on computed tomography (CT) images with 10-mm slices or 10 mm or more on CT images with slices of 5 mm or less.
4. Cases in which a port-catheter system for HAI was placed percutaneously, and arterially infused contrast medium was distributed through the entire liver or at least the entire hepatic lesions and in whom it was confirmed that there was no distribution of the arterially infused contrast medium in the surrounding extrahepatic organs based on CT angiography or MR angiography from the implanted port.
5. Cases aged 20 years or more with an Eastern Cooperative Oncology Group performance status classification of 2 or less.
6. Cases in which major organ function was maintained (white blood cell count $\geq 3000/mm^3$ and $\leq 12{,}000/mm^3$, platelets $\geq 100{,}000/mm^3$, transaminase ≤ 5 times the institution's upper limit of normal, serum total bilirubin ≤ 3.0 mg/dL, serum creatinine ≤ 1.5 mg/dL, electrocardiogram not indicating the need for treatment) and in whom hepatic function was Grade 2 or less on National Cancer Institute-Common Toxicity Criteria (NCI-CTC) (version 2.0) with consideration of the influence of the hepatic lesion.
7. Cases of life expectancy of more than 8 weeks.
8. Cases in which written informed consent was obtained.

Patients excluded from the trial were the patients who scheduled for radiation therapy for the hepatic portal region because of hepatic portal region invasion or lymph node metastasis, or who had previously undergone radiation therapy; patients with concurrent infection excluding viral hepatitis, fever of 38°C or above, or who required antibiotics; patients with serious complications (intestinal paralysis, intestinal obstruction, interstitial pneumonia, pulmonary fibrosis, intractable diabetes mellitus, cardiac failure, renal failure, hepatic failure, etc); patients with other concurrent cancer; patients who could not undergo angiography because of allergy to iodinated contrast material; patients with serious mental disabilities; patients who were pregnant or may have been pregnant, and nursing mothers; and patients whose catheters for HAI chemotherapy were placed via laparotomy.

This study protocol was approved by the ethics committee of the Japanese Society of Interventional Radiology and the institutional review boards of the participating hospitals.

Treatment Protocol and Evaluation Methods

Using a percutaneously placed HAI catheter-port system, 1 course was defined as HAI of GEM on days 1, 8, and 15; a course was performed every 4 weeks for a total of 5 courses.

In phase I portion, the GEM dosage was set at Level −1, 400 mg/m^2; Level 1, 600 mg/m^2; Level 2, 800 mg/m^2; and Level 3, 1000 mg/m^2. Because the approval dosage of GEM is 1000 mg/m^2 in Japan, we defined it as the upper limit in this study. The design called for increase at each level in 3 to 6 patients from Level 1. Three patients were enrolled at each level. The study on the next dose level was not conducted until all 3 patients had completed the first cycle without any problems regarding safety and tolerance. If a DLT of any type was detected in 1 of 3 patients during the first cycle, an additional 3 patients were enrolled. If DLT was detected in more than 2 patients, the dose was defined as the maximum tolerated dose (MTD). RD was estimated to be one level below that judged to be MTD. DLT was defined as follows and judged during the first course: Grade 4 leukopenia or neutropenia; Grade 4 thrombocytopenia; nonhematologic toxicities of Grade 3 or more (excluding that from PD, nausea/vomiting, and alopecia; for patients whose pre-enrollment level of transaminase or serum total bilirubin was Grade 2, DLT was taken to be more than twice the pre-enrollment level); not meeting the criteria to start administration (same as the enrollment criteria) for the next course on day 29 because of toxicity.

In phase II portion, up to 13 patients were added at the dose found to be RD in phase I portion and the tumor response effect was judged using response evaluation criteria in solid tumors. Because HAI was being used, the target lesion was limited to hepatic lesions. Tumor size was measured on intravenous contrast-enhanced CT within 2 weeks before enrollment, and the tumor response effect was judged after the completion of courses 1, 3, and 5, and as needed.

Toxicity assessment was done in all cases using NCI-CTC (version 2.0) and the frequency of the worst grade was obtained during all courses. Physical examination and blood tests were done immediately before the start of each treatment and recorded.

Statistical Analysis

In phase I portion, the number of enrolled patients per level from Level −1 to Level 1 was minimum 6. The maximum number of patients up to Level 3, in case that MTD was reached, was 18 patients in the dose finding stage. In phase II portion, when the threshold tumor response rate was taken to be 20% and the expected efficacy rate was set at 50%, 13 patients would be needed to judge the tumor response effect under conditions of $\alpha = 0.1$ and $\beta = 0.2$, and 7 to 10 cases would need to be added at the estimated RD. For the entire study, a maximum of 25 patients was needed.

RESULTS

Patient Backgrounds

A total of 16 patients were enrolled in the phase I portion (May 2004–November 2005), and 9 patients were added for the phase II portion (February 2006–November 2006). All patients met the eligibility requirements. A summary of all 25 patients is shown in Table 1.

Phase I Portion

In phase I portion, 6 patients were registered at Level 1, 6 at Level 2, and 4 at Level 3. DLT appeared in 2 of the 6 patients at Level 1, and 2 of the 6 patients at Level 2, but DLT did not appear at Level 3. The third and fourth patients at Level 3 were registered at almost the same time. Four patients did not meet the criteria to start administration for the second course on day 29. In these 4 patients, the administration of drugs had been delayed because of Grade 1 and 2 leukopenia (n = 3) or thrombocytopenia (n = 4) in the first course. No Grade 4 hematologic toxicity or nonhematologic toxicity of Grade 3 or more was seen in the first course (Tables 2, 3). MTD was not reached up to Level 3. Accordingly, the RD was assumed to be the Level 3 dose of 1000 mg/m^2.

Phase II Portion

Nine patients were added at GEM 1000 mg/m^2. In these patients, together with the patients at Level 3 in phase I portion (total: 13 patients), the tumor response effect was complete response 0/partial response 1/stable disease 8/progressive disease 3/not evaluated 0 in the liver only, and complete response 0/partial response 1/stable disease 8/progressive disease 4/not evaluated 0 in the whole body. The response rate was 7.7% (95% confidence interval [CI], 0.2%–36.0%). Although disease control was not one of the assessment items, the disease control rate with SD added was 69% (95% CI, 38.6%–90.9%). The tumor response effect and survival in all 25 treated patients are shown in Table 4 and Figure 1.

Toxicity

The incidence of adverse events (NCI-CTC version 2.0) of Grade 3 or more in all treated cases was 20% neutropenia, 8% elevated gamma-glutamyl transpeptidase (GGT), 4% elevated aspartate aminotransferase (AST), 4% elevated alanine aminotransferase (ALT), 4% elevated bilirubin, 4% nausea, and 4% fatigue. The only

TABLE 1. Patients' Characteristics

Phase	Phase I			Phase II	
Level of GEM Dose	Level 1	Level 2	Level 3	Estimated RD	All Patients
GEM dose	600 mg/m^2	800 mg/m^2	1000 mg/m^2	1000 mg/m^2	600, 800, 1000 mg/m^2
No. patients	6	6	4	9	25
Age (yr)					
Median (range)		64 (34–76)		56 (46–74)	58 (34–76)
Gender					
Male	3	5	3	7	18
Female	3	1	1	2	7
ECOG PS					
0	4	5	3	7	19
1	1	1	1	2	5
2	1	0	0	0	1
Previous therapy					
None	4	2	3	4	13
Resection	1	3	1	5	10
Chemotherapy	1	0	1	2	4
Embolization or ablation	0	2	0	1	3
Extrahepatic lesions					
None	3	3	2	8	16
Lymph node	3	3	2	0	8
Peritoneum	1	0	0	0	1
Lung	0	1	2	1	4
Median no. courses administered	5	4.5	4		5
Median no. administrations	15	14	12		15
Relative dose intensity	81.9%	87.3%	84.8%		84.7%

ECOG indicates Eastern Cooperative Oncology Group performance status.

TABLE 2. No. Patients With Hematologic Toxicities (Cycle 1, Phase I Portion, n = 16)

	Level 1 600 mg/m^2 6				Levele 2 800 mg/m^2 6				Level 3 1000 mg/m^2 4			
Level Dose n Grade	1	2	3	4	1	2	3	4	1	2	3	4
Leucocytes	1	2	0	0	1	3	0	0	2	1	0	0
Neutrophils	0	2	1	0	1	1	2	0	1	1	0	0
Hemoglobin	0	1	0	0	0	0	0	0	0	0	0	0
Platelets	2	2	0	0	2	1	0	0	1	1	0	0

Grade 4 event was elevated bilirubin in 1 patient in the second course, but this was accompanied by portal vein tumor thrombosis (Tables 5, 6).

Events related to the HAI procedure included difficulties with the placed catheter-port system in 5 patients (catheter obstruction in 3 patients, port damage in 2 patients), and hepatic artery occlusion in 1 patient. In 2 of the patients with catheter obstruction and the 2 patients with port damage the catheter or port was exchanged and the treatment continued. The remaining patient with catheter obstruction showed an antitumor effect of PD, so the catheter was not replaced and the treatment was stopped. In the patient with hepatic artery occlusion, a left hepatic artery occlusion occurred in the second course, which meant that the drug was not reaching the left lobe of the liver, and the treatment was discontinued.

TABLE 3. No. Patients With Adverse Events (Cycle 1, Phase I Portion, n = 16)

	Level 1 600 mg/m^2 6				Level 2 800 mg/m^2 6				Level 3 1000 mg/m^2 6			
Level Dose n Grade	1	2	3	4	1	2	3	4	1	2	3	4
Nausea	0	2	0	0	2	0	0	0	3	0	0	0
Vomiting	0	1	0	0	0	0	0	0	2	0	0	0
Fatigue	1	1	0	0	3	0	0	0	0	0	0	0
Stomatitis	0	0	0	0	1	0	0	0	0	0	0	0
Headache	0	0	0	0	1	0	0	0	0	0	0	0
Diarrhea	0	0	0	0	0	0	0	0	0	0	0	0
Fever without neutropenia	0	0	0	0	0	0	0	0	1	0	0	0
Anorexia	0	0	0	0	0	0	0	0	0	0	0	0
Alopecia	0	0	0	0	1	0	0	0	0	0	0	0
Alkaline phosphatase	2	0	0	0	1	0	0	0	1	0	0	0
Bilirubin	1	0	0	0	0	0	0	0	0	0	0	0
GGT	1	0	0	0	0	1	0	0	0	0	0	0
Hypoalbuminemia	0	0	0	0	0	0	0	0	1	0	0	0
SGOT (AST)	1	0	0	0	0	0	0	0	1	0	0	0
SGPT (ALT)	0	0	0	0	0	1	0	0	1	0	0	0
Hyperkalemia	0	0	0	0	1	0	0	0	0	0	0	0
Hyponatremia	0	0	0	0	0	0	0	0	1	0	0	0

TABLE 4. Objective Response and Clinical Outcome

GEM Dose	600 mg/m²		800 mg/m²		1000 mg/m² (Phase II)		All Patients	
No. Patients	6		6		13		25	
Evaluation Site	Liver	Whole Body	Liver	Whole Body	Liver	Whole Body	Liver	Whole Body
Best response								
CR	0	0	0	0	0	0	0	0
PR	0	0	2	2	1	1	3	3
SD	4	4	3	3	9	8	16	15
PD	2	2	0	0	3	4	5	6
NE	0	0	1	1	0	0	1	1
Response rate	0%	0%	33.3%	33.3%	7.7%	7.7%	12.0%	12.0%
95% CI	0%–45.9%	0%–45.9%	4.3%–77.7%	4.3%–77.7%	0.2%–36.0%	0.2%–36.0%	2.5%–31.2%	2.5%–31.2%
Disease control rate	66.7%	66.7%	83.3%	83.3%	76.9%	69.2%	76.0%	72.0%
95% CI	22.3%–95.7%	22.3%–95.7%	35.9%–99.6%	35.9%–99.6%	46.2%–95.0%	38.6%–90.9%	54.9%–90.6%	50.6%–87.9%
Median survival time	297 d		298 d		389 d		340 d	
95% CI	140–454 d		0–747 d		158–620 d		198–482 d	

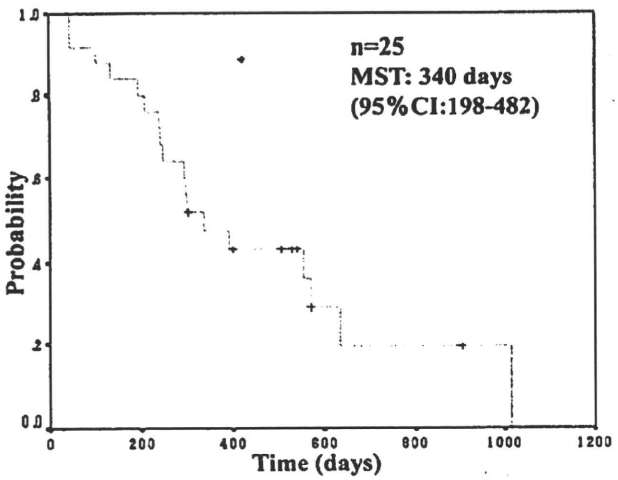

FIGURE 1. Survival time in all 25 patients received hepatic arterial infusion with gemcitabine.

TABLE 5. No. Patients With Hematologic Toxicities (Cycle 1–5, Phase I–II Portion, n = 25)

Dose n	600 mg/m² 6				800 mg/m² 6				1000 mg/m² 13			
Grade	1	2	3	4	1	2	3	4	1	2	3	4
Leucocytes	1	3	0	0	0	4	0	0	4	6	0	0
Neutrophils	0	2	1	0	1	1	2	0	1	7	2	0
Hemoglobin	0	1	0	0	0	1	0	0	2	1	0	0
Platelets	2	2	0	0	2	1	0	0	6	3	0	0

DISCUSSION

ICC originates in the biliary epithelium and is almost always adenocarcinoma. In Japan, it has been reported to account for 5% to 15% of primary hepatic cancers. The only curative treatment is surgical resection. However, at the time of detection, the cancer is often judged to be unresectable because of liver metastasis, vascular invasion, lymph node metastasis, or other distant metastasis.[1–3]

TABLE 6. No. Patients With Adverse Events (Cycle 1–5, Phase I–II Portion, n = 25)

	600 mg/m² 6				800 mg/m² 6				1000 mg/m² 13			
Dose n Grade	1	2	3	4	1	2	3	4	1	2	3	4
Nausea	0	2	0	0	3	0	1	0	7	1	0	0
Vomiting	0	0	0	0	1	1	0	0	3	0	0	0
Fatigue	1	1	0	0	3	0	1	0	3	2	0	0
Stomatitis	0	0	0	0	1	0	0	0	0	0	0	0
Headache	0	0	0	0	1	0	0	0	1	0	0	0
Diarrhea	1	0	0	0	0	0	0	0	0	0	0	0
Fever without neutropenia	0	0	0	0	1	0	0	0	4	1	0	0
Anorexia	0	0	0	0	0	0	0	0	4	1	0	0
Alopecia	0	0	0	0	1	0	0	0	1	0	0	0
Alkaline phosphatase	3	0	0	0	1	0	0	0	2	4	0	0
Bilirubin	1	0	0	0	3	0	0	0	1	1	0	1
GGT	1	0	0	0	0	1	0	0	1	0	2	0
Hypoalbuminemia	0	0	0	0	0	0	0	0	3	2	0	0
SGOT (AST)	1	0	0	0	1	0	0	0	4	2	1	0
SGPT (ALT)	0	0	0	0	1	1	0	0	3	2	1	0
Hyperkalemia	0	0	0	0	1	0	0	0	1	0	0	0
Hyponatremia	0	0	0	0	1	0	0	0	1	0	0	0

Chemotherapy is the treatment option for unresectable ICC but no standard therapy has been established.[4,5] Multiagent treatment has been reported with drugs such as 5-FU, mitomycin C (MMC), adriamycin, and epirubicin hydrochloride similar to biliary tract cancer (extrahepatic bile duct cancer, gallbladder cancer). Combined use of cisplatin and 5-FU is reportedly effective but all of these reports are from case studies only.[11,12] HAI chemotherapy has also been attempted for unresectable intrahepatic bile duct cancer and regimens such as FAM (5-FU + adriamycin + MMC), FEM (5-FU + epirubicin hydrochloride + MMC), high-dose 5-FU, and low-dose FP (5-FU + cisplatin) have been reported to be effective.[13] Again, however, all of these reports are from case studies only.

A new anticancer agent of GEM has been introduced for pancreatic cancer and biliary tract cancer, which has no standard therapy like ICC.[6] For pancreatic cancer chemotherapy, it is the drug of choice.[14,15] In treating ICC with GEM, good results were reported in 2001 from a phase II trial in Germany in which the tumor response effect was reported to be 30% and the median survival time (MST) was 9.3 months.[16] Because ICC is classified as a primary hepatic cancer in Japan, HAI of GEM has also been attempted. Tsujino et al performed HAI of GEM at the recommended dose of 1000 mg/m^2 with intravenous infusion, and they observed tumor size and tumor marker reductions.[17]

Whereas no consensus has been reached with regard to the contribution of HAI to extending survival in cases of hepatic metastasis of colorectal cancer, the local tumor response effect is considered to be superior to that with systemic chemotherapy.[18–20] Moreover, in hepatocellular carcinoma which is a primary hepatic cancer like ICC, the intra-arterial local therapy for hepatic arterial chemoembolization is thought to significantly prolong survival in unresectable cases compared with the results of symptomatic treatment.[21,22] It is possible that local therapy can also prolong survival in cases of ICC.

This study was designed with consideration of the above to establish the DLT for HAI of GEM and estimate the RD; the tumor response effect with the estimated RD was then determined and safety was evaluated. In phase 1 portion, GEM was increased from 600 mg/m^2 to 800 mg/m^2 and 1000 mg/m^2. A delay in the start of the second course because of Grade 1 and 2 leukopenia or thrombocytopenia as DLT was seen in 4 cases (25%). MTD was not reached up to dosage Level 3. Thus, RD was estimated to be 1000 mg/m^2, and more patients were added in phase II portion.

The incidence of adverse events of Grade 3 or more in all courses was 20% neutropenia, 8% elevated GGT, 4% elevated AST, 4% elevated ALT, 4% elevated bilirubin, 4% nausea, and 4% fatigue. The only Grade 4 event was elevated bilirubin in 1 case during the second course. However, this was a case of portal vein tumor thrombosis, which was thought to have caused the elevated bilirubin. Toxicity with HAI of GEM was generally tolerable throughout all courses and it was milder than in reports of systemic administration.[23]

Events related to the HAI itself or the implanted catheter-port system occurred in 6 cases (24%). Most were dealt with by replacing the port in order that HAI could be continued. Hepatic artery occlusion occurred in only 1 case. Compared with other reports,[8–10] more of the present cases were within the tolerable range. No catheter or port infection or induced thrombosis was observed.

The response rate of HAI of GEM at the estimated RD of 1000 mg/m^2 in 13 cases of unresectable ICC was 7.7% (CR, n = 0; PR, n = 1), which was below the established threshold efficacy rate of 20%. Although disease control was not one of the items investigated in this study, the disease control rate including SD (n = 8) was 69% and MST in all 25 patients was 340 days (95% CI: 198–482 days).

In conclusion, DLT was the delay in the start of the second course because of Grade 1 and 2 leukopenia or thrombocytopenia and RD was estimated to be 1000 mg/m^2 in HAI of GEM for unresectable ICC. Toxicity was within the tolerable range. However, the tumor response effect of HAI of GEM at 1000 mg/m^2 was low, and it was judged that no improvement in treatment results can be expected with HAI. The disease control rate and MST were acceptable, but, considering that the subjects in this study were patients whose hepatic lesions were predominant and that the implanted catheter-port system was required for HAI as a painful procedure, it cannot be claimed that this protocol has an advantage over systemic treatment.

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Insulin-like growth factor-binding protein 7 alters the sensitivity to interferon-based anticancer therapy in hepatocellular carcinoma cells

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BACKGROUND: A striking efficiency of interferon (IFN)-based anticancer therapy for advanced hepatocellular carcinoma (HCC) has been reported. Because its clinical efficiency greatly depends on each patient's local response, prediction of local response is crucial.
METHODS: Continuous exposure of IFN-α to parental PLC/PRF/5 cells (PLC-P) and a limiting dilution method resulted in the establishment of IFN-resistant cell clones (PLC-Rs). Microarray analyses of PLC-P and PLC-Rs identified insulin-like growth factor-binding protein 7 (IGFBP7) as one of the most significantly downregulated genes in PLC-Rs. Changes in anticancer effects of IFN-α were examined in HCC cells after genetic manipulation of IGFBP7 expression. The correlation between immunohistochemically determined IGFBP7 expression and the response to IFN-α/5-fluorouracil (5-FU) therapy was investigated in surgically resected HCC specimens.
RESULTS: PLC-R cells showed a remarkable downregulation of IGFBP7 and resistance to IFN-α, compared with PLC-P. Parental PLC/PRF/5 cells transfected with short hairpin RNA against IGFBP7 showed a significant resistance to IFN-α relative to control cells (IC_{50} fold increase = 14.38 times). Insulin-like growth factor-binding protein 7 transfection into PLC-R restored sensitivity to IFN-α. In resected specimens, IGFBP7 expression significantly correlated with the response to IFN-α/5-FU therapy.
CONCLUSION: IGFBP7 could be a useful predictor of the response to IFN-based therapy in advanced HCC.
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The prognosis of patients with advanced hepatocellular carcinoma (HCC) remains poor, particularly in patients with tumour thrombi in the major trunk of the portal vein, even after curative resection of the tumour (Tanaka *et al*, 1996; Yamakado *et al*, 1999; Asahara, 1999 no. 47). In such a situation, conventional therapies have no clinical impact because of poor efficacy and possible complications (Furuse *et al*, 1997; Lee *et al*, 1997). Therefore, a new strategy is required for patients with advanced HCC.

Several studies have reported strong antitumour activity of interferon (IFN)-based combination chemotherapy to HCC, irrespective of the lack of satisfactory results of IFN-α monotherapy (Urabe *et al*, 1998; Leung *et al*, 1999; Chung *et al*, 2000; Patt *et al*, 2003; Obi *et al*, 2006). We have also reported the clinical efficiency of IFN-α and 5-fluorouracil (5-FU) (IFN-α/5-FU) therapy for advanced HCC and the underlying mechanisms of antitumour effects (Eguchi *et al*, 2000; Kondo *et al*, 2000, 2005; Sakon *et al*, 2002; Yamamoto *et al*, 2004; Ota *et al*, 2005; Nakamura *et al*, 2007; Wada *et al*, 2007, 2009; Damdinsuren *et al*, 2007a, b; Nagano *et al*, 2007a, b). These previous studies showed that IFN-α suppresses the proliferation of HCC cells that express type I IFN receptor type 2 (IFNAR2), and that the expression of IFNAR2 in HCC tissues was significantly associated with a clinical response to IFN-α/5-FU therapy, suggesting that IFNAR2 expression might be useful in predicting the clinical response to such therapy (Ota *et al*, 2005; Nagano *et al*, 2007a). However, even a portion of patients expressing IFNAR2 showed resistance to the therapy, indicating the necessity of finding novel biological markers that can more accurately predict the clinical response to IFN-α/5-FU therapy. Because the clinical outcome between responders and non-responders is markedly different, and to avoid the potentially debilitating adverse effects of this therapy in non-responders, finding the predictive biomarker is crucial.

In this study, IFN-resistant HCC cell clones were established and an oligonucleotide microarray analysis was applied to these IFN-resistant cells and their parental cells. The microarray analysis identified that insulin-like growth factor (IGF)-binding protein 7 (IGFBP7), which has been reported to have a tumour-suppressive activity through the induction of apoptosis in some cancers, was a key gene related to the response to this therapy (Burger *et al*, 1998; Landberg *et al*, 2001; Mutaguchi *et al*, 2003; Sato *et al*, 2007; Lin *et al*, 2008; Wajapeyee *et al*, 2008). Furthermore, we confirmed that IGFBP7 significantly correlated with the response to IFN-α/5-FU therapy in genetic manipulation experiments and to the clinical

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response in HCC tissue samples. These results indicate that IGFBP7 could be a suitable marker for predicting the clinical response to IFN-α/5-FU therapy.

MATERIALS AND METHODS

Cell lines

Human HCC cell lines, PLC/PRF/5 and HLE, were obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan), and Hep3B was obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin at 37°C in a humidified incubator with 5% CO_2 in air.

Establishment of IFN-resistant cells

Parental PLC/PRF/5 cells (PLC-P) were exposed to IFN-α at an initial concentration of 50 IU ml^{-1}. At 2 weeks after exposure, surviving cells were continuously exposed to sequentially increasing doses of 100 IU ml^{-1} (2 weeks), 200 IU ml^{-1} (2 weeks), 500 IU ml^{-1} (2 weeks), 1000 IU ml^{-1} (2 weeks), and 2000 IU ml^{-1}. Through this process, we successfully established IFN-resistant cells. By limiting the dilution of the established cells, 10 clones of PLC/PRF/5 cells resistant to IFN-α were established. The clones were confirmed as being resistant to IFN-α stably over 20 passages. Among the 10 clones, three clones (PLC-Rs; PLC-R1, PLC-R2, and PLC-R3) were used in the experiments of this study.

Drugs and reagents

Purified human IFN-α was kindly supplied by Otsuka Pharmaceutical Co. (Tokyo, Japan) and 5-FU and doxorubicin (DXR) by Kyowa Hakko Kirin Co. (Tokyo, Japan). Cisplatin (CDDP), insulin, and IGF-1 were purchased from Nippon Kayaku Co. (Tokyo, Japan), Sigma-Aldrich Co. (St Louis, MO, USA), and Peprotech (Rocky Hill, NJ, USA), respectively. As for primary antibodies, polyclonal goat anti-human IGFBP7 antibody and polyclonal rabbit anti-human IGFBP7 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) were used for immunohistochemistry and western blot analysis, respectively. Antibodies to IFNAR2 and phosphotyrosine (p-Tyr) were purchased from Santa Cruz Biotechnology Inc; antibodies to signal transducer and activator of transcription factor (STAT) 1, phosphorylated (Tyr 701) STAT (pSTAT) 1, Akt, and phosphorylated (Ser 473) Akt were from Cell Signaling Technology (Beverly, MA, USA); antibodies to STAT2, pSTAT2, and insulin receptor substrate-1 (IRS-1) were from Millipore (Milford, MA, USA); and antibody to actin was from Sigma-Aldrich Co.

Plasmid and transfection

Plasmid coding for short hairpin RNA (shRNA) against *IGFBP7* and *IGFBP7* expression plasmids was purchased from OriGene Technologies Inc. (Rockville, MD, USA). They were transfected into HCC cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions provided by the manufacturer. After transfection of the shRNA plasmid and *IGFBP7* expression plasmid, stable transfectants were selected and maintained by adding 1.0 μg ml^{-1} of puromycin (Sigma-Aldrich Co.) and 600 μg ml^{-1} of G418 (Gibco-BRL, Grand Island, NY, USA), respectively. The control vector plasmid expressing non-effective shRNA was similarly introduced into cells to establish negative control cells for the shRNA plasmid. Empty vector plasmid was also similarly used to establish negative control cells for the *IGFBP7* expression plasmid. Successful transfection was confirmed by the coexpression of GFP.

PLC-P transfected by shRNA plasmid against *IGFBP7* and by the negative control vector plasmid was named as PLC-P/shRNA (no. 1 and no. 2) and PLC-P/shRNA-NC, respectively. Short hairpin RNA no. 1 and no. 2 were different in sequence to shRNA. The PLC-Rs transfected with the *IGFBP7* expression plasmid and the negative control vector plasmid were named PLC-Rs/IGFBP7 and PLC-Rs/IGFBP7-NC, respectively.

Patients and specimens

The study subjects were 30 patients with advanced HCC and recruited as described previously (Nagano *et al*, 2007a). All patients had multiple liver tumours in both lobes and tumour thrombi in the main trunk of the portal vein, and each underwent palliative reduction surgery with tumour thrombectomy of the main trunk of the portal vein at the Osaka University Hospital between October 1999 and December 2004. The IFN-α/5-FU therapy for remnant multiple liver tumours was applied postoperatively, as described previously (Ota *et al*, 2005; Nagano *et al*, 2007a). Patients were followed up after surgery, with a postoperative follow-up period of 18.2 ± 19.7 months. Clinical response to therapy was evaluated according to the criteria of the Eastern Cooperative Oncology Group (Oken *et al*, 1982). On the basis of the clinical response, responders were defined as patients with a complete response or partial response and non-responders were defined as patients with a stable disease or progressive disease. The study protocol was approved by the Human Ethics Review Committee of Osaka University Hospital and a signed consent form was obtained from each patient.

Real-time quantitative reverse transcription-PCR

For reverse transcriptase reaction, the extracted RNA, random hexamers, and Superscript II reverse transcriptase (Invitrogen) were used according to the instructions supplied by the manufacturer. Real-time quantitative reverse transcription-PCR (qRT-PCR) was performed using designed oligonucleotide primers and Light Cycler (Roche Diagnostics, Mannheim, Germany), and the amount of target gene expression was calculated. The expression of the target gene was normalised relative to the expression of *porphobilinogen deaminase* (*PBGD*), which was used as an internal control. The designed PCR primers were as follows: *IGFBP7* forward primer 5′-CTGGGTGCTGGTATCTCCTC-3′, *IGFBP7* reverse primer 5′-TATAGCTCGGCACCTTCACC-3′; *PBGD* forward primer 5′-TGTCTGGTAACGGCAATGCGGCTGCAAC-3′, *PBGD* reverse primer 5′-TCAATGTTGCCACCACACTGTCCGTCT-3′.

Microarray experiments

Microarray experiments were conducted according to the method described previously (Noda *et al*, 2009). In brief, total RNA was purified by TRIzol reagent (Invitrogen) according to the instructions provided by the manufacturer. The integrity of the purified RNA was assessed as being of high quality by Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and RNA 6000 LabChip kits (Yokokawa Analytical Systems, Tokyo, Japan). The purified RNAs obtained from PLC-P, PLC-R1, PLC-R2, and PLC-R3 were used as samples, and all samples were examined in duplicate. The samples were mixed and hybridised on a microarray covering 30 336 human probes (AceGene Human 30 K; DNA Chip Research Inc and Hitachi Software Engineering Co, Kanagawa, Japan). The ratio of the expression level of each gene was converted into a logarithmic scale (base 2) and the data matrix was normalised. In each sample, genes with missing values in more than two samples were excluded from the analysis. A total of 28 761 genes out of 30 336 genes were finally available for the analysis.

Western blot analysis

Cells grown to semiconfluence were lysed in RIPA buffer (25 mM Tris (pH 7.5), 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulphate, 1 mM phenylmethylsulphonyl fluoride, 1.6 $\mu g\,ml^{-1}$ aprotinin). Western blot analysis was carried out as described previously (Kondo *et al*, 2005).

Growth inhibitory assay

The growth inhibitory assay was assessed by the 3-(4-, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich Co.) assay, as described previously (Eguchi *et al*, 2000). Briefly, cells were incubated for 72 h under several concentrations of IFN-α and 5-FU. After reincubation for 4 h with MTT solution, acid–isopropanol mixture was added to dissolve the resultant formazan crystals. The absorbance of the plate was measured in a microplate reader at a wavelength of 570 nm with a 650 nm reference, and the results were expressed as a percentage of absorbance relative to that of untreated controls.

Annexin V assay

The binding of annexin V was used as a sensitive method for measuring apoptosis, as described previously (Nakamura *et al*, 2007). At 24 h after treatment with IFN-α, PLC-P/shRNA and PLC-P/shRNA-NC cells were stained by Annexin V-APC and propidium iodide (PI) (BD Biosciences, Franklin Lakes, NJ, USA), and analysed on a FACS Aria (BD Biosciences). Annexin V-positive and PI-negative cells, considered as early apoptotic cells, were used for the assessment of apoptosis in this study (Lugli *et al*, 2005).

Measurement of caspase activities

Caspase-3, caspase-8, and caspase-9 activities were measured using caspase-3, caspase-8, and caspase-9 colorimetric assay kits (Chemicon International Inc, Temecula, CA, USA). The measurement was performed in cell lysates obtained from each cell 24 h after treatment with IFN-α, using the instructions provided by the manufacturer.

IRS-1 immunoprecipitation

After incubation for 12 h in serum-free medium, cells were stimulated with 1 nM insulin or 10 nM IGF-1. The stimulated cells were lysed in lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1.0% Triton-X-100, 1.0 mM EGTA, 1 mM phenylmethylsulphonyl fluoride, 1.6 $\mu g\,ml^{-1}$ aprotinin, 10 $\mu g\,ml^{-1}$ leupeptin). Solubilised proteins were immunoprecipitated with anti-IRS-1 antibody, and tyrosine phosphorylation was detected with anti-p-Tyr antibody.

Immunohistochemical staining

Immunohistochemical staining for IGFBP7 in 30 HCC samples was performed by the method described previously (Kondo *et al*, 1999). Briefly, formalin-fixed, paraffin-embedded 4 μm-thick sections were deparaffinised in xylene, then treated with an antigen retrieval procedure and incubated in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase. After incubation with normal protein block serum, the sections were incubated overnight at 4°C with an anti-IGFBP7 antibody as the primary antibody. Thereafter, the sections were incubated with a biotin-conjugated secondary antibody (horse anti-goat antibody for IGFBP7) and with peroxidase-conjugated streptavidin. The peroxidase reaction was then developed with 0.02% 3, 30-diaminobenzidine tetrachloride (Wako Pure Chemicals, Osaka, Japan) solution with 0.03% hydrogen peroxide. Finally, the sections were counterstained with Meyer's haematoxylin. The IGFBP7 expression was defined as the presence of specific staining in the cytoplasm of cancer cells. Insulin-like growth factor-binding protein 7 expression was evaluated as positive or negative. Two investigators (Y.T. and H.E.) independently assessed IGFBP7 expression without knowledge of the corresponding clinicopathological data. The assessments were similar by the two investigators for all samples.

Statistical analysis

Data are expressed as mean ± s.d. Clinicopathological parameters were compared using the χ^2-test and continuous variables were compared using Student's *t*-test. Survival curves were computed using the Kaplan–Meier method, and differences between survival curves were compared using the log-rank test. A *P*-value <0.05 denoted the presence of a statistically significant difference. Statistical analysis was performed using StatView (version 5.0, SAS Institute Inc, Cary, NC, USA).

RESULTS

Characteristics of established IFN-resistant cells

The morphology of PLC-Rs resembled that of PLC-P. Although PLC-Rs showed similar growth curves compared with PLC-P in the absence of IFN-α (data not shown), PLC-Rs were significantly resistant to IFN-α compared with PLC-P, which was confirmed by MTT assays (Figure 1A). The expression levels of IFNAR2 were not different between PLC-P and PLC-Rs (Figure 1B). The protein level of STAT1 and STAT2, which directly bind to the intracellular domain of IFNAR2 and function as key molecules for signal transduction, was also not different between PLC-P and PLC-Rs treated with 1000 IU ml^{-1} of IFN-α for 20 min (Figure 1B). Moreover, the phosphorylation of STAT1 and STAT2 (pSTAT1 and pSTAT2), active forms of STATs, were also not different between these cells.

IGFBP7 is significantly downregulated in IFN-resistant cells

To investigate the candidate genes involved in the response to IFN-α, microarray analysis was carried out with PLC-P and PLC-Rs. The analysis showed that, among the 28,761 genes, 579 (2.0%), 646 (2.2%), and 567 genes (2.0%) altered more than 1.5-fold in PLC-R1, PLC-R2, and PLC-R3, respectively. As shown in Figure 1C, 107 genes including 92 upregulated genes and 15 downregulated genes (listed in Supplementary Table S1) were common among the above 579, 646, and 567 genes. Among these 107 genes, *IGFBP7* was identified as one of the most downregulated genes with a 2.963-fold decrease. The downregulation of IGFBP7 in PLC-Rs compared with PLC-P was validated by real-time qRT-PCR and western blot analysis (Figure 1D).

Knockdown of *IGFBP7* induces resistance to IFN-α

To evaluate the biological effect of *IGFBP7*, two kinds of plasmids coding for shRNA against IGFBP7 (no. 1 and no. 2) were transfected into PLC-P and named as PLC-P/shRNA no. 1 and PLC-P/shRNA no. 2. The IGFBP7 expression was suppressed at both mRNA and protein levels in the established PLC-P/shRNAs, which was confirmed by qRT-PCR and western blot analysis, respectively (Figure 2A). The MTT assay showed that PLC-P/shRNAs were significantly more resistant to IFN-α than PLC-P/shRNA-NC (Figure 2B). On the basis of the measurement of IC_{50}, the fold increase of IC_{50} to IFN-α was much larger than that to other drugs, including 5-FU, CDDP, and DXR, suggesting that chemoresistance acquired by IGFBP7 is specific to IFN-α (Table 1). IFNAR2, STAT1, and STAT2 were similarly expressed in PLC-P/shRNA and PLC-P/shRNA-NC, and the IFN-α-induced pSTAT1

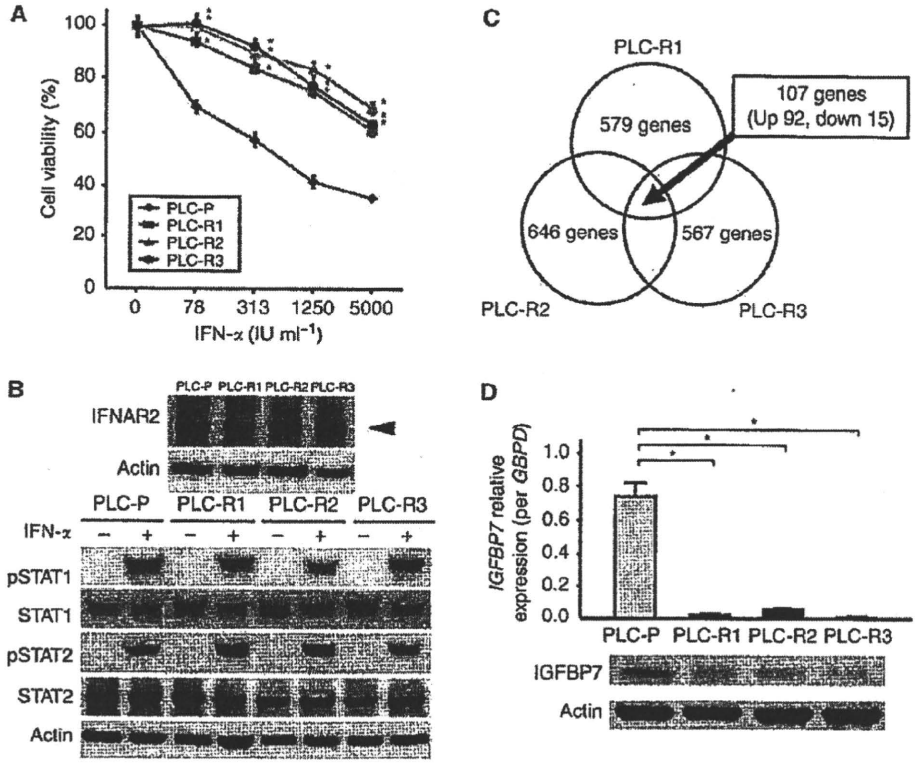

Figure 1 Characteristics of IFN-resistant PLC/PRF/5 cell clones (PLC-Rs). **(A)** MTT assay showed that the antitumour effect of interferon-α (IFN-α) in PLC-Rs was significantly lower than that in parental PLC/PRF/5 cells (PLC-P). Data are mean ± s.d. $^*P<0.05$ compared with PLC-P. **(B)** Western blot analysis revealed that the expression levels of type I IFN receptor type 2 (IFNAR2), signal transducer and activator of transcription factor 1 (STAT1), STAT2, phosphorylated STAT1 (pSTAT1), and pSTAT2 were similar in PLC-P and PLC-Rs. **(C)** Schematic of the results of the performed microarray analysis and identified 107 genes. The 107 genes were up- or downregulated by more than 1.5-fold and were commonly identified in the three types of cells. **(D)** Quantitative reverse transcriptase-PCR and western blot analysis confirmed the significant suppression of insulin-like growth factor-binding protein 7 (IGFBP7) expression in PLC-Rs compared with PLC-P. Data are mean ± s.d. $^*P<0.05$.

and pSTAT2 expressions were also not similar in the two cells (Supplementary Figure S1A).

As IGFBP7 has been shown to suppress tumour activity through induction of apoptosis (Landberg *et al*, 2001; Mutaguchi *et al*, 2003; Sato *et al*, 2007; Wajapeyee *et al*, 2008), we evaluated the extent of apoptosis induced at 24 h after treatment of PLC-P/shRNA with 1000 IU ml^{-1} IFN-α. Annexin V assay using flow cytometry showed a significantly lower percentage of early apoptotic cells in PLC-P/shRNA than in PLC-P/shRNA-NC (Figure 3C). Moreover, the activity of caspase-3, caspase-8, and caspase-9 induced by IFN-α in PLC-P/shRNA was significantly lower than that by PLC-P/shRNA-NC (Figure 3D). A plasmid coding for shRNA against IGFBP7 was transfected in other liver cancer cell lines (HLE and Hep3B). Both these cell lines showed downregulated *IGFBP7* expression (Supplementary Figure S2A). The transfected HLE and Hep3B cells were also resistant to IFN-α treatment (500 IU ml^{-1}) (Supplementary Figure S2B).

As IGFBP7 has been reported to bind insulin and IGF (Oh, 1998; Subramanian *et al*, 2007), it could be conceivable that IGFBP7 induces resistance by interfering with insulin and/or IGF signalling. To verify this possibility, we examined the effect of IGFBP7 on the phosphorylation of IRS-1 and Akt, major transducers of insulin and IGF signalling. As shown in Supplementary Figure S1B, there were no significant differences in the phosphorylation of IRS-1 or Akt between PLC-P/shRNA and PLC-P/shRNA-NC. This result suggests that IGFBP7-related resistance occurs in an insulin- and IGF-independent manner.

Transfection of IGFBP7 restores sensitivity to IFN-α

Next, *IGFBP7* expression plasmid was transfected into PLC-R1 (PLC-R1/IGFBP7). Upregulation of IGFBP7 in PLC-R1/IGFBP7 compared with PLC-R1/IFGBP7-NC was confirmed by qRT-PCR and western blot analysis (Figure 3A). By the MTT assay, PLC-R1/IGFBP7 partially but significantly restores sensitivity to IFN-α compared with PLC-R1/IGFBP7-NC (Figure 3B).

IGFBP7 is a useful predictor of clinical response to IFN-α/5-FU therapy

To confirm whether IGFBP7 expression is associated with the clinical response to IFN-α/5-FU therapy, HCC samples of 30 patients who underwent IFN-α/5-FU therapy postoperatively were immunohistochemically stained for IGFBP7 expression. Whereas the expression levels of IGFBP7 in cancerous lesions varied among the patients, a homogenous staining for IGFBP7 was observed in the cytoplasm of cells in non-cancerous sections (Figure 4). Among the 30 patients examined, 12 (40.0%) showed positive staining, whereas 18 (60.0%) patients were negative for IGFBP7. Of the IGFBP7-positive patients, 66.7% (8 of 12) were histologically evaluated as responders to the therapy, whereas only 11.1% (2 of 18) of IGFBP7-negative patients were responders, suggesting that IGFBP7 expression was significantly associated with response to therapy ($P<0.05$) (Table 2). The sensitivity, specificity, and accuracy for the prediction to IFN-α/5-FU therapy by IGFBP7

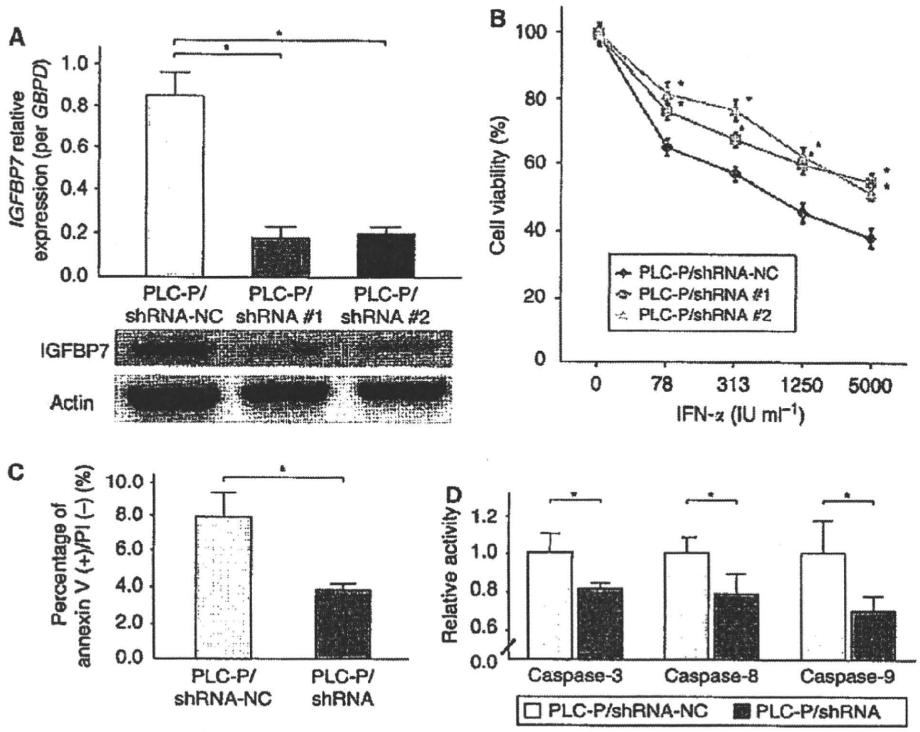

Figure 2 Characteristics of parental PLC/PRF/5 cell (PLC-P)/short hairpin RNA (shRNA) (no. 1 and no. 2). **(A)** Insulin-like growth factor-binding protein 7 (IGFBP7) was confirmed to be significantly suppressed in PLC-P/shRNA compared with PLC-P/shRNA-negative control (NC) in quantitative reverse transcriptase-PCR and western blot analysis. **(B)** MTT assay revealed that PLC-P/shRNA was significantly more resistant to interferon-α (IFN-α) than was PLC-P/shRNA-NC. **(C)** The percentage of early apoptotic PLC-P/shRNA cells assessed by annexin V assay was significantly lower than that of PLC-P/shRNA-NC. **(D)** The activity of caspase-3, caspase-8, and caspase-9 induced by IFN-α in PLC-P/shRNA was significantly lower than that in PLC-P/shRNA-NC. Data are mean ± s.d. *$P<0.05$.

Table 1 IC_{50} for IFN-α, 5-FU, CDDP, and DXR in PLC-P/shRNA-NC and PLC-P/shRNA

Drug	IC_{50} PLC-P/ shRNA-NC	IC_{50} PLC-P/ shRNA	Fold increase (shRNA/shRNA-NC)
IFN-α (IU ml^{-1})	807 ± 96.5	11 608.0 ± 1179.4	14.38
5-FU (μg ml^{-1})	41.4 ± 5.5	53.1 ± 10.3	1.28
CDDP (μg ml^{-1})	3.9 ± 0.3	4.8 ± 0.4	1.24
DXR (μg ml^{-1})	1.4 ± 0.2	2.2 ± 0.4	1.52

Abbreviations: 5-FU = 5-fluorouracil; CDDP = cisplatin; DXR = doxorubicin; IC = inhibitory concentration; IFN-α = interferon-α; NC = negative control; PLC-P = Parental PLC/PRF/5 cell; sh-RNA = short hairpin RNA. Data are mean ± s.d.

were 80.0% (8 of 10), 80.0% (16 of 20), and 80.0% (24 of 30). None of the other clinicopathological factors tested, apart from IFNAR2, were associated with response to the therapy (Supplementary Table S2).

Finally, we examined the correlations between postoperative prognosis and various clinicopathological factors including IGFBP7 status. The postoperative overall survival in IGFBP7-positive patients was significantly better than that in IGFBP7-negative patients ($P<0.05$, Figure 5). Furthermore, multivariate analysis of overall survival using two significant factors identified in the univariate analyses showed that, in addition to IFNAR2, IGFBP7 status was an independent and significant determinant of overall survival (Table 3), indicating that IGFBP7 is a potentially useful marker for the prediction of clinical response to IFN-α/5-FU therapy.

DISCUSSION

In this study, gene expression profiling identified significant suppression of IGFBP7 in PLC-Rs compared with PLC-P. Insulin-like growth factor-binding protein 7, also known as IGFBP-rP1 and MAC25, can inhibit the proliferation of cancer cells, and its expression is downregulated in certain cancers (Burger *et al*, 1998; Landberg *et al*, 2001; Mutaguchi *et al*, 2003; Sato *et al*, 2007; Lin *et al*, 2008; Wajapeyee *et al*, 2008). It is also reported that IGFBP7 suppression is associated with rapid tumour growth and tumour invasiveness (Burger *et al*, 1998; Sato *et al*, 2007; Lin *et al*, 2008). However, there are no reports of the association between IGFBP7 expression and sensitivity to chemotherapeutic drugs.

In this study, IGFBP7 was suppressed by shRNA transfection in HCC cells and the transfected cells acquired resistance to IFN-α. The association between IGFBP7 expression and response to IFN-α was also confirmed in experiments using IGFBP7-overexpressing cells. Considering that IGFBP7-suppressed cells showed a smaller percentage of apoptosis than control cells, the acquired resistance was thought to result from the impediment of apoptosis. The suppression of apoptosis by downregulation of IGFBP7 was consistent with that found in previous studies (Burger *et al*, 1998; Landberg *et al*, 2001; Mutaguchi *et al*, 2003; Sato *et al*, 2007; Lin *et al*, 2008; Wajapeyee *et al*, 2008). In addition to resistance to IFN-α, IGFBP7-suppressed cells showed modest but significant resistance to other drugs. Taking into consideration the fact that IGFBP7 promotes apoptosis even in the absence of any drugs, the acquisition of resistance to both IFN-α and other drugs may be quite natural. However, the fold increase in acquired resistance to IFN-α was much larger than that to other drugs as confirmed by measurements of IC_{50}, suggesting that IGFBP7 is specifically

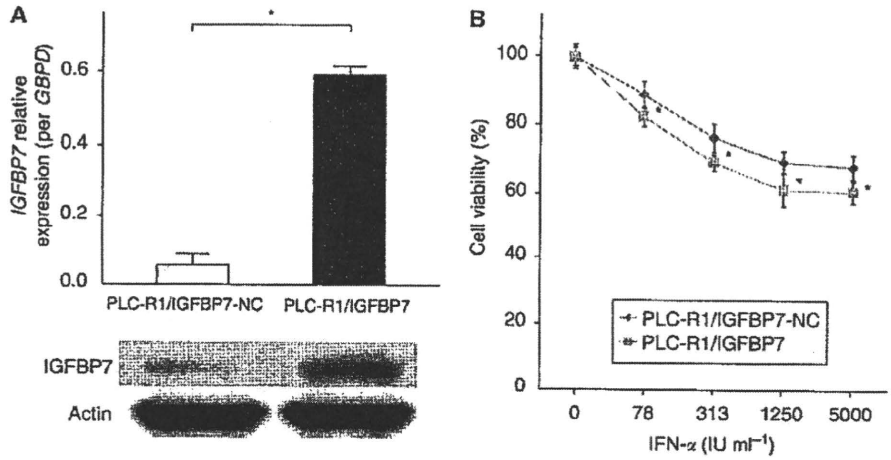

Figure 3 Characteristics of IFN-resistant PLC/PRF/5 cell clones (PLC-R1) transfected with *Insulin-like growth factor-binding protein 7* (*IGFBP7*) expression plasmid. (**A**) Quantitative reverse transcriptase-PCR and western blot analysis showed that the IGFBP7 expression level in PLC-R1/IGFBP7 was significantly higher than that in PLC-R1/IGFBP7-negative control (NC). (**B**) MTT assay showed that PLC-R1/IGFBP7 were significantly more sensitive to IFN-α than was PLC-R1/IGFBP7-NC. Data are mean ± s.d. $^*P<0.05$.

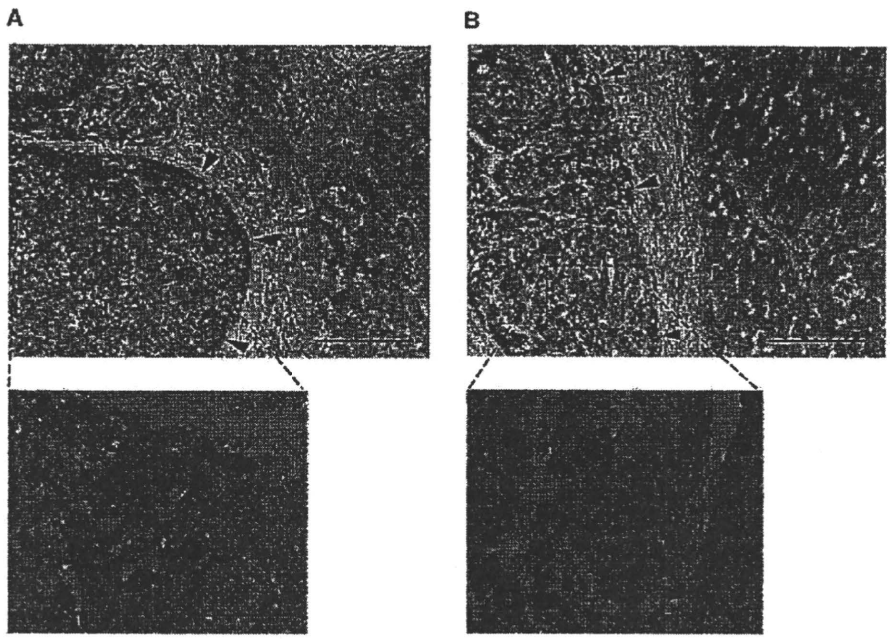

Figure 4 Immunohistochemistry for Insulin-like growth factor-binding protein 7 (IGFBP7) in representative hepatocellular carcinoma cases (**A**) A representative IGFBP7-positive case. The IGFBP7 expression was shown in the cytoplasm of normal liver cells and in the majority of tumour cells. (**B**) A representative IGFBP7-negative case. The IGFBP7 expression was not identified in tumour cells. Upper panel, low-power field (Bar = 200 μm); lower panel, high-power field (Bar = 50 μm); T, tumour lesion (arrowheads); N, non-tumour lesion.

Table 2 Association between immunohistochemically determined IGFBP7 expression and clinical response to IFN-α/5-FU therapy

	Responders	Non-responders	*P*-value
IGFBP7(+)	8	4	0.0057
IGFBP7(−)	2	16	

Abbreviations: 5-FU = 5-fluorouracil; IFN-α = interferon-α; IGFBP7 = insulin-like growth factor-binding protein 7.

related to the resistance to IFN-α. Moreover, from the experiments of insulin- and IGF signalling, this effect of IGFBP7 was suggestive to occur in an insulin- and IGF-independent manner.

Furthermore, to clarify the mechanism of IGFBP7-specific IFN resistance, we examined IFNAR2 expression and IFN signalling and compared them between PLC-P and PLC-Rs and between PLC-P/shRNA and PLC-P/shRNA-NC. The IFN signalling was evaluated by the expression of STAT1 and STAT2, and by IFN-α-induced expression of pSTAT1 and pSTAT2. The results showed no

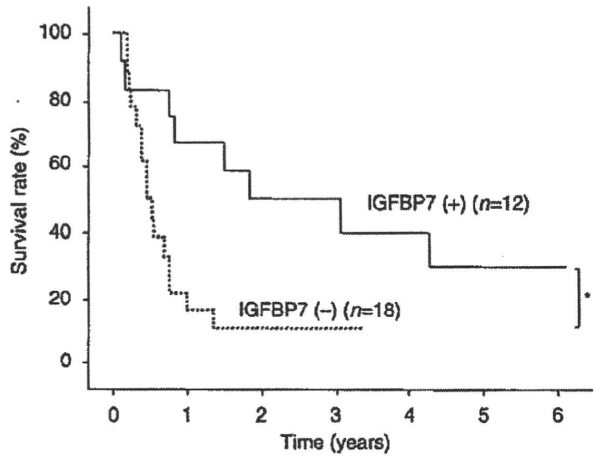

Figure 5 Postoperative overall survival curves showed a significantly better survival rate for Insulin-like growth factor-binding protein 7 (IGFBP7)-positive patients than for IGFBP7-negative patients (*$P<0.05$).

Table 3 Statistical analyses of overall survival of 30 patients with advanced hepatocellular carcinoma

	Univariate	Multivariate		
	P-value	OR	95%CI	*P*-value
Age (<60/⩾60 years)	0.6846			
Gender (male/female)	0.5975			
Cirrhosis (–/+)	0.7014			
Child-Pugh classification (A/B)	0.1825			
AFP (<400/⩾400 ng ml^{-1})	0.7459			
PIVKA-II (<1000/⩾1000 mAU l^{-1})	0.6637			
Histological grade (mod, poor/undifferentiated)	0.1705			
IFNAR2 status (–/+)	0.0010	2.645	1.024–6.831	0.0056
IGFBP7 status (–/+)	0.0170	4.096	1.511–11.108	0.0445

Abbreviations: AFP = 95% CI = 95% confidence interval; α-fetoprotein; IFNAR2 = type I interferon receptor 2; IGFBP7 = insulin-like growth factor-binding protein 7; mod = moderately differentiated; OR = odds ratio; PIVKA-II = protein induced by vitamin K absence; poor = poorly differentiated.

significant differences in the expression of IFNAR2 and IFN signalling between PLC-P and PLC-Rs or between PLC-P/shRNA and PLC-P/shRNA-NC. On the other hand, Wajapeyee *et al* (2008) reported that IGFBP7 induces apoptosis through increased SMARCB1 upregulation by the recruitment of STAT1 to the binding site of the SMARCB1 promoter. Another study reported that STAT1 is recruited to the SMARCB1 promoter by IFN, suggesting that IFN-induced STAT1 recruitment to the SMARCB1 promoter is possibly one of the mechanisms of IFN-induced apoptosis (Hartman *et al*, 2005). It might therefore be possible that STAT1 recruitment could be prevented antagonistically when IGFBP7 is suppressed, leading to a higher resistance to IFN-α than to other drugs. In this study, however, pSTAT1 expression was not different between PLC-P and PLC-Rs or between PLC-P/shRNA and PLC-P/shRNA-NC, and there were no significant differences in the SMARCB1 expression evaluated by the result of microarray between PLC-P and PLC-Rs. These results indicate that IGFBP7-related IFN resistance is based not on SMARCB1 but on a novel mechanism, which should be clarified in the future.

The present study revealed that, in addition to the significant association between IGFBP7 status and the clinical response to IFN-α/5-FU therapy, the IGFBP7 status as well as IFNAR2, was an independent prognostic factor in HCC patients undergoing IFN-α/5-FU therapy. Because our 30 patients in this study are those with far advanced HCC, it is quite reasonable that the clinical response to the therapy correlates well with the prognosis after the therapy. These results indicate that prediction of response and prognosis by evaluating IGFBP7 and IFNAR2 is useful in this clinical setting.

In summary, IGFBP7 was selected on the basis of the results of the microarray analysis using established IFN-resistant HCC cell lines. The expression of IGFBP7 in tumour tissue correlated significantly with the response to IFN-α/5-FU therapy. This correlation was also confirmed in genetic manipulation experiments. Our findings suggest that IGFBP7 could be a novel marker for the prediction of the clinical response to IFN-α/5-FU therapy.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

Translational Therapeutics

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Effects of Preceding Interferon Therapy on Outcome After Surgery for Hepatitis C Virus-Related Hepatocellular Carcinoma

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Background and Objectives: Interferon (IFN) can eradicate hepatitis C virus (HCV)-RNA from serum and hepatic tissue, and suppress the development of hepatocellular carcinoma (HCC). Despite such effectiveness, HCC develops even in HCV patients successfully treated with IFN therapy.

Methods: HCV-related HCC patients who underwent curative hepatectomy for HCC were divided into three groups according to preceding IFN for HCV infection therapy and the therapeutic effect: responders group (n = 23), non-responders group (n = 46), and no-IFN group (n = 215). Postoperative outcome was retrospectively examined in the three groups.

Results: AST and ALT were significantly lower in responders group than non-responders group ($P < 0.001$, $P = 0.001$) and no-IFN group ($P = 0.001$, $P = 0.002$). Platelet count was significantly higher in responders group than other groups ($P = 0.008$, $P = 0.001$). The percentage of cirrhotic patients in responders group was significantly lower than other groups ($P = 0.017$, $P = 0.014$). Multivariate analysis identified preceding IFN therapy to be associated with disease-free survival at marginal significance ($P = 0.086$), and as a significant independent factor for overall survival ($P = 0.042$).

Conclusions: Preceding IFN therapy for HCV infection improves postoperative outcome in HCV-related HCC patients treated successfully with IFN.

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KEY WORDS: **hepatocellular carcinoma (HCC); interferon (IFN); hepatitis C virus (HCV); hepatic resection**

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Approximately 80% of Japanese HCC patients have a history of chronic infection with hepatitis C virus (HCV), which is a known cause of HCC [1,2]. Recent advances in imaging modalities and treatment have brought some improvement to the prognosis of patients with HCV-related HCC, but the outcome remains unsatisfactory. Even after curative hepatic resection for HCV-related HCC, the rate of tumor recurrence within 1 year is 20–40%, rising to about 80% by 5 years [3,4]. This high recurrence rate and the progression of the underlying hepatic damage due to HCV-related chronic hepatitis (CH) or cirrhosis result in unfavorable postoperative outcome in patients with HCV-related HCC.

Interferon (IFN) is the only agent known to be effective against HCV infection [5–10]. It can eradicate HCV-RNA from peripheral blood and hepatic tissue, prevent deterioration of liver dysfunction in patients with HCV infection, and suppress the development of HCC. HCV-infected patients treated with IFN, especially those who develop sustained virological response (SVR), defined as negative HCV-RNA polymerase chain reaction at 6 months after the end of treatment, enjoy the benefits of such treatment [11,12]. However, despite such effectiveness of IFN therapy, there have been recently some reports of development of HCC even in HCV patients who had gained SVR following IFN therapy [13,14]. With regard to the HCC development in patients treated successfully with IFN, HCV-related HCC patients can be divided into three groups according to the clinical background of preceding IFN therapy; successfully treated, unsuccessfully treated, or not treated with IFN. However, to date, there have been few studies on the correlation between clinical background of previous IFN therapy for HCV infection and surgical outcome of HCV-related HCC [15,16].

In the present retrospective study, we reviewed HCV-related HCC patients who had undergone surgery in our hospital. We analyzed the factors that affected postoperative outcome including history of previous IFN therapy and the effect of such therapy.

MATERIALS AND METHODS

The present study included 284 patients with HCC who had undergone curative hepatic resection at the Department of Surgery, Osaka University Hospital between January 1990 and December 2008. Patients with HCC grade Vp3, Vp4, Vv2, and Vv3, defined according to the classification system of the Liver Cancer Study Group of Japan, were excluded from this study [17]. Curative resection was defined as complete removal of all macroscopically evident tumors. Patients who had undergone surgery for recurrent HCC were also excluded from this

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study. Among the 284 patients, 215 patients were not treated with IFN (no IFN group). The remaining 69 patients received IFN therapy for HCV infection. In the latter group, HCC had not been detected at the IFN therapy, and was detected after the IFN therapy. The IFN therapy was performed not for HCC, but for HCV-related hepatitis. The administration of IFN therapy was determined based on the informed consent between each physician and patient. The response to IFN therapy was assessed retrospectively based on changes in HCV-RNA. Based on the response, patients were divided into the responders group and non-responders group; 23 patients whose HCV-RNA disappeared after IFN therapy were categorized as the responders group, and 46 patients whose HCV-RNA did not disappear after IFN therapy in non-responders group. Figure 1 summarizes the classification of the enrolled patients. The type, dosage, and duration of IFN administration before surgery varied, though all patients received IFN-α.

Hospital records were retrospectively reviewed for clinical factors including previous history of IFN therapy, tumor- and surgery-related factors. The surgical procedure was selected based on the extent of the tumor and residual liver function. The HCC staging was performed according to the classification system of the Liver Cancer Study Group of Japan [17]. The histological grade of differentiation of HCC was determined according to the Edmondson–Steiner classification, and was based on the areas of the tumor with the highest grade [18]. Non-cancerous lesion of the liver was divided histopathologically into chronic hepatitis CH and liver cirrhosis (LC).

Patients were followed up after hepatic resection at regular intervals of 3–4 months with physical examination, tumor markers including alpha-fetoprotein (AFP), and protein induced by vitamin K absence or antagonists-II (PIVKA-II), liver biochemical tests, abdominal ultrasonography, and abdominal computed tomography (CT) to check for intrahepatic recurrence, and chest radiography and bone scintigraphy for extra-hepatic recurrence. The median duration of clinical follow-up after the initial hepatectomy was 51.2 months.

Data were expressed as mean ± standard deviation. Differences between groups were assessed by the chi-square test, Fisher's exact test, or the Mann–Whitney U test. Survival rates were calculated according to the Kaplan and Meier method and compared using the log-rank test. Statistical analysis was performed using StatView (version 5.0, SAS Institute Inc., Cary, NC). A P-value <0.05 was considered statistically significant. The study protocol was approved by the Human Ethics Review Committee of Osaka University Hospital and a signed consent form was obtained from each patient.

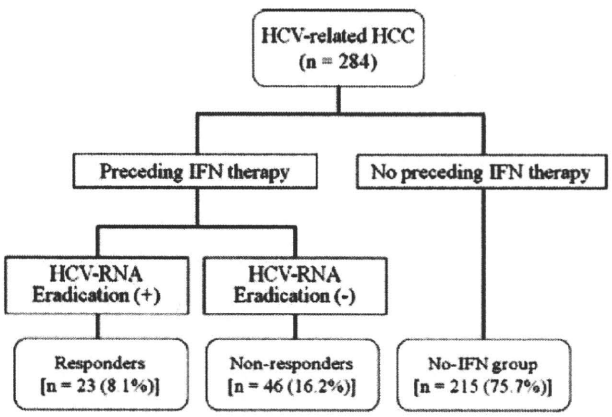

Fig. 1. Distribution of patients enrolled in this study according to the clinical background of preceding IFN therapy. HCV, hepatic C virus; HCC, hepatocellular carcinoma; IFN, interferon.

RESULTS

The study group comprised 222 (78.2%) men and 62 (21.8%) women, with a mean age of 65 (range, 39–79). Table I summarizes the clinicopathological characteristics of the responders group, the non-responders group, and the no-IFN group. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly lower in the responders group than the non-responders group ($P < 0.001$, $P = 0.001$) and no-IFN group ($P = 0.001$, $P = 0.002$). There were no significant differences in the levels of AST and ALT between the non-responders group and the no-IFN group. Platelet count was significantly higher in the responders than that in the non-responders ($P = 0.008$) and that in the no-IFN group ($P = 0.001$). In the responders group, histopathological status of the non-cancerous liver tissue obtained at surgery was CH in 16 patients (69.6%) and LC in seven patients (30.4%). The percentage of patients of the responders group with LC was significantly lower than that of the non-responders group (28/46, 60.9%; $P = 0.017$) and that of the no-IFN group (123/215, 57.2%; $P = 0.014$). Liver function assessed by Child–Pugh classification was not different among the three groups. Other clinical factors listed in Table I were also not different among the three groups, including tumor- and surgical-related factors. Adjuvant therapy of IFN was administered in a small number of patients (n = 14, 4.9%), and the frequency of such patients was not different among the three groups.

For all the 284 patients, the 1-, 3-, and 5-year disease-free survival (DFS) rates were 70.8%, 36.7%, and 22.8%, respectively. There was no significant difference in DFS between the IFN group (the responders group and the non-responders group) and the no-IFN group ($P = 0.396$). However, the DFS of the responders group (1 year: 89.2%, 3 years: 59.4%, 5 years: 59.4%) was significantly better than that of the no-IFN group (1 year: 70.8%, 3 years: 35.7%, 5 years: 21.6%; $P = 0.039$), and tended to be better than that of the non-responders group (1 -year: 60.4%, 3 years: 32.3%, 5 years: 16.9%; $P = 0.051$; Fig. 2). However, there was no significant difference in DFS between the non-responders group and the no-IFN group ($P = 0.673$). The 1-, 3-, and 5-year overall survival rates for all patients were 94.5%, 80.4%, and 66.9%, respectively. The overall survival rates of the IFN group (responders group and non-responders group) tended to be higher than those of the no-IFN group ($P = 0.093$). The 1-, 3-, and 5-year overall survival rates for the responders group were 100%, 100%, and 100%, respectively, and were significantly higher than the non-responders group (1-year: 94.4%, 3 years: 78.6%, 5 years: 55.4%; $P = 0.026$) and the no-IFN group (1 year: 94.0%, 3 years: 79.0%, 5 years: 66.1%; $P = 0.009$; Fig. 3). There was no significant difference in overall survival between the non-responders group and the no-IFN group ($P = 0.904$).

Univariate analysis was performed between DFS and various clinicopathological factors (Table II). Microscopic vascular invasion (negative vs. positive), tumor stage (I, II vs. III, IV), number of nodules (single vs. multiple), the diameter of largest tumor nodules (<5 cm vs. ≥5 cm), AFP level (<5 ng/m vs. ≥5 ng/m), and preceding IFN therapy (responders vs. non-responders, no-IFN) were significant factors ($P < 0.001$, $P = 0.006$, $P = 0.008$, $P = 0.021$, $P = 0.017$, $P = 0.037$). Multivariate analysis for DFS using the above six factors identified the number of nodules and microscopic vascular invasion as significant independent factors ($P = 0.014$, $P = 0.041$; Table III). In the same analysis, preceding IFN therapy showed a borderline significance with DFS ($P = 0.086$). The diameter of the largest tumor nodules and AFP level also tended to be associated with DFS ($P = 0.090$, $P = 0.098$).

Univariate analysis for overall survival using various clinicopathological factors demonstrated that microscopic vascular invasion (negative vs. positive), preceding IFN therapy (responders vs. non-responders, no-IFN), number of nodules (single vs. multiple), diameter of largest nodules (<5 cm vs. ≥5 cm), and AFP level (<5 ng/m vs. ≥5 ng/m) were significant factors ($P = 0.004$, $P = 0.009$, $P = 0.015$,

TABLE I. Clinicopathological Characteristics of Patients With HCV-Related HCC

	IFN group			*P*-value		
	Responders (n = 23)	Non-responders (n = 46)	No-IFN (n = 215)	Responders versus non- responders	Responders versus no-IFN	Non- responders versus no-IFN
Clinical factors						
Gender (male/female)	19/4	33/13	170/45	0.323	0.793	0.278
Age (years)	66 ± 7	64 ± 7	65 ± 7	0.355	0.653	0.424
Alcohol abuse (+/−)	14/9	27/19	132/83	0.862	0.961	0.733
HCV serotype (1/2/unknown)	19/4/0	35/5/6	166/29/20	>0.999	0.795	0.969
HBs Ag (+/−)	1/22	1/45	7/208	>0.999	0.562	>0.999
AST (IU/L)	28 ± 13	49 ± 27	46 ± 21	<0.001	0.001	0.184
ALT (IU/L)	26 ± 16	52 ± 36	47 ± 29	0.001	0.002	0.233
Platelet count ($\times 10^4/\mu l$)	16.4 ± 3.3	13.0 ± 5.3	13.2 ± 4.5	0.008	0.001	0.867
Albumin (g/dl)	4.0 ± 0.4	3.8 ± 0.6	3.8 ± 0.5	0.142	0.175	0.975
Total bilirubin (mg/dl)	0.6 ± 0.2	0.7 ± 0.2	0.7 ± 0.3	0.114	0.104	0.362
Prothrombin time (%)	77 ± 10	77 ± 8	76 ± 9	0.719	0.888	0.442
Hepaplastin test (%)	81 ± 13	78 ± 12	77 ± 12	0.244	0.217	0.834
Child-Pugh (A/B)	22/1	37/9	187/28	0.148	0.326	0.265
Non-cancerous lesion (CH/LC)	16/7	18/28	92/123	0.017	0.014	0.648
Tumor-related factors						
AFP (ng/ml)	1,791 ± 6,654	545 ± 1,444	851 ± 4,004	0.226	0.332	0.610
PIVKA-II (mAU/ml)	1,773 ± 5,433	1,418 ± 4,733	2,006 ± 4,879	0.786	0.837	0.459
Preoperative TAE (+/−)	10/13	23/23	119/96	0.609	0.278	0.509
Postoperative IFN (+/−)	1/22	1/45	12/203	>0.999	>0.999	0.476
Number of nodules (single/multiple)	17/6	33/13	152/63	0.849	0.747	0.888
Tumor diameter (cm)	3.5 ± 1.9	3.2 ± 2.0	3.6 ± 2.6	0.287	0.725	0.171
Vascular invasion (+/−)	2/21	2/44	18/197	0.596	>0.999	0.543
Stage (I/II/III/IV)	5/12/4/2	12/24/7/3	45/109/49/12	0.967	0.890	0.671
Edmondson-Steiner grade (I, II/III, IV/unknown)	13/10/0	26/16/4	117/89/9	0.672	0.980	0.541
Surgery-related factors						
Hr (0/S/1/2)	13/3/3/4	29/5/7/5	116/41/37/21	0.869	0.615	0.543
Volume of resection (g)	152 ± 118	137 ± 151	165 ± 162	0.393	0.682	0.186
Blood loss (ml)	1,022 ± 1,583	996 ± 702	1,167 ± 1,217	0.770	0.571	0.197
Operation time (min)	253 ± 128	236 ± 99	236 ± 99	0.337	0.940	0.174
Transfusion (+/−)	4/19	7/39	52/163	>0.999	0.465	0.187

Data are expressed as mean ± standard deviation.
IFN, interferon; HCV, hepatic C virus; HBs Ag, hepatitis B surface antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CH, chronic hepatitis; LC, liver cirrhosis; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; TAE, transcatheter arterial chemoembolization; Hr, hepatic resection; 0: partial resection; S, subsegmentectomy; 1, one segmentectomy; 2, two segmentectomies.

$P = 0.034$, $P = 0.045$; Table II). Multivariate analysis for overall survival using the above five factors identified number of nodules, microscopic vascular invasion, and preceding IFN therapy as significant independent factors ($P = 0.025$, $P = 0.037$, $P = 0.042$; Table III).

HCC recurred postoperatively in nine (39.1%) patients of the responders group, 29 (63.0%) of the non-responders group, and in 157 (73.0%) of the no-IFN group. Table IV summarizes the clinical characteristics of patients with recurrent HCC at diagnosis of the recurrence. AST and ALT levels in the responders group were

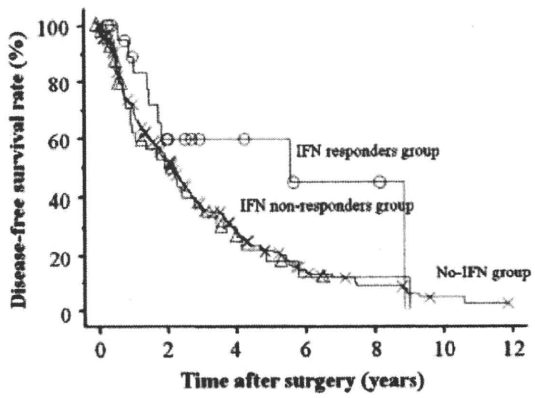

Fig. 2. Disease-free survival after initial surgery for HCC in the responders group, the non-responders group, and the no-IFN group. Open circles: responders (n = 23), open triangles: non-responders (n = 46), crosses: no-IFN (n = 215). IFN: interferon.

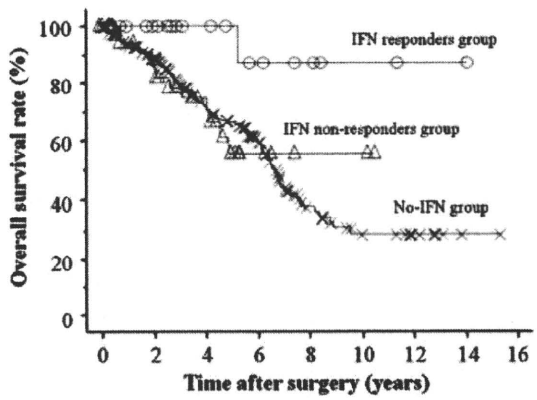

Fig. 3. Overall survival calculated from the initial surgery for HCC for the responders group, the non-responders group, and the no-IFN group. Open circles: responders (n = 23), open triangles: non-responders (n = 46), crosses: no-IFN (n = 215). IFN: interferon.

TABLE II. Univariate Analysis of Disease-Free Survival and Overall Survival of Patients With HCV-Related HCC

	Number of patients	Disease-free survival	Overall survival
Clinical factors			
Gender (male/female)	222/62	0.732	0.789
Age, years (<66/≥67)	143/141	0.682	0.842
Alcohol abuse (+/−)	172/112	0.955	0.572
HCV genotype (1/2/unknown)	220/40/25	0.612	0.427
AST (IU/L) (<40/≥40)	126/158	0.496	0.547
ALT (IU/L) (<40/≥40)	122/162	0.216	0.301
Total bilirubin (mg/dl) (<1.0/≥1.0)	252/32	0.890	0.587
Albumin (g/dl) (<3.5/≥3.5)	114/170	0.174	0.171
Prothrombin time (%) (<70/≥70)	77/207	0.693	0.875
Hepaplastin test (%) (<70/≥70)	75/209	0.427	0.398
Platelet count ($\times 10^4/\mu l$) (<10/≥10)	83/201	0.176	0.123
Child-Pugh (A/B)	246/38	0.866	0.594
Non-cancerous lesion (CH/LC)	126/158	0.247	0.177
Tumor-related factors			
AFP (ng/ml) (<5/≥5)	54/230	0.021	0.045
PIVKA-II (mAU/ml) (<400/≥400)	190/83	0.130	0.142
Preceding IFN (responders/non-responders, no-IFN)	23/261	0.037	0.009
Preoperative TAE (+/−)	152/132	0.863	0.562
Postoperative IFN (+/−)	14/270	0.222	0.253
Number of nodules (single/multiple)	202/82	0.008	0.015
Tumor diameter (cm) (<5/≥5)	232/52	0.017	0.034
Vascular invasion (+/−)	22/262	<0.001	0.004
Stage (I, II/III, IV)	207/77	0.006	0.197
Edmondson-Steiner grade (I, II/III, IV)	156/115	0.328	0.265
Surgery-related factors			
Hr (0/S, 1, 2)	158/126	0.313	0.893
Intraoperative blood loss (L) (<1/≥1)	151/133	0.289	0.270
Operation time (min) (<240/≥240)	141/143	0.221	0.493
Transfusion (+/−)	63/221	0.756	0.180

IFN, interferon; HCV, hepatic C virus; HBs Ag, hepatitis B surface antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CH, chronic hepatitis; LC, liver cirrhosis; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; TAE, transcatheter arterial chemoembolization; Hr, hepatic resection; 0: partial resection; S, subsegmentectomy; 1, one segmentectomy; 2, two segmentectomies.

significantly lower than those in the non-responders group ($P = 0.047$, $P = 0.045$) and those in the no-IFN group ($P = 0.028$ and $P = 0.034$). There were no significant differences in AST and ALT levels between the non-responders and no-IFN groups. Platelet count was significantly higher in the responders group than that in the no-IFN group ($P = 0.029$) and tended to be higher than that in the non-responders group ($P = 0.079$). Figure 4A shows the distribution of interval between initial hepatectomy and recurrence. In most patients, HCC recurred within 2 years in the three groups, and the distribution of the interval was not different among the three groups. In all groups, the

TABLE III. Multivariate Analysis of Disease-Free Survival and Overall Survival of Patients With HCV-Related HCC

	OR	95% CI	*P*-value
Disease-free survival			
AFP (ng/ml) (<5/≥5)	1.427	0.937–2.174	0.098
Preceding IFN (responders/non-responders, no-IFN)	1.809	0.919–3.561	0.086
Number of nodules (single/multiple)	1.707	1.022–2.850	0.041
Tumor diameter (cm) (<5/≥5)	1.391	0.951–2.037	0.090
Vascular invasion (−/+)	2.331	1.186–4.587	0.014
Stage (I, II/III, IV)	1.287	0.715–2.315	0.401
Overall survival			
AFP (ng/ml) (<5/≥5)	1.689	0.847–3.367	0.137
Preceding IFN (responders/non-responders, no-IFN)	7.750	1.076–55.798	0.042
Number of nodules (single/multiple)	1.622	1.062–2.476	0.025
Tumor diameter (cm) (<5/≥5)	1.381	0.842–2.268	0.200
Vascular invasion (−/+)	2.247	1.049–4.808	0.037

IFN, interferon; HCV, hepatic C virus; HBs Ag, hepatitis B surface antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CH, chronic hepatitis; LC, liver cirrhosis; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; TAE, transcatheter arterial chemoembolization; Hr, hepatic resection; 0: partial resection; S, subsegmentectomy; 1, one segmentectomy; 2, two segmentectomies. OR, odds ratio, 95% CI, 95% confidence interval.

TABLE IV. Clinicopathological Characteristics of Patients With Recurrent HCC in the Responders Group, the Non-Responders Group, and the No-IFN Group

	IFN group			*P*-value		
	Responders (n = 9)	Non-responders (n = 29)	No-IFN (n = 157)	Responders versus non-responders	Responders versus no-IFN	Non-responders versus no-IFN
Clinical factors						
Gender (male/female)	9/0	22/7	126/31	0.164	0.212	0.590
Age (years)	67 ± 7	66 ± 6	67 ± 7	0.641	0.971	0.378
AST (IU/L)	30 ± 25	50 ± 28	55 ± 28	0.047	0.028	0.786
ALT (IU/L)	32 ± 26	53 ± 33	54 ± 34	0.045	0.034	0.902
Platelet count ($\times 10^4/\mu l$)	14.8 ± 3.3	12.2 ± 3.7	11.8 ± 3.5	0.079	0.029	0.720
Albumin (g/dl)	3.9 ± 0.3	3.7 ± 0.4	3.6 ± 0.4	0.122	0.085	0.782
Total bilirubin (mg/dl)	0.7 ± 0.2	0.7 ± 0.2	0.8 ± 0.3	0.216	0.242	0.757
Prothrombin time (%)	76 ± 8	75 ± 12	75 ± 11	0.894	0.942	0.918
Hepaplastin test (%)	75 ± 11	74 ± 11	73 ± 13	0.872	0.817	0.907
Child-Pugh (A/B)	8/1	25/4	130/27	>0.999	>0.999	0.791
Tumor-related factor						
AFP (ng/ml)	51 ± 112	60 ± 98	81 ± 305	0.983	0.848	0.757
PIVKA-II (mAU/ml)	90 ± 83	258 ± 712	200 ± 696	0.491	0.640	0.744
Latency to recurrence (years)	2.6 ± 2.8	2.0 ± 2.0	2.2 ± 2.1	0.497	0.561	0.707
Recurrence site (intrahepatic/extrahepatic)	8/1	29/0	150/7			
Intrahepatic recurrence (single/multiple)	6/2	11/18	57/93			

Data are expressed as mean ± standard deviation.
IFN, interferon; HCV, hepatic C virus; HBs Ag, hepatitis B surface antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CH, chronic hepatitis; LC, liver cirrhosis; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; TAE, transcatheter arterial chemoembolization; Hr, hepatic resection; 0: partial resection; S, subsegmentectomy; 1, one segmentectomy; 2, two segmentectomies.

majority of first recurrence sites were residual liver [responders group: 89% (8/9), non-responders group: 100% (29/29), no-IFN group: 94% (150/157)] (Fig. 4B). In the responders group, among eight patients with intrahepatic recurrence, solitary recurrence was seen in six patients (75.0%). On the other hand, the percentage of solitary intrahepatic recurrence was 37.9% (11/29) in the non-responders group and 38.0% (57/150) in the no-IFN group. In the responders group, surgery, percutaneous therapy, and transarterial chemoembolization (TACE) was selected in three, four, and two patients for treatment of recurrence, respectively (Fig. 4C). The proportion of patients in whom surgery or percutaneous therapy was selected for treatment in the responders group (7/9, 77.8%) was significantly higher than that of the non-responders group (7/29, 24.1%, $P = 0.006$) and the no-IFN group (28/157, 17.8%, $P < 0.001$).

Figure 5 shows the overall survival from diagnosis of the first HCC recurrence in the three groups. The overall survival rate of the

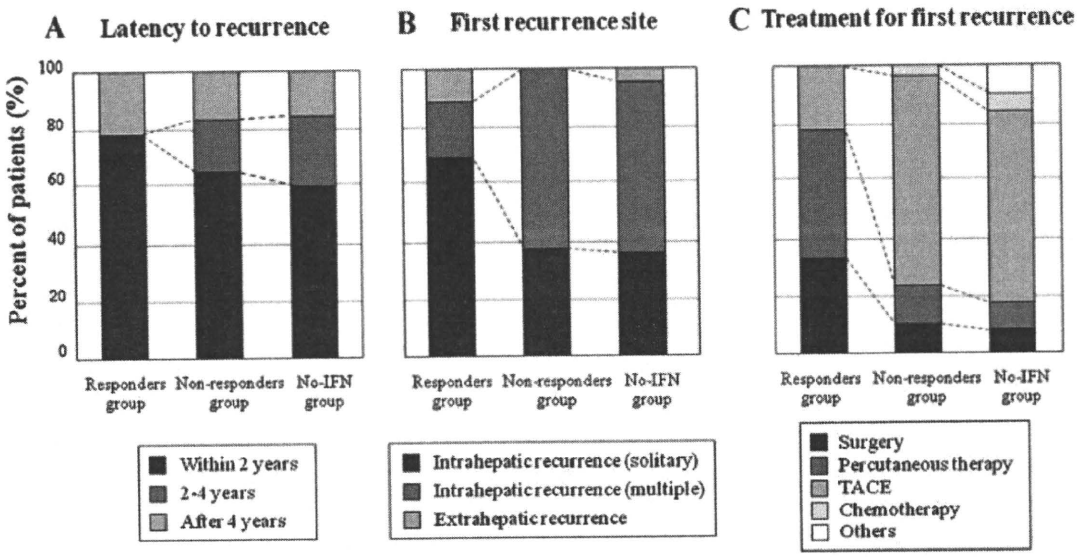

Fig. 4. **A**: Distribution of the latency from the initial hepatectomy to HCC recurrence for the responders, the non-responders, and the no-IFN group. **B**: Distribution of the first recurrence site in patients with HCC recurrence of the responders, the non-responders, and the no-IFN group. **C**: Distribution of selected treatment for first HCC recurrence in the responders, the non-responders, and the no-IFN group. IFN: interferon, TACE: transcatheter arterial chemoembolization.

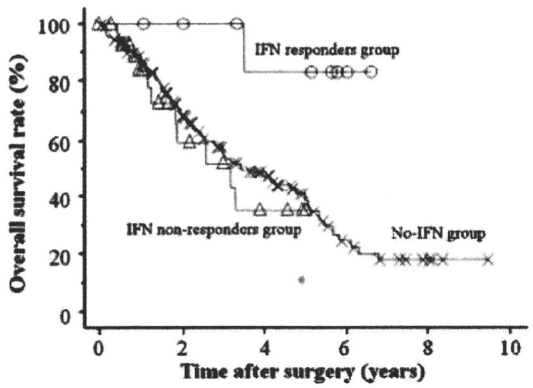

Fig. 5. Overall survival rates calculated from the diagnosis of first HCC recurrence in the responders group, the non-responders group, and the no-IFN group. Open circles: responders (n = 9), open triangles: non-responders (n = 29), crosses: no-IFN group (n = 157). IFN: interferon.

responders group was significantly higher than that of the non-responders group ($P = 0.012$) and that of no-IFN group ($P = 0.011$).

DISCUSSION

The present study demonstrated that a significantly better DFS from the initial hepatectomy in the responders group than the other two groups. This result was similar to that reported previously by Uenishi et al. [16]. Based on the pattern of the DFS curve of the responders group in this study, the recurrence rate appeared to decrease mainly in 2 years later. We have reported that DFS curves for postoperative HCC patients in the early (within 2 years) and late (4 years after surgery) represented both intrahepatic metastasis and multicentric carcinogenesis, respectively [19]. Based on this viewpoint, the decrease in recurrence in the responders group was probably mainly due to the suppression of new multicentric carcinogenesis. A number of investigators have reported that suppression of increased liver inflammation, as assessed by AST and ALT, contributes to inhibition of hepatocarcinogenesis and postoperative intrahepatic recurrence after HCC surgery, which is more likely to originate from multicentric carcinogenesis [20,21]. IFN has been reported also to be effective in eradication of HCV-RNA from the serum and hepatic tissue and prevention of deterioration of liver dysfunction in patients with HCV infection [5–8,10]. It is possible that the suppression of new multicentric carcinogenesis seen in the IFN responders group of this study was due to these effects of IFN therapy. This speculation is supported by the findings of the present study that the levels of aminotransferases and platelet count in the responders group were significantly lower and higher, respectively, than those of the other groups, at the initial hepatectomy and first recurrence, and that the frequency of LC in the responders group was significantly lower than that of the other groups.

On the other hand, IFN has been reported to have anti-tumor effects [22–24]. These anti-tumor effects of IFN had been actually verified also in IFN-alpha/5-fluorouracil combination therapy for advanced HCC in a series of studies by our group [25–32]. Additionally, in a previous report by Uenishi et al. [16], only one patient developed postoperative recurrence about 5 years after the initial surgery among 11 patients of the responders group, and the recurrence pattern of the responders group was also suggestive of the inhibitory effect of IFN on metastasis originating from the primary HCC. Taken together, also in the present study, the decrease of recurrence might be potentially derived from the suppression of intrahepatic metastasis by IFN.

In the present study, overall survival from the initial hepatectomy in the responders group was also significantly better than those of the other two groups. This improvement of overall survival was caused by the aforementioned decrease of HCC recurrence rate in the responders group. In addition, in the responder group, the percentage of patients who underwent selective surgery or percutaneous therapy for the treatment of recurrent HCC was higher than other groups. In general, the treatment for the postoperative HCC recurrence is frequently restricted for the residual liver function, which is one of the reasons for the unfavorable postoperative outcome [3,4]. Considering such restriction of the treatment, the improved liver function by IFN therapy was also speculated to contribute to the better overall survival. Finally, it could be argued that IFN therapy was the main reason for the improvement in both DFS and overall survival rates in the responders group.

To date, several studies examined the impact of IFN therapy after curative loco-regional treatment for HCC [33–37]. For example, in a randomized controlled trial, Ikeda et al. [33] reported that IFN therapy suppressed tumor recurrence after surgery or ethanol injection for HCV-related HCC. Kubo et al. [36] also reported that postoperative IFN therapy significantly decreased recurrence after resection of HCV-related HCC in a randomized controlled trial. That several randomized controlled trials indicated improved posttreamtemt outcome in patients with HCV-related HCC who received postoperative IFN therapy, adds support to our conclusion of the effectiveness of preceding IFN therapy.

Since the present study is retrospective in nature, few details of IFN therapy are unavailable. For example, the duration of HCV-RNA clearance was not clear in several patients treated with IFN. Therefore, in this study, we could not divide patients of the responders group into those with SVR or not. In order to examine more strictly the effectiveness of preceding IFN therapy for surgical outcome, is prospectively designed study is necessary.

CONCLUSIONS

The present study demonstrated the effectiveness of IFN therapy for HCV infection administered before HCC resection as assessed by evaluating the disease-free and overall survival. IFN therapy for HCV might be essential not only for the treatment of HCV infection but also for improvement of prognosis of patients who are susceptible to the development of HCC.

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BRIEF ARTICLE

Fresh frozen plasma transfusion does not affect outcomes following hepatic resection for hepatocellular carcinoma

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Abstract

AIM: To investigate whether fresh frozen plasma (FFP) transfusion affects outcomes following hepatic resection for hepatocellular carcinoma (HCC) in terms of liver function, postoperative complications and cancer prognosis.

METHODS: We retrospectively compared the incidence of postoperative complications between 204 patients who underwent hepatectomy for HCC with routine FFP transfusion in an early period (1983-1993, Group A) and 293 with necessity for FFP transfusion during a later period (1998-2006, Group B), and also between two subgroups of Group B [22 patients with FFP transfusion (Group B1) and 275 patients without FFP transfusion (Group B2)]. Additionally, only in limited patients in Group B1 and Group B2 with intraoperative blood loss ≥ 2000 mL (Group B1≥ 2000 mL and Group B2≥ 2000 mL), postoperative complications, liver function tests, and cancer prognosis were compared.

RESULTS: No mortality was registered in Group B, compared to 8 patients (3.9%) of Group A. The incidence of morbidity in Group B2 [23.2% (64/275)] was not significantly different from Group B1 [40.9% (9/22)] and Group A [27.0% (55/204)]. The incidence of complications and postoperative liver function tests were comparable between Group B1≥ 2000 mL *vs* Group B2≥ 2000 mL. Postoperative prognosis did not correlate with administration of FFP, but with tumor-related factors.

CONCLUSION: The outcome of hepatectomy for HCC is not influenced by FFP transfusion. We suggest FFP transfusion be abandoned in patients who undergo hepatectomy for HCC.

Key words: Fresh frozen plasma; Hepatocellular carcinoma; Surgery; Transfusion

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