (years)			0.002			0.001			0.04
≤59	10/883	1.13 (0.54–2.07)		0/1,197	0.0 (0.00–0.30)		45/3,418	1.32 (0.96–1.76)	
60–69	40/1,703	2.35 (1.68–3.18)		2/3,162	0.06 (0.01–0.23)		112/9,797	1.14 (0.94–1.37)	
70–79	76/2,261	3.36 (2.66–4.19)		19/5,471	0.35 (0.21–0.54)		163/17,515	0.93 (0.79–1.08)	
≥80	11/423	2.60 (1.31–4.61)		8/1,858	0.43 (0.19–0.85)		63/6,457	0.98 (0.75–1.25)	
Procedure type			<0.001						
Partial hepatectomy	42/2,163	1.94 (1.40–2.62)		–			–		
Segmentectomy	45/1,921	2.34 (1.71–3.12)		–			–		
Lobectomy	31/869	3.57 (2.44–5.03)		–			–		
Extended lobectomy	19/317	6.00 (3.65–9.20)		–			–		
Hospital volume[a]			<0.001			0.26			<0.001
High	27/1,744	1.55 (1.02–2.24)		8/3,875	0.21 (0.09–0.41)		97/12,101	0.80 (0.65–0.98)	
Intermediate	38/1,742	2.18 (1.55–3.00)		8/3,896	0.21 (0.09–0.40)		126/12,497	1.01 (0.84–1.20)	
Low	72/1,784	4.04 (3.17–5.06)		13/3,917	0.33 (0.18–0.57)		160/12,589	1.27 (1.08–1.48)	
Comorbidities									
Diabetes mellitus			0.97			0.68			0.76
No	107/4,108	2.60 (2.14–3.14)		25/9,743	0.26 (0.17–0.38)		311/30,416	1.02 (0.91–1.14)	
Yes	30/1,162	2.58 (1.75–3.67)		4/1,945	0.21 (0.06–0.53)		72/6,771	1.06 (0.83–1.34)	
Cardiac diseases			0.93			<0.001			0.79
No	129/4,953	2.60 (2.18–3.09)		24/11,323	0.21 (0.14–0.32)		369/35,731	1.03 (0.93–1.14)	
Yes	8/317	2.52 (1.10–4.91)		5/365	1.37 (0.45–3.17)		14/1,456	0.96 (0.53–1.61)	
Chronic renal diseases			0.02			0.52			0.29
No	132/5,198	2.54 (2.13–3.00)		29/11,527	0.25 (0.17–0.36)		375/36,649	1.02 (0.92–1.13)	
Yes	5/72	6.94 (2.29–15.5)		0/161	0.0 (0.00–2.27)		8/538	1.49 (0.00–1.70)	

CI confidence interval, *RFA* radiofrequency ablation, *TAE* trans-catheter arterial embolization

[a] Hospital volume was defined according to the number of cases per year. High hospital volume represents hospitals with more than 57 cases per year for hepatectomy, 105 cases per year for RFA, and 183 cases per year for TAE. Low hospital volume represents hospitals with fewer than 21 cases per year for hepatectomy, 38 cases per year for RFA, and 76 cases per year for TAE

In-hospital complications occurred in 763 (14.48 %), 531 (4.54 %) and 1,668 (4.49 %) for hepatectomy, RFA, and TAE patients, respectively. The details of the complications are listed in Table 3. Postoperative hemorrhage was the commonest complication of hepatectomy and was seen in 361 (6.85 %) admissions. The most significant complication of both RFA and TAE was related to bile duct stenosis, which occurred in 152 (1.30 %) and 657 (1.77 %) cases, respectively.

Discussion

Hepatectomy, RFA, and TAE are widely applied therapeutic procedures which account for more than 90 % of all procedures performed in patients with primary HCC in Japan [23]. When deciding on a treatment strategy, both therapeutic efficacy and the risks associated with each treatment modality should be considered. Whereas the effectiveness of a procedure can be evaluated based on established criteria such as survival, local recurrence, and tumor necrosis, the complication rate as an index of safety varies greatly among reports, mainly because of differences in the definitions used. In-hospital mortality is a more reliable indicator of safety, but a large number of samples are required to obtain an accurate estimate of mortality, because death is a relatively rare event. In the present study, we investigated the mortalities and morbidities associated with various therapeutic procedures for HCC, using information from the nationally representative Japanese DPC database. It is particularly worth noting that the data for in-hospital mortality were expected to be 100 %

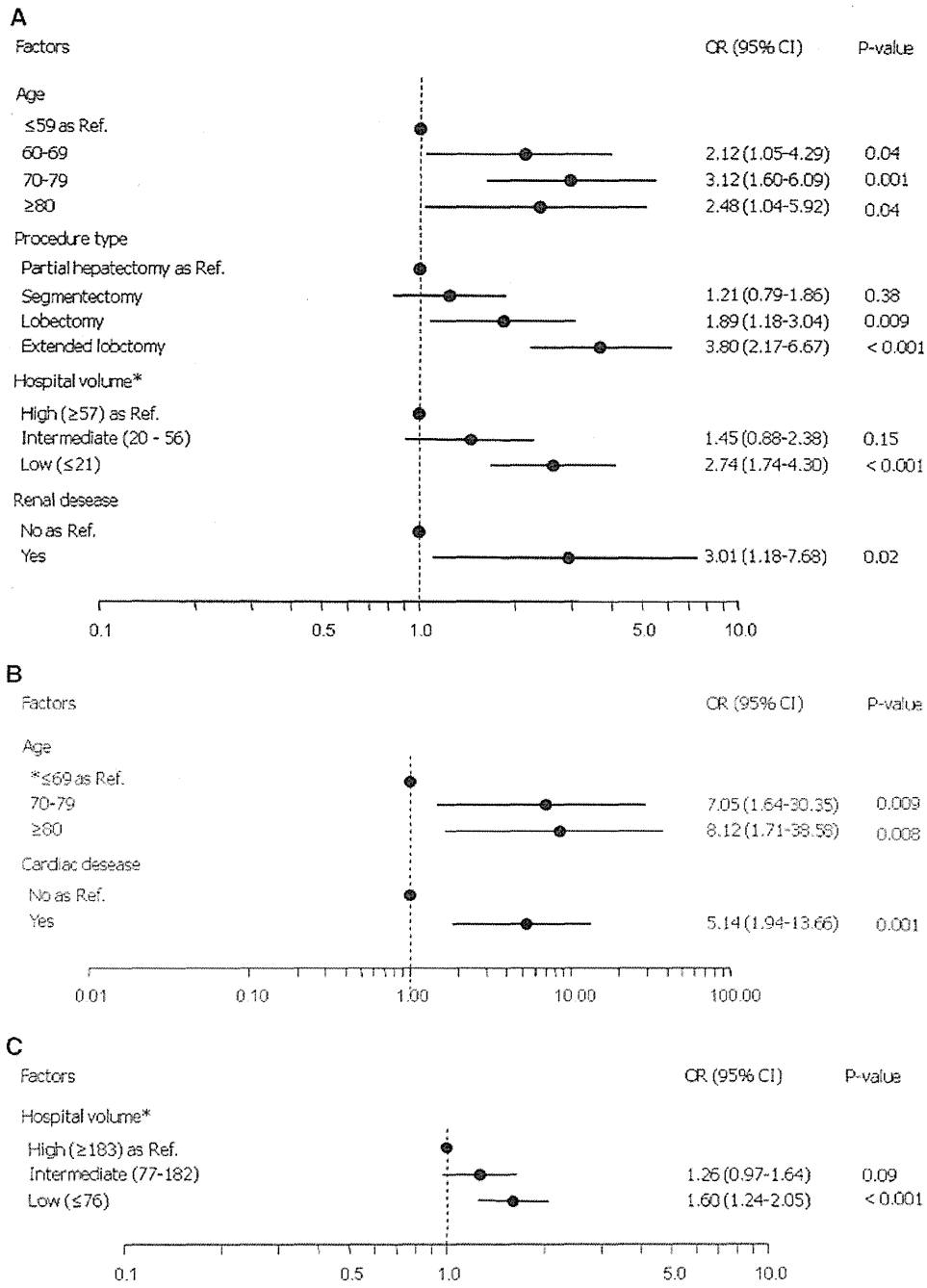

Fig. 1 **a** Multivariate logistic regression analyses for in-hospital mortality of hepatectomy. **b** Multivariate logistic regression analyses for in-hospital mortality of radiofrequency ablation (RFA). *Asterisk* indicates that patients aged 60–69 years and those aged ≤59 years are combined because the mortality of the patients aged ≤59 years was 0. **c** Multivariate logistic regression analyses for in-hospital mortality of trans-catheter embolization (TAE). *OR* odds ratio, *CI* confidence interval, *Ref.* reference value

reliable and free from recall bias because outcome was a required item on discharge.

In this study, the mortality following hepatectomy was 2.60 %, out of a total of 54,145 patients with HCC. Previously reported in-hospital mortalities following hepatectomy for primary and metastatic liver tumors at major high-volume centers were 3.8–8 and 0–7.0 % [4, 5, 24–30], respectively. For example, one report from the United States, using data from a nationwide inpatient sample over a 9-year period, showed a mortality of 6 % [31]. However, the background of that study may be different from that of the present study; for example, lower invasive procedures such as partial hepatectomy and segmentectomy accounted for the major part of the present study. This kind of factor may have influenced the lower mortality rate in the present study.

The mortality associated with RFA in the present study was 0.25 %, which was similar to that noted in previous reports (0.2–0.6 %) [32–39]. Multivariate analysis identified older age and cardiac comorbidity as factors

Table 3 Complications related to each procedure

	Hepatectomy ($n = 5270$)	RFA ($n = 11688$)	TAE ($n = 37187$)
Overall, *n* (%)[a]	763 (14.48)	531 (4.54)	1,668 (4.49)
Hemorrhage, *n* (%)	361 (6.85)	56 (0.48)	102 (0.27)
Bile duct stenosis, *n* (%)	59 (1.12)	152 (1.30)	657 (1.77)
Liver abscess, *n* (%)	19 (0.36)	36 (0.31)	201 (0.54)
Pneumothorax, *n* (%)	1 (0.02)	16 (0.14)	–
Perforation of gastrointestinal tract, *n* (%)	2 (0.04)	3 (0.03)	–
Peritonitis, *n* (%)	98 (1.86)	98 (0.84)	–
Heat burn, *n* (%)	–	7 (0.06)	–
Hepatic infarction, *n* (%)	–	7 (0.06)	3 (0.00)
Liver failure, *n* (%)	122 (2.31)	131 (1.12)	617 (1.66)
Cardiac complication, *n* (%)	35 (0.66)	15 (0.13)	37 (0.10)
Ruptured suture, *n* (%)	40 (0.76)	2 (0.02)	2 (0.00)
Renal failure, *n* (%)	26 (0.49)	2 (0.02)	35 (0.09)
Pulmonary embolism, *n* (%)	7 (0.13)	4 (0.03)	14 (0.04)
Wound infection, *n* (%)	12 (0.23)	11 (0.09)	11 (0.03)
Pneumonia, *n* (%)	57 (1.08)	30 (0.26)	117 (0.31)
Allergy to anesthetic agents, *n* (%)	2 (0.04)	5 (0.04)	86 (0.23)

[a] More than one complication during hospitalization was counted as one

significantly related to high mortality in patients undergoing RFA.

A recent systematic review of the safety of TAE, based on 37 trials with 2,858 patients, reported a median periprocedural mortality (≤30 days) of 2.4 % (range 0–9.5 %) [40], which was higher than the in-hospital mortality for TAE in the present study (1.03 %). Some previous reports defined mortality as death within the 30 days following the procedure. In the present study, the 30-day mortalities for hepatectomy, RFA, and TAE were 1.08, 0.14, and 0.45 %, respectively.

A number of studies identified hospital procedure volume as an important determinant of postoperative mortality following advanced surgical procedures [31, 41–46]. In the present study, hospital procedure volume was significantly associated with in-hospital mortality for hepatectomy and TAE, but although the RFA-associated in-hospital mortality tended to be lower in high-volume hospitals, the difference was not significant. Despite the large sample size, it is still possible that this study was too underpowered to show any significant association between hospital volume and RFA mortality, because of the exceptionally low mortality rate of RFA. These results suggest that concentrating patients indicated for hepatectomy in high-volume centers should be considered on safety grounds.

In the present study, the mortality rate for hepatectomy was lower in patients over 80 years old. The indication for hepatectomy is determined on the basis of several factors. Although there is no specific age limitation for hepatectomy in Japan, older patients have shorter long-term survival after hepatectomy compared to younger patients, because of their expected life span. Thus, the indication for hepatectomy in older patients, especially those over 80, is stricter in clinical practice. Taking these factors into consideration, it is possible that the patients over 80 years old from the DPC database who did undergo hepatectomy were in generally better than average health. That could explain the lower mortality associated with hepatectomy in patients of 80 years and over in the present study. Similarly, the in-hospital mortality rate for TAE was significantly lower in older patients according to the univariate analysis. This result also may be related to the indications for TAE in Japan. Moreover, the intensity and area of embolization for TAE can be regulated, and embolization is likely to be less intensive in older patients, possibly accounting for the lower mortality in older patients who underwent TAE. Cardiac comorbidity was significantly associated with in-hospital mortality for RFA. According to the database, three out of the five deceased patients with cardiac comorbidities were speculated to have died as a result of cardiac complications (e.g., myocardial infarction, angina pectoris, and heart failure). RFA is thought to be less invasive than hepatectomy, and is sometimes considered as an alternative therapy to hepatectomy in patients with relatively severe cardiac comorbidities. The cardiac comorbidities were thus likely to have been severe in the RFA group, which could account for the higher mortality after RFA in the present study.

The complication rates for hepatectomy, RFA, and TAE in the present study were 14.48, 4.54, and 4.49 %, respectively. Previously reported complication rates have varied among studies, ranging from 28.4 to 47.7 % [47–

52], from 0 to 12.7 % [52–58], and from 4.3 to 10.8 % [53–55] for hepatectomy, RFA, and TAE, respectively. Our multivariate logistic regression analysis demonstrated that the complication rate was significantly higher after more invasive procedures in patients treated in hospitals with lower procedure volumes, in patients with diabetes mellitus, and in patients with cardiac diseases in the case of hepatectomy; and in patients treated in hospitals with higher procedure volumes, patients with diabetes mellitus, and patients with cardiac diseases for RFA and TAE (data not shown). However, complications are usually reported in the DPC database in relation to the reimbursement of medical fees, and the reported complications were therefore less objective than the reported mortality, and could have been underestimated. The complication rate was relatively low for hepatectomy, and the rates for the other procedures were similar to those in previous reports.

The present study had several limitations. First, although the DPC database represents approximately 40 % of all admissions to secondary and tertiary care hospitals in Japan, participating hospitals tend to be medium-to-large-sized institutions. The mortality could therefore have been underestimated by potentially excluding low-procedure-volume hospitals. Second, some important clinical data that may affect the risk of death related to treatments, such as the size and location of the tumor, and severity indexes of liver disease [e.g., the Child–Pugh and model for end-stage liver disease (MELD) scores] were unavailable in this database. Third, data on late-onset complications that appeared after discharge (i.e., biloma, biliary injury, or hepatic abscess) were also unavailable, because the database covers only inpatient data. This may have led to an underestimation of the complication rate in this study. However, according to previous reports, late-onset complications appear to have minimal effects on the mortality rates. Fourth, as noted above, as the DPC system was basically designed for assessing reimbursement, co-existing diseases are usually reported when a specific treatment is needed,; e.g., the proportion of patients with diabetes was higher in the hepatectomy group than that with the other procedures. This may be because patients who underwent hepatectomy were more likely to have been treated with intensive insulin therapy before surgery. Fifth, the immediate cause of death is not a required item in the DPC database. Accordingly, the in-hospital deaths recorded in this database have room for treatment unrelated deaths, the procedure-related mortality could therefore have been overestimated. However in-hospital mortality is more robust in terms of objectivity compared to treatment-related mortality assessed by operators. Finally, some complications, such as tumor seeding, were not covered by the ICD codes and could therefore not be evaluated.

In conclusion, this study confirmed that the therapeutic procedures used to treat liver tumors in Japan were associated with low mortalities and low complication rates. However, procedure-related mortality can be affected by patient and therapeutic backgrounds.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, et al. Cancer statistics, 2005. CA Cancer J Clin. 2005;55:10–30.
2. Kamangar F, Dores GM, Anderson WF. Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. J Clin Oncol. 2006;24:2137–50.
3. Arii S, Yamaoka Y, Futagawa S, Inoue K, Kobayashi K, Kojiro M, et al. Results of surgical and nonsurgical treatment for small-sized hepatocellular carcinomas: a retrospective and nationwide survey in Japan. The Liver Cancer Study Group of Japan. Hepatology. 2000;32:1224–9.
4. Fong Y, Sun RL, Jarnagin W, Blumgart LH. An analysis of 412 cases of hepatocellular carcinoma at a Western center. Ann Surg. 1999;229:790–9 (discussion 799–800).
5. Grazi GL, Ercolani G, Pierangeli F, Del Gaudio M, Cescon M, Cavallari A, et al. Improved results of liver resection for hepatocellular carcinoma on cirrhosis give the procedure added value. Ann Surg. 2001;234:71–8.
6. Llovet JM, Fuster J, Bruix J. Intention-to-treat analysis of surgical treatment for early hepatocellular carcinoma: resection versus transplantation. Hepatology. 1999;30:1434–40.
7. Takayama T, Makuuchi M, Hirohashi S, Sakamoto M, Yamamoto J, Shimada K, et al. Early hepatocellular carcinoma as an entity with a high rate of surgical cure. Hepatology. 1998;28:1241–6.
8. Abdalla EK, Vauthey JN, Ellis LM, Ellis V, Pollock R, Broglio KR, et al. Recurrence and outcomes following hepatic resection, radiofrequency ablation, and combined resection/ablation for colorectal liver metastases. Ann Surg. 2004;239:818–25. discussion 825–17.
9. Choti MA, Sitzmann JV, Tiburi MF, Sumetchotimetha W, Rangsin R, Schulick RD, et al. Trends in long-term survival following liver resection for hepatic colorectal metastases. Ann Surg. 2002;235:759–66.
10. Fernandez FG, Drebin JA, Linehan DC, Dehdashti F, Siegel BA, Strasberg SM. Five-year survival after resection of hepatic metastases from colorectal cancer in patients screened by positron emission tomography with F-18 fluorodeoxyglucose (FDG-PET). Ann Surg. 2004;240:438–47 (discussion 447–50).
11. Rossi S, Di Stasi M, Buscarini E, Quaretti P, Garbagnati F, Squassante L, et al. Percutaneous RF interstitial thermal ablation in the treatment of hepatic cancer. AJR Am J Roentgenol. 1996;167:759–68.
12. Livraghi T, Goldberg SN, Lazzaroni S, Meloni F, Solbiati L, Gazelle GS. Small hepatocellular carcinoma: treatment with radio-frequency ablation versus ethanol injection. Radiology. 1999;210:655–61.

13. Curley SA, Izzo F, Delrio P, Ellis LM, Granchi J, Vallone P, et al. Radiofrequency ablation of unresectable primary and metastatic hepatic malignancies: results in 123 patients. Ann Surg. 1999;230:1–8.
14. Tateishi R, Shiina S, Teratani T, Obi S, Sato S, Koike Y, et al. Percutaneous radiofrequency ablation for hepatocellular carcinoma. An analysis of 1000 cases. Cancer. 2005;103:1201–9.
15. Bruix J, Sherman M. Management of hepatocellular carcinoma. Hepatology. 2005;42:1208–36.
16. Chang MH, You SL, Chen CJ, Liu CJ, Lee CM, Lin SM, et al. Decreased incidence of hepatocellular carcinoma in hepatitis B vaccinees: a 20-year follow-up study. J Natl Cancer Inst. 2009;101:1348–55.
17. Llovet JM, Bruix J. Systematic review of randomized trials for unresectable hepatocellular carcinoma: chemoembolization improves survival. Hepatology. 2003;37:429–42.
18. Imamura H, Seyama Y, Kokudo N, Maema A, Sugawara Y, Sano K, et al. One thousand fifty-six hepatectomies without mortality in 8 years. Arch Surg. 2003;138:1198–206 (discussion 1206).
19. Jarnagin WR, Gonen M, Fong Y, DeMatteo RP, Ben-Porat L, Little S, et al. Improvement in perioperative outcome after hepatic resection: analysis of 1,803 consecutive cases over the past decade. Ann Surg. 2002;236:397–406 (discussion 397–406).
20. Sumitani M, Uchida K, Yasunaga H, Horiguchi H, Kusakabe Y, Matsuda S, et al. Prevalence of malignant hyperthermia and relationship with anesthetics in Japan: data from the diagnosis procedure combination database. Anesthesiology. 2011;114:84–90.
21. Yasunaga H, Shi Y, Takeuchi M, Horiguchi H, Hashimoto H, Matsuda S, et al. Measles-related hospitalizations and complications in Japan, 2007–2008. Intern Med. 2010;49:1965–70.
22. Yasunaga H, Yanaihara H, Fuji K, Horiguchi H, Hashimoto H, Matsuda S. Impact of hospital volume on postoperative complications and in-hospital mortality after renal surgery: data from the Japanese Diagnosis Procedure Combination Database. Urology. 2010;76:548–52.
23. Ikai I, Arii S, Okazaki M, Okita K, Omata M, Kojiro M, et al. Report of the 17th nationwide follow-up survey of primary liver cancer in Japan. Hepatol Res. 2007;37:676–91.
24. Belghiti J, Regimbeau JM, Durand F, Kianmanesh AR, Dondero F, Terris B, et al. Resection of hepatocellular carcinoma: a European experience on 328 cases. Hepatogastroenterology. 2002;49:41–6.
25. Biasco G, Gallerani E. Treatment of liver metastases from colorectal cancer: what is the best approach today? Dig Liver Dis. 2001;33:438–44.
26. Cromheecke M, de Jong KP, Hoekstra HJ. Current treatment for colorectal cancer metastatic to the liver. Eur J Surg Oncol. 1999;25:451–63.
27. Poon RT, Fan ST. Hepatectomy for hepatocellular carcinoma: patient selection and postoperative outcome. Liver Transpl. 2004;10:S39–45.
28. Ruers T, Bleichrodt RP. Treatment of liver metastases, an update on the possibilities and results. Eur J Cancer. 2002;38:1023–33.
29. Vivarelli M, Guglielmi A, Ruzzenente A, Cucchetti A, Bellusci R, Cordiano C, et al. Surgical resection versus percutaneous radiofrequency ablation in the treatment of hepatocellular carcinoma on cirrhotic liver. Ann Surg. 2004;240:102–7.
30. Yoon SS, Tanabe KK. Multidisciplinary management of metastatic colorectal cancer. Surg Oncol. 1998;7:197–207.
31. Kohn GP, Nikfarjam M. The effect of surgical volume and the provision of residency and fellowship training on complications of major hepatic resection. J Gastrointest Surg. 2010;14:1981–9.
32. Bilchik AJ, Wood TF, Allegra DP. Radiofrequency ablation of unresectable hepatic malignancies: lessons learned. Oncologist. 2001;6:24–33.
33. Curley SA, Izzo F, Ellis LM, Nicolas Vauthey J, Vallone P. Radiofrequency ablation of hepatocellular cancer in 110 patients with cirrhosis. Ann Surg. 2000;232:381–91.
34. de Baere T, Risse O, Kuoch V, Dromain C, Sengel C, Smayra T, et al. Adverse events during radiofrequency treatment of 582 hepatic tumors. AJR Am J Roentgenol. 2003;181:695–700.
35. Giorgio A, Tarantino L, de Stefano G, Coppola C, Ferraioli G. Complications after percutaneous saline-enhanced radiofrequency ablation of liver tumors: 3-year experience with 336 patients at a single center. AJR Am J Roentgenol. 2005;184:207–11.
36. Jansen MC, van Duijnhoven FH, van Hillegersberg R, Rijken A, van Coevorden F, van der Sijp J, et al. Adverse effects of radiofrequency ablation of liver tumours in the Netherlands. Br J Surg. 2005;92:1248–54.
37. Livraghi T, Solbiati L, Meloni MF, Gazelle GS, Halpern EF, Goldberg SN. Treatment of focal liver tumors with percutaneous radio-frequency ablation: complications encountered in a multicenter study. Radiology. 2003;226:441–51.
38. Wood TF, Rose DM, Chung M, Allegra DP, Foshag LJ, Bilchik AJ. Radiofrequency ablation of 231 unresectable hepatic tumors: indications, limitations, and complications. Ann Surg Oncol. 2000;7:593–600.
39. Kasugai H, Osaki Y, Oka H, Kudo M, Seki T. Severe complications of radiofrequency ablation therapy for hepatocellular carcinoma: an analysis of 3,891 ablations in 2,614 patients. Oncology. 2007;72(Suppl 1):72–5.
40. Marelli L, Stigliano R, Triantos C, Senzolo M, Cholongitas E, Davies N, et al. Transarterial therapy for hepatocellular carcinoma: which technique is more effective? A systematic review of cohort and randomized studies. Cardiovasc Interv Radiol. 2007;30:6–25.
41. Begg CB, Cramer LD, Hoskins WJ, Brennan MF. Impact of hospital volume on operative mortality for major cancer surgery. JAMA. 1998;280:1747–51.
42. Birkmeyer JD, Siewers AE, Finlayson EV, Stukel TA, Lucas FL, Batista I, et al. Hospital volume and surgical mortality in the United States. N Engl J Med. 2002;346:1128–37.
43. Birkmeyer JD, Stukel TA, Siewers AE, Goodney PP, Wennberg DE, Lucas FL. Surgeon volume and operative mortality in the United States. N Engl J Med. 2003;349:2117–27.
44. Birkmeyer JD, Sun Y, Goldfaden A, Birkmeyer NJ, Stukel TA. Volume and process of care in high-risk cancer surgery. Cancer. 2006;106:2476–81.
45. Kuwabara K, Matsuda S, Fushimi K, Ishikawa KB, Horiguchi H, Fujimori K. Impact of hospital case volume on the quality of laparoscopic colectomy in Japan. J Gastrointest Surg. 2009;13:1619–26.
46. Schrag D, Cramer LD, Bach PB, Cohen AM, Warren JL, Begg CB. Influence of hospital procedure volume on outcomes following surgery for colon cancer. JAMA. 2000;284:3028–35.
47. Benzoni E, Cojutti A, Lorenzin D, Adani GL, Baccarani U, Favero A, et al. Liver resective surgery: a multivariate analysis of postoperative outcome and complication. Langenbecks Arch Surg. 2007;392:45–54.
48. Delis SG, Bakoyiannis A, Dervenis C, Tassopoulos N. Perioperative risk assessment for hepatocellular carcinoma by using the MELD score. J Gastrointest Surg. 2009;13:2268–75.
49. Laurent C, Sa Cunha A, Couderc P, Rullier E, Saric J. Influence of postoperative morbidity on long-term survival following liver resection for colorectal metastases. Br J Surg. 2003;90:1131–6.
50. Schiesser M, Chen JW, Maddern GJ, Padbury RT. Perioperative morbidity affects long-term survival in patients following liver resection for colorectal metastases. J Gastrointest Surg. 2008;12:1054–60.
51. Sim HG, Ooi LL. Results of resections for hepatocellular carcinoma in a new hepatobiliary unit. ANZ J Surg. 2003;73:8–13.

52. Ziparo V, Balducci G, Lucandri G, Mercantini P, Di Giacomo G, Fernandes E. Indications and results of resection for hepatocellular carcinoma. Eur J Surg Oncol. 2002;28:723–8.
53. Kiely JM, Rilling WS, Touzios JG, Hieb RA, Franco J, Saeian K, et al. Chemoembolization in patients at high risk: results and complications. J Vasc Interv Radiol. 2006;17:47–53.
54. Kothary N, Weintraub JL, Susman J, Rundback JH. Transarterial chemoembolization for primary hepatocellular carcinoma in patients at high risk. J Vasc Interv Radiol. 2007;18:1517–26 (quiz 1527).
55. Pietrosi G, Miraglia R, Luca A, Vizzini GB, Fili D, Riccardo V, et al. Arterial chemoembolization/embolization and early complications after hepatocellular carcinoma treatment: a safe standardized protocol in selected patients with Child class A and B cirrhosis. J Vasc Interv Radiol. 2009;20:896–902.

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Silencing of microRNA-122 enhances interferon-α signaling in the liver through regulating SOCS3 promoter methylation

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Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. Although novel drugs against HCV are under development, the current standard therapy consists principally of interferon (IFN). To improve the response to IFN treatment by enhancing interferon-stimulated response element (ISRE)-mediated gene transcription, we screened 75 microRNAs highly expressed in hepatocytes for their ability to modulate ISRE activity. Overexpression of microRNA-122 (miR122) significantly suppressed ISRE activity. Conversely, silencing of miR122 function enhanced IFN-induced ISRE activity, by decreasing expression of suppressor of cytokine signaling 3 (SOCS3). This decrease in SOCS3 level was not mediated by microRNA target gene suppression, but rather by enhanced methylation at SOCS3 gene promoter. Taken together, our data, along with the fact that antisense oligonucleotides of miR122 also directly inhibit HCV replication, suggest that a combination therapy comprising IFN and silencing of miR122 function may be a promising therapeutic option in the near future.

More than 170 million individuals worldwide are chronically infected with hepatitis C virus (HCV), which results in hepatic inflammation, hepatic fibrosis, and liver cirrhosis[1]. End-stage liver diseases as well as hepatocellular carcinoma attributable to chronic hepatitis C are increasingly serious problems[2]. For almost a decade, the standard of care in patients with chronic hepatitis C has consisted of pegylated interferon-α2a (pegIFN-α2a) or pegIFN-α2b in combination with the guanosin analog ribavirin. However, this eradicates HCV in only about half of those infected with HCV genotype 1, the most common genotype globally. Moreover, severe adverse events are associated with IFN therapy, such as myelo-suppression and flu-like syndrome. Because these effects are dose-limiting, many patients are unable to receive a higher dose of IFN that might more effectively inhibit HCV replication[3]. While recent licensing of HCV protease inhibitors for the treatment of patients with chronic hepatitis C as part of a triple therapy with pegIFN-α and ribavirin is expected to increase the sustained viral response (SVR) rate, IFN currently remains the principal drug for the eradication of HCV.

Type I interferons (IFNs), such as IFN-α and IFN-β, bind to the type I IFN receptor[4]. One major pathway in type I IFN signaling involves the Jak-STAT signaling cascade[5]. Activated tyrosine kinases phosphorylate STAT-1 and STAT-2 proteins, which bind to p48, a member of the IFN regulatory family (IRF), to form interferon-stimulated gene factor-3 (ISGF3). ISGF3 translocates to the nucleus and binds to the interferon-stimulated response element (ISRE) in the promoter region of IFN target genes, which code for antiviral proteins such as double-stranded RNA-activated protein kinase (PKR) and 2′5′-oligoadenylate synthethase (OAS1). On the other hand, regulatory molecules are also involved in the IFN pathway. The suppressor of cytokine signaling (SOCS) protein is a negative regulator of the Jak-STAT cascade[6]. The SOCS family includes eight members (SOCS-1 to SOCS-7 and CIS), all sharing a central SH2 domain and a C-terminal SOCS box. SOCS-1 and SOCS-3 are the most effective members of this family, and act as negative regulators of several intracellular pathways, particularly the Jak–STAT pathway. In hepatic cells, inhibition of IFN-α-induced STAT-1 activation by HCV core protein overexpression is associated with induction of SOCS-3 mRNA expression[7]. Therefore, increased SOCS3 protein expression during HCV infection may be a mechanism of IFN resistance[8–10]. Such regulatory functions may also be important determinants of the efficacy of anti-HCV IFN therapy.

MicroRNAs are short, single-stranded, non-coding RNAs. They are expressed in most organisms, ranging from plants to vertebrates[11], and are involved in the regulation of target gene expression. Different microRNAs are responsible for the control of various biological processes[12–14]. In this context, a number of microRNAs have

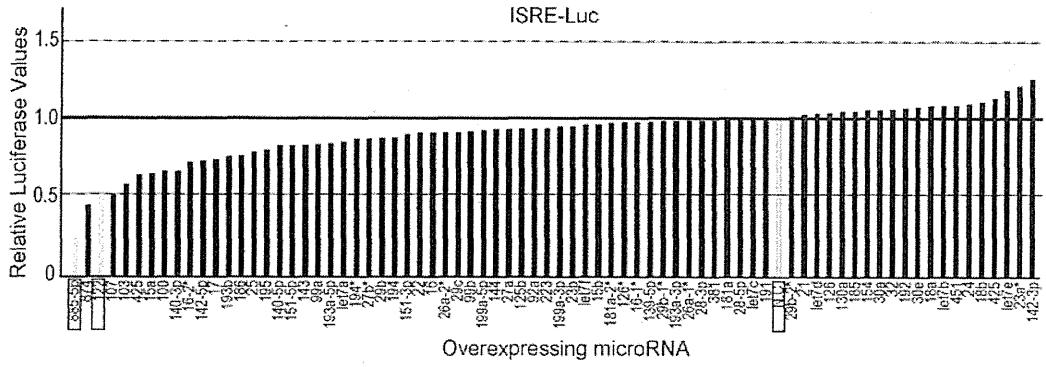

Figure 1 | MiR122 and miR885-5p suppress ISRE activity. Functional screening for liver microRNAs that modulate ISRE activity. Seventy-five mature microRNA oligonucleotides known to be highly expressed in the liver were transiently reverse-transfected into stable 293T cell-derived ISRE reporter cell lines, followed by IFN-α stimulation. Reporter activities were normalized to the values of negative control RNA oligonucleotides (N.C. in green) and ranked in ascending order. Determinations were performed in duplicate, and a representative result is shown. MiR122 and miR885-5p, which were chosen for further analyses, are in blue and represented as rectangles.

recently been shown to regulate the function of intracellular signaling intermediates, such as p53 and NF-κB pathways, by regulating expression of their target genes[15–18].

Primary microRNAs, which possess stem-loop structures, are processed into mature microRNAs by Drosha and Dicer RNA polymerase III. These mature microRNAs then associate with the RNA-induced silencing complex (RISC), and the resulting complex binds directly to the 3′-untranslated regions (3′-UTRs) of target mRNAs to suppress translation and gene expression post-transcriptionally. While this is undoubtedly the main action of microRNAs, recent studies have demonstrated that microRNAs can enter the nucleus[19], and are involved in establishing DNA methylation[20–22]. In addition, microRNAs may also regulate chromatin structure by regulating key histone modifiers[23]. Taken together, these results suggest that microRNAs are important players in epigenetic and post-transcriptional control of gene expression[20].

The aim of this study was to determine the possible role of microRNAs in IFN signaling. We focused on microRNAs expressed in the liver because we were interested in regulators of IFN signaling during HCV treatment. We screened a subset of microRNAs for their ability to modulate ISRE activity to develop a more effective IFN-based therapy against chronic hepatitis C infection.

Results

Screening for microRNAs regulating ISRE activities. We initially screened for microRNAs that affected ISRE-mediated gene transcription using stable ISRE activity reporter cell lines and by transiently overexpressing 75 mature synthetic microRNAs, as we did previously to screen for microRNAs that affect NF-κB activity[15]. Because we were interested in IFN-mediated intracellular signaling in the liver, the microRNAs examined were selected on the basis of their hepatic expression level[24]. In addition, we used non-liver 293T cells for the initial screening to determine the effects of the microRNA overexpression. The data suggested differential effects of microRNAs on ISRE activity in response to IFN-α stimulation (Fig. 1). Of the microRNAs examined, we chose miR122 and miR885-5p for further investigation because they suppressed ISRE activity significantly and reproducibly in two independent screens.

Silencing of miR122 enhances ISRE activities. To confirm the suppressive effects of miR122 and miR885 overexpression on ISRE activities, we first performed a reporter assay to monitor ISRE activities with plasmid-based miR-overexpressing constructs. While both miR122 and miR885 suppressed ISRE activities induced by IFN-α stimulation in 293T cells, the effect of miR122 was more significant (Fig. 2a). For this reason, and because miR122 is the most abundant microRNA in the liver[24], we further focused on miR122. The suppressive effect of miR122 was ISRE-specific, because it had no effect on p53-mediated transcriptional activities (Fig. 2b). Next, to examine the effects of silencing miR122 function on ISRE activity in hepatoma cell lines, we transiently transfected plasmid-based anti-miR122 constructs into Huh7 cells, in which miR122 is highly expressed[25]. The silencing of miR122 function resulted in about two-fold augmentation of IFN-α-induced ISRE activity (Fig. 2c), suggesting that miR122 is also involved in ISRE activity in hepatoma cell lines during IFN-α treatment. To further confirm these effects, we examined Hela-Tet-Off-miR122 cells, in which the expression of miR122 precursors can be shut off by doxycyclin treatment. In these cells, ISRE activity was more highly induced by IFN-α treatment when the expression of miR122 precursors was suppressed by doxycyclin treatment (Fig. 2d). Interferon stimulated genes, such as ISG15 and IFNAR1, were induced to a greater extent by IFN-α treatment in miR122-silenced Huh7 cells than in control cells (Fig. 2e and 2f). These data suggest that silencing miR122 can enhance IFN-α-ISRE activities.

Silencing miR122 suppresses SOCS3 expression by methylation of its promoter. To gain insight into the mechanisms underlying the suppression of ISRE activity by miR122, we searched the Gene Expression Omnibus (GEO) database regarding the effect of silencing miR122 on changes in IFN pathway-related gene expression (DataSet Record GDS1729)[26]. Consistent with our results (Fig. 2), the expression of several known ISRE-mediated IFN-stimulated genes, such as OAS1, interferon α and β receptor 1 (Ifnar1), interferon α-inducible protein 27-like 2A (Ifi27l2a), and interferon regulatory factor 6 (IRF6), were indeed up-regulated by silencing miR122 function in the mouse liver. However, the expression of regulatory genes involved in the IFN signaling pathway from the receptor to the nucleus, such as STAT1, STAT2, JAK1, and JAK2, were unchanged. Although we searched for potential miR122 target genes related to IFN signaling in several microRNA target databases, including TargetScan (http://www.targetscan.org), no major IFN-related genes were found.

Because epigenetic changes induced by microRNAs have been reported[20–22], we compared the comprehensive methylation levels of 27,578 promoter-associated CpG sites using an Illumina Infinium methylation assay (Human Methylation27 BeadChip) between control and stably miR122-silenced Huh7 cell lines. Although the methylation levels of most CpG sites were unchanged, those of a number of CpG sites were altered by silencing miR122 (Table 1). While the methylation of most of these decreased, the CpG sites of a few genes were more methylated by miR122 silencing. The most

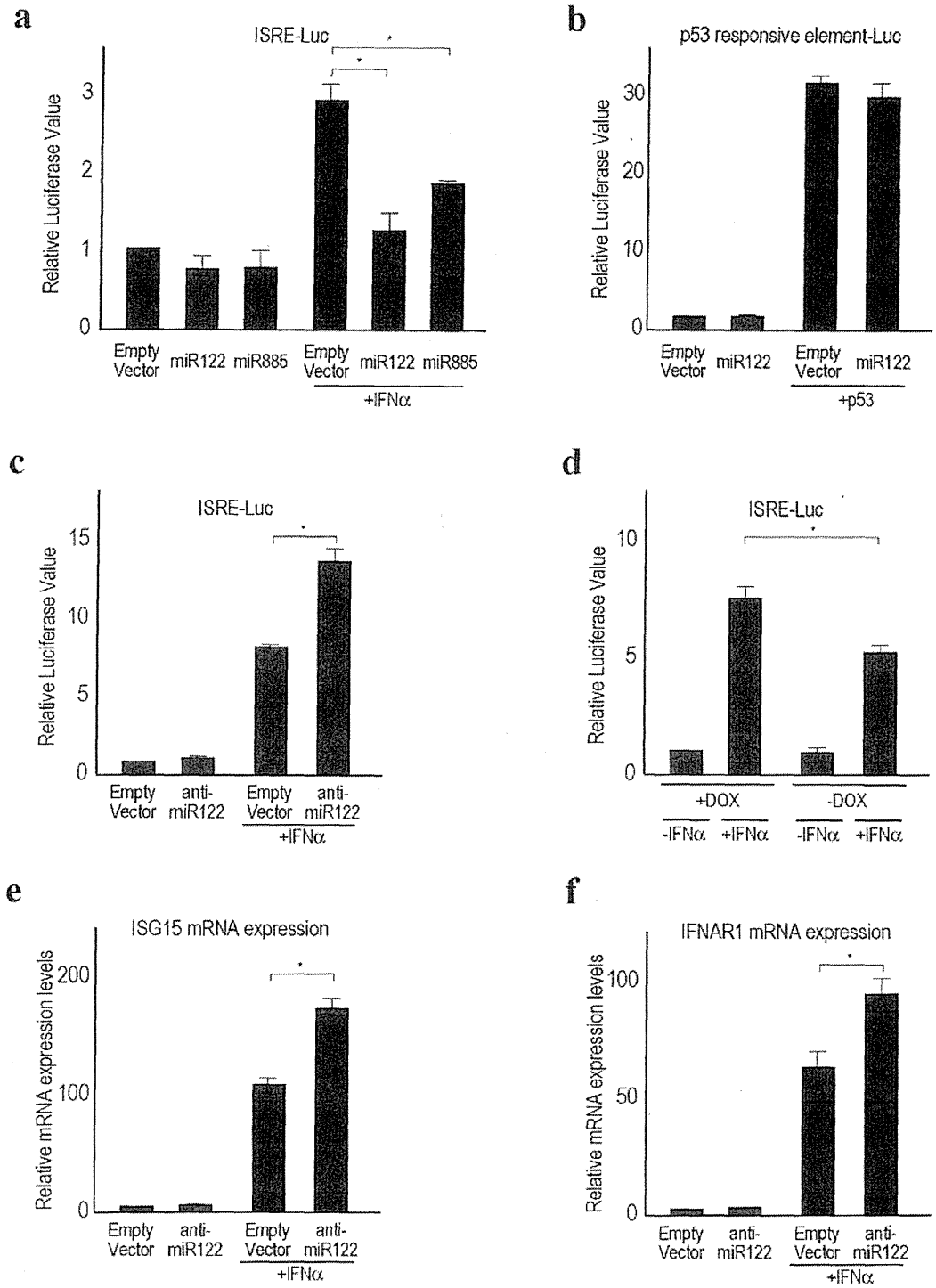

Figure 2 | MiR122 modulates ISRE activities. (a) Overexpression of the selected microRNA precursors suppresses ISRE activity following IFN-α stimulation. ISRE reporter plasmids were transiently transfected, with or without selected microRNA precursor-expressing plasmids, into Huh7 cells. Luciferase values were normalized to those of cells transfected with an empty vector and without IFN, which were set to 1. *, $p < 0.05$. Data represent the means ± standard deviations (SD) of three independent determinations. Similar results were obtained using HepG2 cells. (b) p53 activities were unaffected by miR122 expression. Reporter assays were performed with p53 reporter and p53 expressing plasmids with or without miR122 precursor expression in Huh7 cells. Data represent the means ± SD of three independent determinations. (c) Silencing of miR122 function enhances ISRE activity. Anti-miR122 expressing plasmids were used to perform an ISRE reporter assay in Huh7 cells. *, $p < 0.05$. Data represent the means ± SD of three independent determinations. (d) Hela-Tet-Off-miR122 precursor cell lines were used to measure ISRE activity. Cells were transfected with ISRE reporter constructs with (DOX+) or without (DOX-) doxycyclin. DOX+ shut off the miR122 expression in these cells. The reporter activities after 6 h of IFN-α stimulation were compared. *, $p < 0.05$. Data represent the means ± SD of three independent determinations. (e, f) IFN-inducible genes (e, ISG15; f, IFNAR1) were more induced in miR122-silenced Huh7 cells, determined by quantitative RT-PCR using RNA after 6 h of IFN-α stimulation. *, $p < 0.05$. Data represent the means ± SD of three independent determinations.

Table 1 | Top 30 genes with promoters differentially methylated in control and miR122-silenced Huh7 cells. Values indicate methylation levels. Values in 'Differences' indicate quantitative differences in methylation levels of target gene CpG sites. Higher values indicate greater methylation levels. Genes in bold and highlighted in gray means greater methylation; others were less methylated in miR122-silenced Huh7 cells

SYMBOL	Control	miR122-silenced	Difference	CpG ISLAND LOCATIONS
GPC3	0.4242135	0.11596	0.3082535	X:132946499-132947763
FLJ30058	0.4155016	0.1428133	0.2726883	X:130019792-130020537
EIF3S6IP	0.672594	0.4012034	0.2713906	22:36574299-36576077
BHLHB9	0.3481071	0.07687688	0.27123022	X:101862184-101862707
PORCN	0.6389685	0.3697215	0.269247	
MSI2	0.4593301	0.1955636	0.2637665	17:52688030-52689554
DNTT	0.6114385	0.3489618	0.2624767	10:98054238-98054478
SEMA3B	0.3901392	0.1301293	0.2600099	3:50285369-50286362
RYR2	0.4331115	0.1756537	0.2574578	1:235271651-235273428
TCEAL7	0.6519198	0.4006093	0.2513105	
NR0B2	0.3711949	0.1234481	0.2477468	
SOCS3	**0.1831344**	**0.4273662**	**0.2442318**	17:73866027-73868731
TAS2R16	0.5821649	0.3380581	0.2441068	
BHLHB9	0.3021148	0.06171004	0.24040476	X:101862184-101862707
OR12D3	0.5266486	0.2884241	0.2382245	
ADAMTSL1	0.4537211	0.2196532	0.2340679	
TBX6	0.5031447	0.2695281	0.2336166	16:30010427-30011656
ICAM4	0.4990334	0.2668367	0.2321967	19:10258558-10259935
C9orf125	**0.2387415**	**0.4707038**	**0.2319623**	9:103287963-103289481
ELN	**0.1332373**	**0.3629701**	**0.2297328**	7:73080116-73080605
ANKRD30A	0.898423	0.6711555	0.2272675	10:37453955-37454965
OR10J1	0.5324367	0.3094136	0.2230231	
TP73	0.3596564	0.1386476	0.2210088	1:3596889-3597535
CNNM1	0.6313883	0.4106784	0.2207099	10:101078809-101080801
DHH	0.2955738	0.07619943	0.21937437	12:47774151-47774653
CTAG2	0.5303309	0.3110451	0.2192858	X:153536438-153536702
GNB4	0.3091451	0.09039365	0.21875145	3:180651316-180652496
PNPLA2	0.4458646	0.2272328	0.2186318	11:808884-809164
FGF7	0.6298472	0.4128944	0.2169528	
NTE	0.4557684	0.2400911	0.2156773	19:7504300-7507429

significantly increased methylation levels were observed in the promoter of the SOCS3 gene, which is a negative regulator of IFN signaling. The enhanced methylation levels in silencing miR122 were confirmed by bisulphite sequencing at the CpG island in the SOCS3 promoter, from −556 to −335 relative to the transcriptional start site (Fig. 3a and b). Thus, we hypothesized that the greater methylation of SOCS3 induced by miR122 silencing results in decreased expression of SOCS3 protein, which may, in turn, enhance IFN-α signaling.

MiR122 silencing enhances STAT3 activation by decreasing SOCS3 expression. To confirm the above results, we examined SOCS3 expression and IFN signaling-related molecules in miR122-silenced Huh7 cells (Fig 4a). While the GEO database contained no direct data regarding SOCS3 cDNA expression, we found decreased SOCS3 protein levels in miR122-silenced cells, consistent with promoter hyper-methylation (Fig. 4a). Although the mechanisms underlying the altered methylation induced by miR122 silencing remain unknown, it did not depend on DNA methyltransferase 1 (Dnmt1), which is a key mediator of DNA methylation that catalyzes the methylation of CpG dinucleotides in genomic DNA[27], because the decreased SOCS3 expression by miR122 silencing was also present in Dnmt1 knockdown cells (Fig. 4b).

Because SOCS3 is a potent inhibitor of STAT3 activation[6], and because type I IFNs induce STAT3 as well as STAT1 and STAT2 activation[5,28], we examined the phosphorylation status of STAT proteins after IFN treatment in Huh7 control and miR122-silenced cells (Fig. 4a). While the STAT1, 2, and 3 protein levels and the phosphorylation levels of STAT1 before and after IFN-α stimulation in these two cell lines did not differ significantly, STAT3 phosphorylation levels were higher in miR122-silenced cells 1 and 6 h after IFN-α stimulation, consistent with the decreased expression of SOCS3 (Fig. 4a). Furthermore, STAT2 phosphorylation was slightly higher in miR122-silenced cells (Fig. 4a). Similar tendencies were also observed after treatment with IFN-β, another type I IFN. These data suggest that IFN-α treatment induced greater STAT3 activation in miR122-silenced cells, probably due to decreased SOCS3 expression.

In contrast, whereas IFN-γ (type II IFN) and IFN-λ (type III IFN) induced slight phosphorylation of STAT1 and STAT3, the levels in control and miR122-silenced cells were comparable. STAT2 protein levels were significantly lower in miR122-silenced cells than in controls after treatment with these cytokines (Fig. 4a). No induction of SOCS3 after treatment with IFN-α/β, IFN-γ, or IFN-λ was detected in miR122-silenced cells, probably due to promoter methylation (Fig. 4a), whereas SOCS3 protein was induced by all IFNs in control cells (Fig. 4a). Because increased expression of miR122 was detected after IFN-λ stimulation (Fig. 4c), this might be responsible for the increased SOCS3 expression induced by IFN-λ stimulation.

To confirm whether the induction of the enhanced ISRE activity in miR122-silencing was dependent on the decreased expression of SOCS3, we investigated whether the restoration of SOCS3 expression in miR122-silencing could reduce the ISRE activity in a reporter assay. The overexpression of SOCS3 in miR122-silenced Huh7 cells reduced the induction of ISRE activity caused by miR122-silencing (Fig. 5a and b), although we could not fully exclude the possibility that other mechanisms were also involved because the reversal of ISRE activity did not completely reach the level of the control. To support this, we confirmed the restoration of STAT2 and STAT3 phosphorylation levels induced by IFN-α treatment in miR122-silenced cells that stably overexpressed SOCS3 (Fig. 5c). These results suggest that the enhanced ISRE activity in miR122-silenced cells is

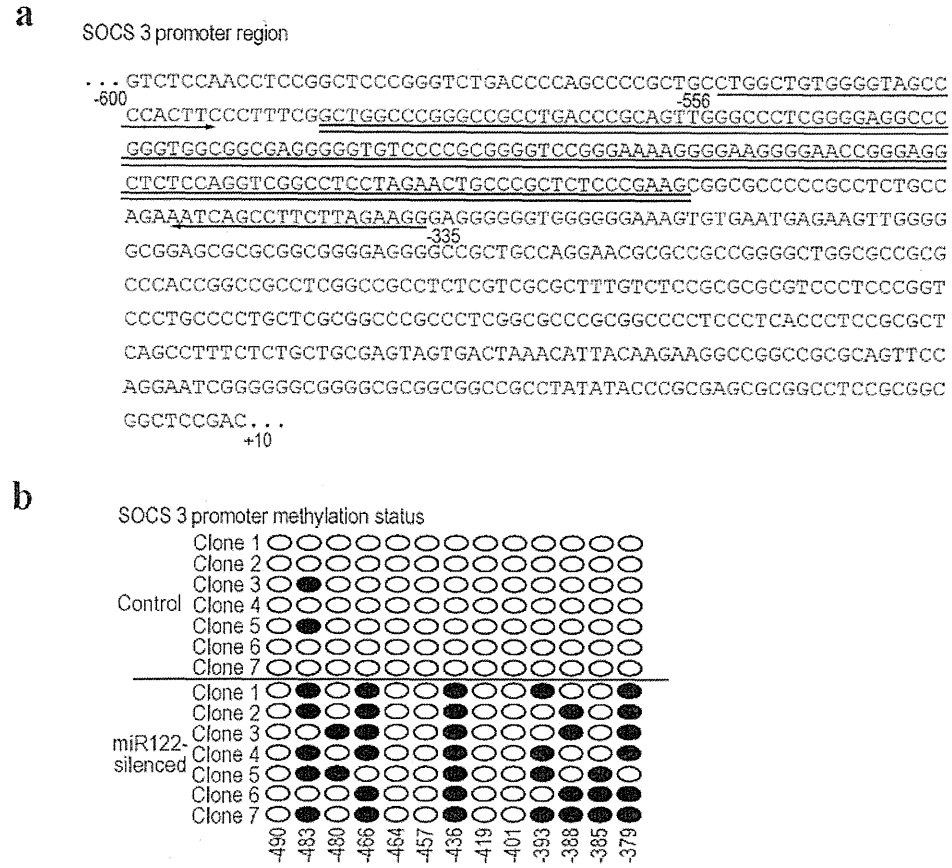

Figure 3 | CpG methylation status in the SOCS3 promoter. (a) The CpG island in the SOCS3 promoter is indicated by double underlines. The numbers are the positions relative to the transcription start site. Primers used for the bisulphate sequences in this study are indicated by arrows. (b) Methylation status in the CpG island of the SOCS3 promoter in control and miR122-silenced Huh7 cells, determined by the bisulphate sequences. Seven clones each were sequenced. Circles represent CpG sites. Black circles, methylated CpG sites.

mostly, if not completely, dependent on the reduced expression of SOCS3.

MiR122 silencing enhances ISGF3-DNA binding. Type I IFNs (IFN-α and -β) activate STATs by phosphorylation, followed by formation of the ISGF3 complex, which is composed of STAT1, STAT2, and IRF9. The importance of ISGF3 in antiviral responses is well established[29]. In contrast, the precise role of STAT3 in type I IFN signaling is not completely understood[30]. However, numerous clinicopathological results suggest that increased SOCS3 expression in the liver is closely related to a poor response to IFN therapy for HCV eradication[8,9]. This in turn suggests that high SOCS3 expression and low STAT3 activation may be related to impaired ISGF3 complex activation. In addition, STAT3 activation supports the ISGF3-dependent induction of antiviral genes *in vitro*[31]. Based on these reports, and because SOCS3 expression was lower in miR122-silenced cells (Fig. 4), we hypothesized that the level of ISGF3 complex after IFN-α treatment is higher in miR122-silenced cells, leading to greater ISRE activation (Fig. 2). While the levels of Oct-1-DNA binding as a loading control were not changed, activation of ISGF3 binding to an ISRE-containing oligonucleotide after IFN-α treatment was significantly greater in miR122-silenced cells (Fig. 6a, b, c), consistent with the fact that ISRE activities were enhanced in miR122-silenced cells in a reporter assay (Fig. 2). These data suggest that miR122-silencing in hepatocytes results in low SOCS3 expression via promoter methylation, which may subsequently enhance the induction of IFN-stimulated gene expression by increasing ISGF3-ISRE binding activities triggered by type I IFN treatment.

Discussion

Although the treatment options for HCV infection are changing due to the introduction of HCV protease inhibitors and DAAs, the principal drug for HCV therapy remains IFN. In this study, we demonstrated that the reduced expression of miR122 contributes to decreased SOCS3 expression via promoter methylation and, subsequently, enhanced ISRE activity results after IFN-α stimulation. These data provide a molecular rationale for, and a method for increasing the efficacy of, IFN therapy for HCV infection.

MicroRNAs are involved in various biologically important intracellular signaling pathways[15–18]. Regarding the convergence of microRNAs and IFN signaling, some microRNAs are reported to be involved in endogenous IFN production in the innate immune response induced by pathogen infection[32]. In addition, several microRNAs that may regulate genes that have anti-pathogen effects are induced by IFN stimulation[33]. In this study, however, the level of miR122 expression seemed to determine the efficacy of the signaling triggered by the exogenous IFN used as an anti-HCV therapy.

MiR122 is the most abundant microRNA in the liver[24], where it has many important biological roles, such as in fatty acid metabolism[26,34] and circadian rhythms[35] under normal conditions. Further, it is also a determinant of the biological aggressiveness of hepatocellular carcinoma[36] in the pathological state. In general, microRNAs act as repressors of target gene expression[37]. However, regarding miR122 and HCV, miR122 somehow enhanced HCV RNA replication in an *in vitro* replicon system[38]. Although the precise molecular mechanisms underlying this phenomenon remain unknown, antisense miR122 has been developed as a therapeutic drug for HCV based

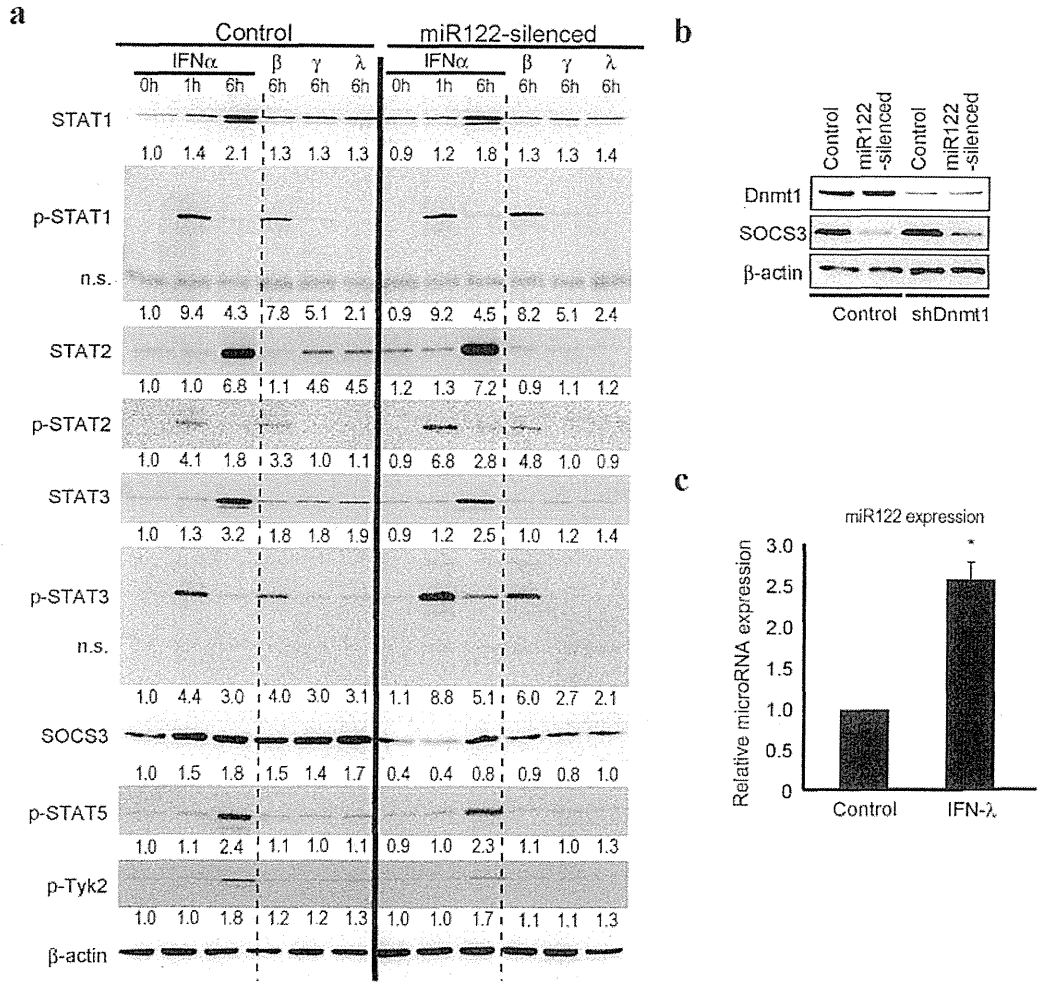

Figure 4 | SOCS3 expression is decreased by miR122 silencing. (a) Protein levels of the indicated IFN signaling-related genes were determined using control and miR122-silenced Huh7 cells after IFN-α, β, γ, or λ stimulation at the indicated time points. A representative of three independent determinations is shown. n.s. indicates non-specific bands. The band intensities were quantitated and adjusted by the expression levels of β-actin. The calculated ratios are indicated below each panel after setting the value of control cells at 0 h as 1.0. (b) Dnmt1 was not involved in the SOCS3 promoter methylation induced by miR122 silencing. SOCS3 protein levels were determined using control, miR122-silenced, Dnmt1 knocked-down, and Dnmt1 knocked-down with miR122-silenced Huh7 cell lysates. A representative of three independent determinations is shown. (c) MiR122 expression levels after IFN-λ stimulation for 6 h in Huh7 cells were determined by quantitative RT-PCR. Results were calculated by normalizing to U6 amounts and the relative ratio was determined by setting the value of unstimulated cells as 1. Data represent the mean ± SD of three independent determinations. *, $p < 0.05$.

on *in vitro* data[39]. Indeed, treatment of chronically HCV-infected chimpanzees with a locked nucleic acid (LNA)-modified oligonucleotide (SPC3649) complementary to miR122 leads to long-lasting suppression of HCV viremia[39]. Development of this drug for human use is now in Phase IIa trials[40]. Because our results showed that lower expression of miR122 leads to augmentation of the intracellular signaling induced by IFN, a combination therapy consisting of an antisense of miR122 and type I IFNs represents a promising and realistic therapeutic option. In addition, because IFN-α/β was reported to suppress miR122 expression, which is considered one of the mechanisms of action of IFN against HCV[41], the effects of exogenous IFN may be self-augmented by the decreased expression of SOCS3 due to decreased miR122 expression. High expression levels of SOCS3 in the liver are negative predictors of IFN treatment of HCV infection[8,9]. This may reflect suppression of IFN signaling by the high miR122 levels in the liver, as suggested by our data.

Our results indicate that reduced miR122 function leads to promoter methylation and decreased SOCS3 expression, which is possibly not the direct target of miR122, because no predictable sites for miR122 interaction in its 3′UTR were found in a computational search. A hypothesis involving an epigenetics-microRNA regulatory circuit has emerged recently[20]. While a number of microRNAs are regulated epigenetically, others simultaneously regulate epigenetic pathway-related molecules. Taken together, these results suggest that post-transcriptional regulation by microRNAs and transcriptional control machinery by epigenetics cooperate to determine the global gene expression profile and to maintain physiological functions in cells[20]. In our genome-wide study, methylation levels of a subset of gene CpG islands were aberrantly induced by miR122 silencing. While our data suggest that SOCS3 methylation was not mediated by Dnmt1, the precise mechanisms of the aberrant methylation induced by miR122, including whether it operates through regulation of the expression of other genes or directly in the nucleus, remains to be elucidated. Nonetheless, our results indicate that aberrant functions of some miRNAs may lead to changes in the methylation levels of a subset of gene CpG islands.

Recent genome-wide association studies have discovered a significant association between the response to pegIFN and ribavirin