C.I., conidence interval.

*Child-Pugh class A includes patients without cirrhosis.

**Evaluated by pathologic examination of resected specimens.

Table 3. Univariate and multivariate analyses of factors associated with post-operative intrahepatic metastasis recurrence in HCC patients (n=77)

Factor		Univariate analysis		Multivariate analysis	
		Risk ratio (95% C.I.)	*p* value	Risk ratio (95% C.I.)	*p* value
Age		0.9825 (0.9265-1.0470)	0.5743	-----	
Sex	Male	1			
	Female	0.9022 (0.4784-1.5192)	0.7148	-----	
Child-Pugh class*	A	1			
	B	0.0242 (0.0059-2.1819)	0.3573	-----	
Tumor size		1.0051 (0.6929-1.3406)	0.9755	-----	
Number of tumors	Single	1			
	Multiple	0.7038 (0.1655-1.5643)	0.4504	-----	
Differentiation**	Well-	1		1	
	Moderately/poorly	1.7843 (1.0185-3.7176)	0.0424	1.6742 (0.9520-3.4993)	0.0769
Growth pattern**	Expansive	1			
	Infiltrative	0.9266 (0.3678-1.7453)	0.8365	-----	
Portal vein invasion**	Absent	1		1	
	Present	2.1224 (1.2405-3.4608)	0.0079	2.0041 (1.1672-3.2828)	0.0138
Non-hypervascular	Absent	1			
hypointense nodules	Present	1.0474 (0.5012-1.8442)	0.8864	-----	

C.I., conidence interval.

*Child-Pugh class A includes patients without cirrhosis.

**Evaluated by pathologic examination of resected specimens.

Table 4. Univariate and multivariate analyses of factors associated with post-operative multicentric recurrence in HCC patients (n=59)

Factor		Univariate analysis		Multivariate analysis	
		Risk ratio (95% C.I.)	*p* value	Risk ratio (95% C.I.)	*p* value
Age		1.0047 (0.9359-1.0823)	0.8985	-----	
Sex	Male	1			
	Female	1.0701 (0.5999-1.7781)	0.8038	-----	
Child-Pugh class*	A	1			
	B	0.0664 (0.0176-5.7947)	0.7029	-----	
Tumor size		0.9517 (0.6300-1.2943)	0.7801	-----	
Number of tumors	Single	1			
	Multiple	1.1331 (0.4469-2.1714)	0.7510	-----	
Differentiation**	Well-	1			
	Moderately/poorly	1.5198 (0.8959-2.8769)	0.1249	-----	
Growth pattern**	Expansive	1			
	Infiltrative	1.3486 (0.7124-2.2884)	0.3270	-----	
Portal vein invasion**	Absent	1			
	Present	1.2908 (0.5077-2.4730)	0.5312	-----	
Non-hypervascular	Absent	1		1	
hypointense nodules	Present	2.8436 (1.6900-4.8407)	0.0002	2.8436 (1.6900-4.8407)	0.0002

C.I., conidence interval.

*Child-Pugh class A includes patients without cirrhosis.

**Evaluated by pathologic examination of resected specimens.

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Transcatheter Arterial Infusion Chemotherapy with a Fine-powder Formulation of Cisplatin for Advanced Hepatocellular Carcinoma Refractory to Transcatheter Arterial Chemoembolization

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Objective: The aim of this study was to assess the safety and efficacy of transcatheter arterial infusion chemotherapy using a fine-powder formulation of cisplatin for patients with advanced hepatocellular carcinoma refractory to transcatheter arterial chemoembolization.
Methods: We retrospectively examined the data of 84 consecutive patients with transcatheter arterial chemoembolization-refractory hepatocellular carcinoma who underwent transcatheter arterial infusion chemotherapy with a fine-powder formulation of cisplatin. Cisplatin was administered at the dose of 65 mg/m^2 into the feeding artery of the hepatocellular carcinoma. The treatment was repeated every 4–6 weeks, until the appearance of evidence of tumor progression or of unacceptable toxicity.
Results: Of the 84 patients, one patient (1.2%) showed complete response and two patients (2.4%) showed partial response, representing an overall response rate of 3.6% (95% confidence interval, 0.7–10.1). Of the remaining, 38 patients (45.2%) showed stable disease and 41 (48.8%) showed progressive disease. The median overall survival, 1-year survival rate and median progression-free survival in the entire subject population were 7.1 months, 27% and 1.7 months, respectively. Major Grade 3 or 4 adverse events included thrombocytopenia in 12 patients (14%) and elevation of the serum aspartate aminotransferase in 33 patients (39%). The gastrointestinal toxicities were mild and reversible.
Conclusions: Transcatheter arterial infusion chemotherapy using a fine-powder formulation of cisplatin appears to have only modest activity, although the toxicity was also only mild, in patients with transcatheter arterial chemoembolization-refractory hepatocellular carcinoma.

Key words: hepatocellular carcinoma – transcatheter arterial infusion chemotherapy – cisplatin – transcatheter arterial chemoembolization

INTRODUCTION

Hepatocellular carcinoma (HCC) is treated by one or more of a wide variety of treatment options available, depending on the tumor characteristics, including the number and size of tumors, and the presence/absence of tumor thrombosis and extrahepatic metastases (1,2). In patients with early-stage HCC, curative therapies can be applied, including resection, liver transplantation or local ablation therapy. However, the prognosis of patients with HCC is still unsatisfactory, mainly because of the high frequency of recurrence post-therapy (3–9). Transcatheter arterial chemoembolization (TACE) has been performed for unresectable advanced HCC in patients who are unsuitable candidates for local ablation therapy or surgical treatment. To date, nine randomized control trials

(RCTs) of transcatherter arterial embolization or TACE versus best supportive care have been reported (10–18). Three of these RCTs and two meta-analyses have demonstrated a survival benefit of this treatment modality in HCC patients (10,16,17,19,20). On the basis of these results, TACE has been the most commonly employed treatment modality in patients with unresectable advanced HCC, especially those with intermediate-stage disease, who are unsuitable candidates for local ablation therapy (21). However, unfortunately, the disease eventually progresses to becoming refractory to TACE.

Transcatheter arterial infusion chemotherapy (TAI) could be expected to have better antitumor efficacy and lesser toxicity than systemic chemotherapy, because it is associated with only a local increase in the concentrations of anticancer drugs, and therefore, a lower incidence of systemic adverse effects. The reported response rates to TAI with a single agent vary in the range of 9–33% (22–25), and those to TAI using combination regimens vary in the range of 44–73% (26–29). Thus, TAI has high antitumor activity and is widely used in clinical practice, especially in Japan, although no survival benefit has been established yet, because no randomized studies of TAI have been conducted until date.

Cisplatin for Intra-arterial Injection (IA-call®, Nippon Kayaku Co., Ltd) is a powder formulation and represents an improvement over the standard liquid type of cisplatin formulation for intra-arterial administration. Since the solubility of this agent is 2.86 times higher than that of standard cisplatin, the injection time can be shortened. In a clinical study of this agent for advanced HCC, a favorable tumor response rate of 33.8% was reported (25), and this agent was approved for use in the treatment of HCC by the Ministry of Health, Labour and Welfare of Japan, in July 2004. However, it has not been clarified whether this agent might also be effective for TACE-refractory HCC. Therefore, we conducted a retrospective investigation of the efficacy and safety of TAI using cisplatin in patients with HCC refractory to TACE.

PATIENTS AND METHODS

Patients and Treatment

From July 2004 to September 2008, 84 consecutive patients with TACE-refractory HCC underwent TAI using cisplatin at the National Cancer Center Hospital, Tokyo, or the National Cancer Center Hospital East, Chiba, Japan. TACE-refractory tumors were defined as those showing an increase in size or <25% reduction in size of the hypervascular lesions visualized on dynamic computed tomography (CT) and/or magnetic resonance imaging (MRI) at 1 month after TACE (30).

TAI was performed by introducing a catheter into the proper, right or left hepatic artery, or another feeding artery by the Seldinger technique, and injecting cisplatin at the dose of 65 mg/m^2 over 20–40 min. Until the appearance of evidence of tumor progression and/or of unacceptable toxicity, the treatment was repeated every 4–6 weeks for up to six cycles. Antiemetic prophylaxis with a 5-hydroxytryptamine3 antagonist (granisetron 1 mg) plus dexamethasone 8 mg was used at the physician's discretion. Patients received adequate hydration for protection against cisplatin-induced renal dysfunction, and the urine output was carefully monitored, especially during the first 3 days after intra-arterial administration of cisplatin, and intravenous furosemide was administered if the output was judged to be inadequate. In principle, the cisplatin dose was reduced if the patient's creatinine clearance decreased to below 50 ml/min.

This retrospective study was conducted with the approval of the Institutional Review Board of the National Cancer Center and conducted in accordance with the ethical principles stated in Japanese ethics guidelines for epidemiologic studies.

Response and Toxicity Evaluations

The antitumor effect was evaluated by dynamic CT and/or MRI performed 1 month after each treatment cycle, and after the completion of six cycles, follow-up examinations were performed every 1–3 months. Responses were evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) (31). The best overall response was recorded for each patient. Progression-free survival was defined as the interval between the date of the initial TAI treatment using cisplatin and either the date of documentation of disease progression (either radiologic or symptomatic progression) or the date of death owing to any cause. Overall survival was measured from the date of the initial TAI treatment using cisplatin to the date of death or last follow-up. Survival curves were estimated using the Kaplan–Meier method. Toxicities were assessed using the Common Terminology Criteria for Adverse Events, version 3.0. Statistical analyses were performed using Dr SPSS II (SPSS Japan Inc., Tokyo, Japan).

RESULTS

Patient Characteristics

The baseline characteristics of the 84 patients enrolled in this study are shown in Table 1. The diagnosis of HCC was made either by histologic examination (44 patients, 52%), or distinctive findings on CT, MRI and/or angiography associated with elevated serum levels of α-fetoprotein or protein induced by vitamin K antagonist II (40 patients, 48%). Of the total, 42 patients each were classified as the Child–Pugh classes A and B, whereas there were no patients of the Child–Pugh class C. Twenty-six patients (31%) had tumor thrombosis in the main and/or first portal vein. Prior therapies other than TACE were hepatectomy (37 patients, 44%), local ablation therapy (33 patients, 39%), TAI (13 patients, 15%) and systemic chemotherapy (10 patients, 12%) with non-platinum-containing regimens. The median number of

Table 1. Patient characteristics ($n = 84$)

Age, median (range)	68 (37–82)
Gender, *n* (%)	
Male	69 (82)
Female	15 (18)
ECOG performance status, *n* (%)	
0	56 (67)
1	26 (31)
2	0 (0)
3	2 (2)
T factor[a]	
T1	2 (2)
T2	34 (40)
T3a	17 (20)
T3b	31 (37)
Portal vein tumor thrombosis, *n* (%)	
Present	26 (31)
Absent	58 (69)
Ascites, *n* (%)	
Present	24 (29)
Absent	60 (71)
Hepatitis virus marker status, *n* (%)	
HBsAg-positive	12 (14)
HCVAb-positive	55 (65)
Child–Pugh class, *n* (%)	
A	42 (50)
B	42 (50)
Number of previous TACE sessions	
Median (range)	4 (1–17)
Reason for TACE-refractory disease, *n* (%)	
Progressive disease	69 (82)
Stable disease (under 25% decrease)	15 (18)
AFP (ng/dl)	
Median (range)	660.2 (1.7–4 06 500)
PIVKA II (mAU/ml)	
Median (range)	600 (11–96 390)

ECOG, Eastern Cooperative Oncology Group; HBsAg, hepatitis B surface antigen; HCVAb, hepatitis C virus antibody; TACE, transcatheter arterial chemoembolization; AFP, α-fetoprotein; PIVKA, protein induced by vitamin K antagonist.
[a]T factor was evaluated according to Sobin et al. (32).

previous sessions of TACE was 4 (range 1–17), and the median period from the first TACE to the date on which the tumors were judged to be TACE-refractory was 15.8 months (range 1.0–78.0). The anticancer agents used for the previous TACE sessions were epirubicin in 79 patients, adriamycin in 17 patients and mitomycin C in 5 patients.

Treatment Delivery and Efficacy

In total, 167 cycles of TAI were administered to the 84 patients, with a median of one cycle (range 1–7) per patient. The median cisplatin dose per treatment session was 100 mg (range 50–135). A total of 83 patients received the standard dose of cisplatin in the first session, and the remaining one patient required a 50% reduction in the dose of cisplatin even from the first treatment cycle because of pre-existing renal dysfunction.

Of the study population, one patient showed complete response and two showed partial response, representing an overall response rate of 3.6% [95% confidence interval (CI), 0.7–10.1]. Stable disease was noted in 38 patients and progressive disease in 41 patients. The remaining two patients were not evaluable as they were lost to follow-up. After treatment discontinuation, 50 (60%) patients received supportive care only, 32 (38%) received additional anticancer therapy and 2 (2%) were lost to follow-up. The additional anticancer therapies were TACE with epirubicin or mitomycin in 18 patients, TAI using non-platinum drugs in 7 patients (including 5-fluorouracil with systemic interferon in 3 patients, epirubicin in 3 patients and zinostatin-stimalamer in 1 patient), systemic chemotherapy in 5 patients (including S-1, i.e. a mixture of tegafur, 5-chloro-2,4-dihydropyrimidine and potassium oxonate, in 3 patients and uracil–tegafur plus mitoxantrone in 2 patients) and immunotherapy in 2 patients. By the time of the analysis, except for eight patients who were still alive but showed disease progression, all of the patients had died. The median progression-free survival was 1.7 months (95% CI, 1.1–2.3) and the median overall survival was 7.1 months (95% CI, 4.9–9.3), with a 1-year survival rate of 27% (Fig. 1).

Adverse Events

Data of all 84 patients were analyzed for adverse events. The adverse events are summarized in Table 2. In regard to the hematologic adverse events, thrombocytopenia was the most common, with 12 (14%) patients developing Grade 3 or 4 thrombocytopenia; however, none of the patients required platelet transfusions. Grade 3 or 4 leukopenia and neutropenia occurred in only 6 and 4% of the patients, respectively. There were no events of febrile neutropenia.

The main non-hematologic adverse events were elevation of the serum aspartate aminotransferase (AST) and serum alanine aminiotransferase (ALT). Grade 3 or 4 elevation of the AST and ALT was observed in 33 (39%) and 5 (6%) patients, respectively. Gastrointestinal adverse events, such as nausea, vomiting and anorexia, were frequently observed after intra-arterial administration of cisplatin, but most were transient and manageable with appropriate medical treatment, such as antiemetic drug administration and intravenous hydration. There was no serious renal toxicity. Four patients died within 30 days of the last treatment session: two of disease progression, one of acute coronary syndrome,

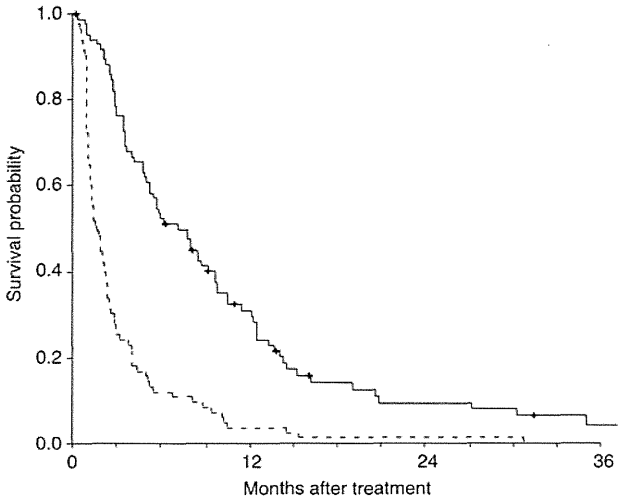

Figure 1. Overall survival (continuous line) and progression-free survival (dotted line) in the 84 patients. The marks on the curve represent censored cases.

Table 2. Adverse events

	No. of patients				Gr 3/4 (%)
	Gr 1	Gr 2	Gr 3	Gr 4	
Hematologic toxicity					
Leukocytopenia	30	29	5	0	6.0
Neutropenia	11	24	3	0	3.6
Anemia	55	18	6	1	8.3
Thrombocytopenia	36	22	12	0	14.3
Non-hematologic toxicity					
Anorexia	45	16	3	0	3.6
Nausea	40	9	3	0	3.6
Vomiting	11	6	0	0	0
Fatigue	59	11	3	0	3.6
Diarrhea	3	1	0	0	0
Constipation	20	0	0	0	0
Hypoalbuminemia	38	41	1	0	1.2
Elevated serum total bilirubin	28	33	4	1	6.0
Elevated serum aspartate aminotransferase	20	26	31	2	39.3
Elevated serum alanine aminotransferase	37	30	4	1	6.0
Elevated serum alkaline phosphatase	53	15	1	0	1.2
Elevated serum creatinine	12	1	0	0	0

Gr, grade.

showing no causal relationship with the treatment, and the remaining one due to known pulmonary artery tumor embolism.

DISCUSSION

In the current study, the response rate to TAI using cisplatin was only 3.6% in patients with TACE-refractory HCC. Moreover, the median progression-free survival of only 1.7 months was extremely disappointing. The efficacy of TAI using cisplatin for advanced HCC limited to TACE-refractory tumors was much worse than that reported from a previous Phase II study in patients with advanced HCC (response rate, 33.8%) (25). One possible explanation for this discrepancy in the response rate may be the differences in the characteristics of the enrolled patients between the two studies. Most patients in the previous Phase II trial were TACE-naïve, whereas only patients with TACE-refractory disease were included in the current study. In our previous study (30), TAI using epirubicin was reported to have unfavorable efficacy in a subset of patients with TACE-refractory HCC (response rate, 5%). When HCC is treated by TACE and/or becomes resistant to TACE, it might acquire resistance to cytotoxic agents, such as cisplatin or epirubicin. Furthermore, to select suitable candidates for this treatment, the predictive factors for disease control and survival for more than 12 months were also investigated, but could not be clarified (data not shown). Therefore, TAI using cisplatin or epirubicin cannot be recommended at present for this patient population in clinical practice.

Recently, systemic chemotherapy has become an important treatment modality for advanced HCC, because two RCTs (the SHARP trial and the Asia-pacific trial) of sorafenib versus placebo demonstrated significantly improved time-to-progression and overall survival in the drug-treated group, although sorafenib yielded a far-from-satisfactory response rate of only 2.3–3.3% (33,34). On the basis of the results of these RCTs, sorafenib is acknowledged as a standard agent for systemic chemotherapy in patients with advanced HCC. The efficacy of sorafenib for advanced HCC refractory to TACE has not yet been clarified, but in both of the aforementioned studies, the results of exploratory subgroup analyses in patients treated previously by TACE were reported. In the subset of patients with a previous history of treatment by TACE in the SHARP trial, the disease control rate (DCR) was significantly greater in the patients who were treated with sorafenib (44.2%) than in those who had received placebo (34.4%) (35). In addition, a trend towards a beneficial effect of sorafenib was also observed in relation to the median overall survival in this subpopulation of patients {11.9 vs. 9.9 months [hazard ratio (HR), 0.75; 95% CI, 0.49–1.14]}. In the Asia-pacific trial, 41% of the enrolled patients had a previous history of undergoing TACE. The DCR for sorafenib (24.6%) in these patients was higher than that for placebo (9.1%) (36). Moreover, a tendency [HR for death was 0.84 (95% CI, 0.52–1.36)] towards favorable overall survival was also noted in the HCC patients with a previous history of TACE treated with sorafenib when compared with that in the same subpopulation of patients who received placebo. Sorafenib appeared to benefit patients with

advanced HCC, regardless of whether or not they had previously been treated by TACE. Thus, molecular-targeted agents, including sorafenib, which exhibit mechanisms of action different from those of cytotoxic agents, may be superior for the treatment of HCC refractory to TACE. Therefore, patients with TACE-refractory HCC are receiving new molecular-targeted agents in clinical trials, and sorafenib is used as the standard agent for the treatment of advanced HCC in clinical practice.

In the current study, the most common Grade 3 and 4 adverse events were elevated AST, thrombocytopenia and anemia, which frequently also reflected the underlying cirrhosis. In terms of the gastrointestinal toxicities, only 4% of the patients experienced Grade 3 anorexia and nausea, and the symptoms resolved within a few days. Thus, the gastrointestinal toxicities were mild and manageable in the current study. There was no need for dose reduction or discontinuation of cisplatin on account of development of toxicities, except in one patient each with Grade 2 elevation of the serum creatinine and Grade 2 fatigue. Thus, advanced HCC patients showed good overall tolerability to TAI using cisplatin, which has also been reported to show favorable efficacy in these patients (25); in our study confined to TACE-refractory patients, however, the treatment showed only modest antitumor activity. TAI using cisplatin may therefore be easy to administer in combination with some molecular-targeted agents, such as sorafenib, since its toxicity is generally mild and its toxicologic profile is distinct from that of sorafenib.

In conclusion, TAI using cisplatin appeared to have only modest activity against TACE-refractory HCC, although this treatment was feasible and well tolerated. Further development of novel treatments is necessary to improve the prognosis of patients with TACE-refractory HCC.

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Conflict of interest statement

None declared.

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Phase I/II Study of Radiologic Hepatic Arterial Infusion of Fluorouracil Plus Systemic Irinotecan for Unresectable Hepatic Metastases from Colorectal Cancer: Japan Clinical Oncology Group Trial 0208-DI

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ABSTRACT

Purpose: Treatment of patients who have metastatic colorectal cancer (CRC) by using a combination of hepatic arterial infusion chemotherapy (HAIC) and systemic chemotherapy has resulted in promising clinical outcomes. Additionally, image-guided HAIC is reported to be less invasive and distribute drugs more accurately than surgical HAIC. The purpose of this study was to assess the combination of image-guided delivery of fluorouracil through HAIC and systemic irinotecan in a multicenter phase I/II study.

Materials and Methods: Twenty-five patients with unresectable liver metastases from CRC were fitted with hepatic arterial catheter and port systems by using image-guided methods. Intraarterial fluorouracil (1,000 mg/m^2) was administered on days 1, 8, and 15 of each treatment cycle. The dose of systemic irinotecan on days 1 and 15 was escalated from 75 mg/m^2.

Results: No dose-limiting toxicity was encountered during phase I, and the recommended dose of irinotecan was set at 150 mg/m^2. Grade 3 or higher adverse events included hyperglycemia (15%), elevated γ-glutamyl transpeptidase levels (15%), and neutropenia (9%). The response rate and median survival time were 72% and 49.8 months (95% CI, 27.5–78.1 mo), respectively.

Conclusions: The combination of image-guided delivery of fluorouracil through HAIC and systemic irinotecan yielded favorable safety, response rate, and survival results. This combination should be evaluated in a large study.

ABBREVIATIONS

AE = adverse event, CRC = colorectal cancer, DLT = dose-limiting toxicity, DSA = digital subtraction angiography, HAIC = hepatic arterial infusion chemotherapy, MTD = maximum tolerated dose, OS = overall survival, RD = recommended dose, WBC = white blood cell

Modern chemotherapy with the use of active agents, such as irinotecan, oxaliplatin, and molecular-targeted therapies, has significantly prolonged the survival of patients with metastatic colorectal cancer (CRC) (1,2). However, achieving complete response and long-term survival is still rare, even with intensive therapy with combinations of these agents.

Although hepatic arterial infusion chemotherapy (HAIC) with fluorinated pyrimidines has demonstrated high local response rates for CRC liver metastases, 10 of 11 randomized

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Table 1. Eligibility Criteria

Inclusion criteria

1. Histologically documented colorectal cancer
2. Unresectable liver-limited metastases as determined by imaging studies
3. Previous resection of primary tumor with D1 or D2 lymph node dissection
4. No previous chemotherapy except adjuvant chemotherapy with fluoropyrimidines completed > 3 mo before study
5. At least one measurable tumor in the liver per RECIST (version 1.0)
6. Between 20 and 70 y of age
7. ECOG performance status of 0–2
8. Adequate hematological, hepatic, renal, and cardiac functions
9. Written informed consent

Exclusion criteria

1. Massive ascites or pleural effusion
2. Active gastrointestinal bleeding
3. Active infection
4. Watery diarrhea
5. Severe comorbid conditions
6. Other untreated cancers
7. Previous abdominal radiotherapy
8. Positive serum hepatitis B antigen or hepatitis C antibody
9. Allergy to iodinated contrast material
10. Severe mental disorder
11. Previous catheter placement into the hepatic artery
12. Pregnancy or nursing

ECOG = Eastern Cooperative Oncology Group, RECIST = Response Criteria in Solid Tumors.

controlled trials published before 2006 did not find any survival benefit of HAIC greater than that of systemic chemotherapy (3–13). Metaanalyses of HAIC studies have also demonstrated that HAIC does not improve survival in patients with CRC (14–16). Consequently, HAIC is not generally considered a first-line treatment or a component of standard treatment regimens.

Laparotomy was employed for HAIC catheter and pump placement in all previous randomized controlled trials of HAIC in Western countries. In Japan, on the contrary, a percutaneous technique for hepatic arterial catheter and port placement was developed in the 1980s and was established in the 1990s as an image-guided interventional radiologic procedure, with drug distribution evaluated by using contrast-enhanced computed tomography (CT) via the indwelling catheter–port system (17–20). The advantages of this technique are that it is minimally invasive and provides accurate periodic evaluation of drug delivery. In addition, HAIC treatment outcomes with this technique are favorable; phase II studies (17,21–23) of intermittent HAIC with fluorouracil in patients with CRC liver metastases with or without extrahepatic metastasis had median survival times of 18.6–26 months. HAIC treatment success requires monitoring of drug distribution to ensure that the administered drug is delivered directly to all liver tumors without reaching extrahepatic organs (20).

Kemeny et al (24) reported a phase I study of HAIC with floxuridine and dexamethasone combined with systemic irinotecan that was or was not followed with cryosurgery. The study demonstrated a response rate of 74% and a median survival time of 17 months in patients who did not undergo cryosurgery. In their study, however, surgical laparotomy was used for implantation instead of a radiologic intervention (24), and the drug and administration schedules were different from those of Japanese phase II studies. Thus, we conducted a multicenter phase I/II study to assess the feasibility, safety, and preliminary efficacy of image-guided delivery of fluorouracil through HAIC combined with systemic irinotecan.

MATERIALS AND METHODS

Patients

Inclusion and exclusion criteria are listed in **Table 1**. A Consolidated Standards of Reporting Trials diagram of this study is shown in **Figure 1**. The study protocol was approved by the institutional review boards of all participating institutions. All patients provided written informed consent. This study was registered to UMIN-CTR (UMIN C000000051, 2005/08/08).

Treatment

Placement of Intraarterial Catheter and Port System. A catheter and port system was implanted within 2 weeks of enrollment in the study. Details of the procedure are described elsewhere (19,25). In brief, percutaneous implantation of a catheter and port system was performed under local anesthesia by using an interventional radiologic technique. Before each cycle of chemotherapy, drug delivery was evaluated by digital subtraction angiography (DSA) and CT angiography through the implanted catheter and port system (**Fig 2**).

Chemotherapy Administration. After implantation of the catheter and port system, chemotherapy was started when the patient's laboratory values were as follows: white blood cell (WBC) count of at least 4,000/mm^3 and no greater than 12,000/mm^3, platelet count of at least 100,000/mm^3, aspartate aminotransferase and alanine aminotransferase levels no greater than three times the upper limit of normal, bilirubin level no greater than 1.5 mg/dL, and serum creatinine level no greater than 1.5 mg/dL. Patients received concurrent systemic chemotherapy and HAIC in 4-week cycles, and the treatment protocol was considered to be complete after five cycles of this regimen. In each cycle, 1,000 mg/m^2 of fluorouracil in saline solution plus 100 mg of hydrocortisone were administered on days 1, 8,

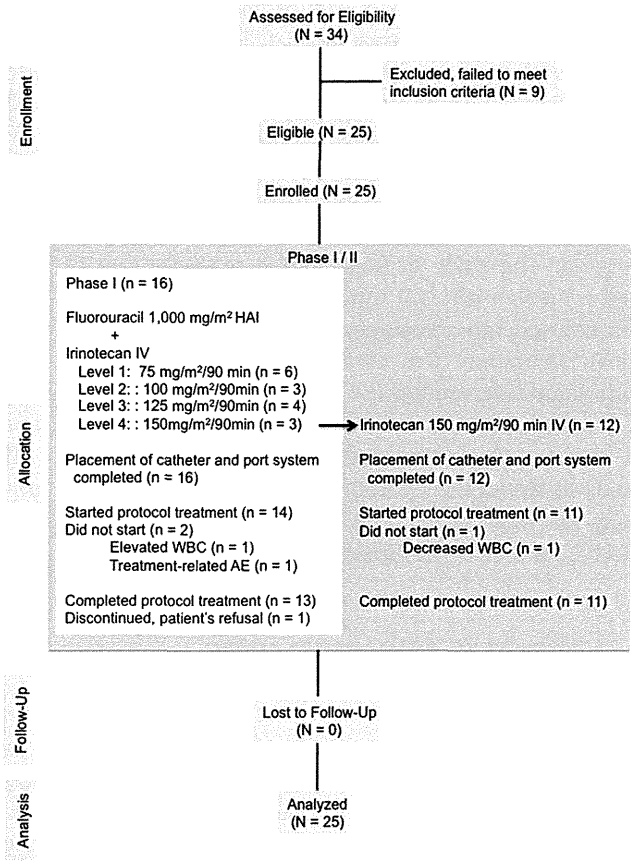

Figure 1. Consolidated Standards of Reporting Trials diagram. HAI = hepatic arterial infusion. (Available in color online at *www.jvir.org.*)

and 15 by continuous 5-hour infusion via a disposable balloon pump system. This dose was determined based on a previous phase I/II study of HAIC with fluorouracil (23). On days 1 and 15, following HAIC, irinotecan diluted in 5% glucose was administered via a 90-minute intravenous drip. The irinotecan doses planned for phase I of the trial were 75, 100, 125, and 150 mg/m^2. After the maximum tolerated dose (MTD) was determined, the study was advanced to phase II.

Patient and Tumor Evaluations

Pretreatment evaluations included medical history, physical examination, and laboratory examinations. Laboratory examinations included evaluation of complete blood counts, total bilirubin, alkaline phosphatase, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, and carcinoembryonic antigen. Baseline evaluation of tumors was performed by contrast-enhanced CT scans of the chest and abdomen. During the course of treatment, each patient was assessed weekly for toxicity, including laboratory determination of complete blood counts, and blood chemistry. CT examination was planned before treatment and after one, three, and five cycles of treatment. Patient responses to treatment were evaluated by three radiologists based on Response Evaluation Criteria In Solid Tumors, version 1.0. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria, version 2.0.

Study Design and Statistical Analysis

This trial was designed as a phase I/II study. The primary endpoints of phase I were to identify dose-limiting toxicities (DLTs), MTD, and the recommended dose (RD) of systemic irinotecan when combined with HAIC that uses a fixed dose of fluorouracil. DLTs in phase I were defined as any grade 4 neutropenia or thrombocytopenia or any nonhematologic toxicity of grade 3 or more severe. We treated patients in cohorts of three to six. The first cohort received the lowest dose (ie, dose level 1) of irinotecan, and doses were escalated in a stepwise fashion. If DLTs were observed in less than one third of the cohort members, subsequent patients were treated at the next dose level. If more than one third of cohort members developed DLTs, the preceding dose level was identified as the MTD.

Based on the results of previous studies, 12 patients were needed in this study with a null proportion of 30%–45% and an alternative proportion of 74% to achieve 80% power, given that the one-sided significance level was 10% (24).

Secondary endpoints of the study included HAIC initiation rate, overall response rate, response rate in the liver, and toxicity. Survival analysis was performed by using the Kaplan–Meier method. Demographics and baseline variables were summarized by using descriptive statistics. Statistical significance was set at 0.05, and differences between groups were examined by using two-tailed *t* tests. We used SPSS software (version 17; SPSS, Chicago, Illinois) to perform all statistical analyses.

RESULTS

Patient Demographics

Twenty-five patients from five participating institutions were enrolled between November 2003 and March 2008. Patient characteristics are listed in **Table 2**. Synchronous liver metastases were seen in 84% of the patients, and 92% of the patients had not received previous adjuvant chemotherapy.

Initiation of HAIC and Systemic Chemotherapy

A catheter and port system was successfully placed in all 25 patients. Catheters were inserted via the left subclavian artery in all patients. Treatment consisting of HAIC and systemic chemotherapy was initiated according to the study protocol in 22 patients (88%; **Fig 1**). Treatment was not started in three patients as a result of elevated WBC count (n = 1), decreased WBC count (n = 1), and cerebral infarction that was presumably caused by catheter placement (n = 1). The elevated WBC count observed in one patient at dose level 1 and the decreased WBC count

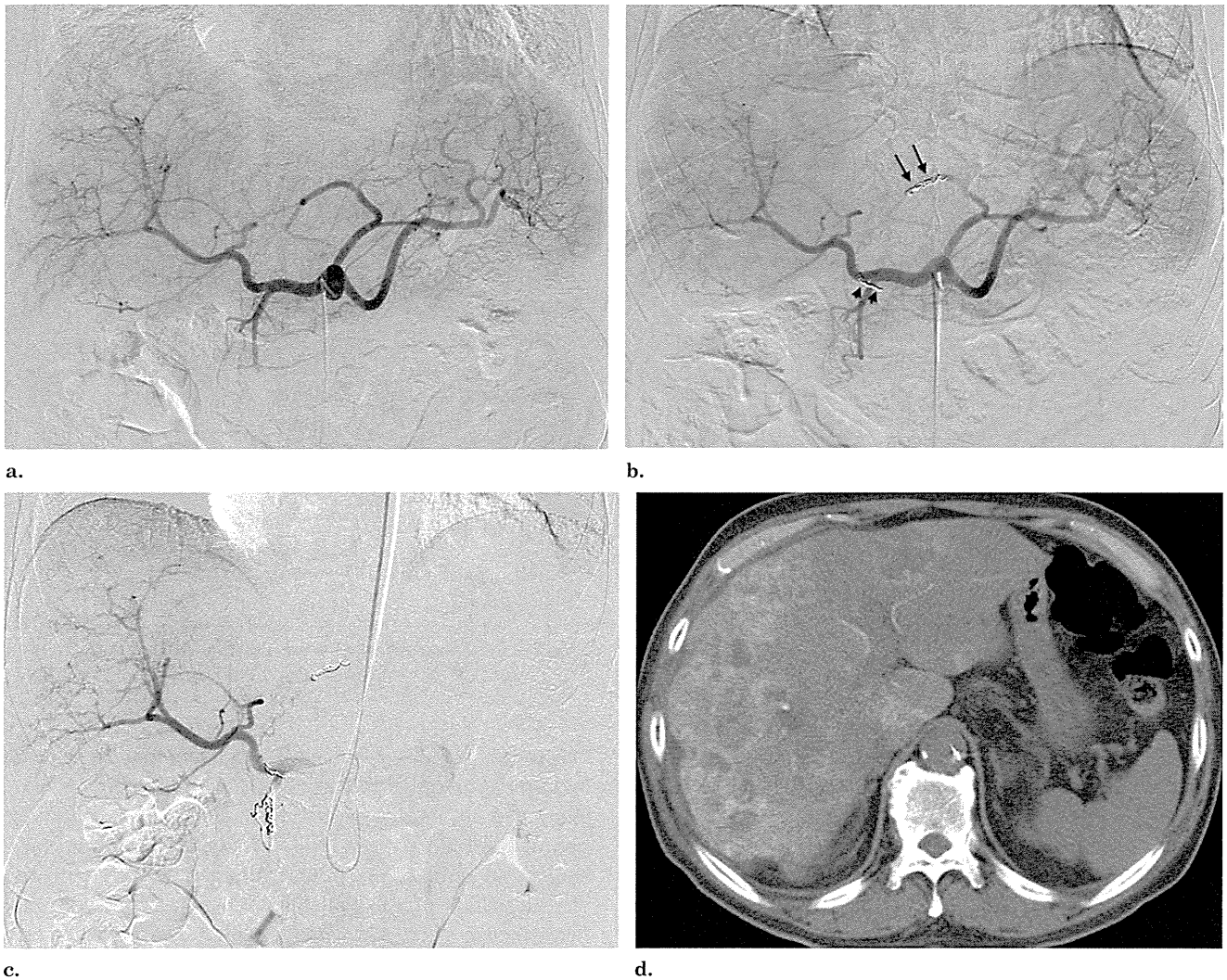

Figure 2. Image-guided insertion of catheter and port system for HAIC. **(a)** DSA of the celiac artery. The left hepatic artery is arising from left gastric artery (ie, replaced left hepatic artery). **(b)** DSA after embolization of the replaced left hepatic artery (long arrows) and right gastric artery (short arrows). The left hepatic artery is visualized from the collateral vessels. **(c)** DSA via the implanted port. The indwelling catheter is implanted via the left subclavian artery. **(d)** CT angiography via the implanted port. Adequate drug distribution is confirmed with enhancement of hepatic metastases by contrast material injected through the implanted port.

observed in one patient at dose level 4 were likely related to the primary disease process, because no clinical findings of infection were found. These two patients were removed from the study and treated by HAIC alone. Details of the patient who developed cerebral infarction are described in the Safety section. The HAIC initiation rate was 96%, including the two patients who were later removed from the study.

Dose-escalation Findings

In phase I, one of six patients developed DLT at dose level 1 (**Fig 1**). Of the 14 patients who started the treatment protocol, 13 patients completed five cycles. Because no DLT was encountered at dose levels 2–4, we were unable to determine the MTD of irinotecan. Dose level 4 (150 mg/m^2) was selected as the RD for phase II of the study.

Safety

There were no treatment-related deaths in this study. The incidence of grade 2 or higher adverse events (AEs) occurring during chemotherapy is shown in **Table 3**. In 106 cycles of protocol treatment, the following grade 3 or higher AEs occurred: leukopenia (2%), neutropenia (9%), elevated γ-glutamyl transpeptidase level (15%), hyperglycemia (15%), and hypokalemia (1%). The only grade 4 AE was neutropenia (2%).

Before the initiation of chemotherapy, one patient (4%) developed central nervous system ischemia. One day after placement of the catheter and port system, the patient developed hemiparesis, and magnetic resonance imaging confirmed multiple cerebral infarctions. The patient subsequently had moderate hemiparesis, but no other neurologic deficits. The indwelling catheter was thought to have caused the cerebral infarctions.

Table 2. Patient Characteristics (N = 25)

Characteristic	Value
Age (y)	
Median	63
Range	45–70
Sex	
Male	21 (84)
Female	4 (16)
ECOG performance status	
0	24 (96)
1	1 (4)
Location of primary tumor	
Colon	13 (52)
Rectum	12 (48)
Differentiation	
Well	7 (28)
Moderate	15 (60)
Poor	3 (12)
Synchronous	
Yes	21 (84)
No	4 (16)
Liver involvement	
< 30%	22 (88)
30%–60%	3 (12)
> 60%	0
Previous adjuvant chemotherapy	
Yes	2 (8)
No	23 (92)

Values in parentheses are percentages. ECOG = Eastern Cooperative Oncology Group.

Response

A total of 25 patients were included in the response analyses. The overall response in the liver was 72%, and included four complete responses (16%) and 14 partial responses (56%). Four patients (16%) exhibited stable disease in the liver, and the responses of three patients (12%) could not be evaluated because the treatment protocol was not initiated. During the course of treatment, no patients developed any observable extrahepatic metastases. Therefore, the overall response rate was 72%.

Survival

Survival analysis was conducted based on all 25 patients (**Fig 3**). With a median follow-up period of 55.0 months (range, 22.8–87.7 mo), the median overall survival (OS) time was 49.8 months (95% CI, 27.5–78.1 mo).

DISCUSSION

The present study is a prospective trial to evaluate image-guided HAIC combined with systemic chemotherapy for patients with unresectable hepatic metastases from CRC.

Table 3. Per-patient Incidence of Grade 2 or Higher Adverse Events in All Cycles of Chemotherapy (N = 106)

Adverse Event	Grade 2	Grade 3	Grade 4
Nausea	3 (3)	0	0
Diarrhea	8 (8)	0	0
Stomatitis	1 (1)	0	0
Fatigue	3 (3)	0	0
Alopecia	12 (11)	0	0
Vertigo	0	1 (1)	0
Glycosuria	2 (2)	0	0
Cystitis	2 (2)	0	0
Leukopenia	21 (20)	2 (2)	0
Neutropenia	15 (14)	7 (7)	2 (2)
Anemia	10 (9)	0	0
Thrombocytopenia	2 (2)	0	0
Hyperbilirubinemia	1 (1)	0	0
GGT	11 (10)	16 (15)	0
ALP	3 (3)	0	0
Hyperglycemia	26 (25)	16 (15)	0
Hypokalemia	0	1 (1)	0

Values in parentheses are percentages. ALP = alkaline phosphatase, GGT = γ-glutamyl transpeptidase.

Our results demonstrate that this treatment may be effective and safe as an initial therapy, as 23 of the 25 patients in the study had not undergone previous chemotherapy. Twenty-one of these 23 patients were enrolled in the study after surgery to remove the primary tumor. Other noteworthy characteristics of the patients in this trial include good performance status (24 patients with a performance status of 0) and moderate tumor involvement in the liver (22 patients with < 30% involvement). To summarize, the characteristics of this patient cohort were resectable primary tumor, synchronous and unresectable liver metastases of moderate intrahepatic extent, good performance status, and limited previous chemotherapy.

We determined the feasibility and the safety of image-guided HAIC combined with systemic irinotecan. Generally, AEs caused by fluorouracil are well tolerated, and bone marrow suppression is not significant. Given that systemic irinotecan has a different toxicity profile than fluorouracil, and the intraarterial effects of fluorouracil were minimal in the present study, it could be surmised that the RD of irinotecan in this study should be the same as the standard RD of 150 mg/m^2 used in Japanese patients. However, modification of the usual treatment schedule of weekly fluorouracil HAIC to a schedule that included a 1-week treatment-free interval in the fourth week of each treatment cycle may account for the minimal hematologic toxicity we observed and the undetermined MTD (23). During phase II of this trial, grade 3 AEs were observed in 21 of 60 treatment cycles (35%), and no grade 4 AEs occurred. Furthermore, all patients, except one who did not meet the criteria for the initiation of chemotherapy, completed the planned five cycles of treatment. Therefore, this

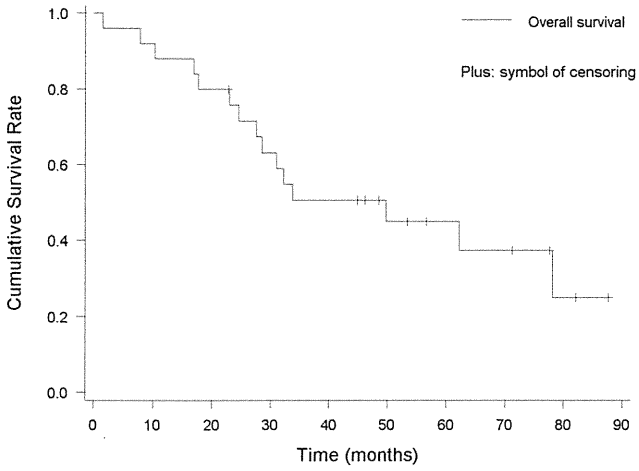

Figure 3. Graph of OS. The median OS time is 49.8 months (95% CI, 27.5–78.1 mo).

study demonstrated that image-guided HAIC with fluorouracil combined with systemic irinotecan (150 mg/m^2) is feasible and safe.

Hyperglycemia was a notable AE in this study. Hyperglycemia occurred in 56% of the 106 treatment cycles. The incidence of hyperglycemia in phase I was almost the same as in phase II. As there have been no reports of hyperglycemia as a result of irinotecan therapy, and intraarterial hydrocortisone (100 mg) was administered with fluorouracil on days 1, 8, and 15 to reduce vascular endothelial injury, this intraarterial hydrocortisone may have influenced the occurrence of hyperglycemia. There is some possibility that intraarterial direct administration of fluorouracil to the liver leads to deterioration of the glucose tolerance of the liver. Hyperglycemia does not directly alter the short-term patient prognosis, but it may become more important if longer survival is achieved with this treatment.

The HAIC initiation rate in the present study is comparable to rates seen in previous studies of image-guided HAIC. Tanaka et al (26) reported a technical success rate of 99.8% among 426 patients undergoing image-guided HAIC. Deschamps et al (27) reported a technical success rate of 94% among 93 patients. Moreover, Ganeshan et al (25) mentioned in their review of HAIC that interventional radiology played a vital role in establishing vascular access and assessing outcomes. On the contrary, the technical success rates of surgical HAIC, a technique widely employed in published randomized controlled trials, are not included in the reports of these trials or result in lower HAIC initiation rates than seen with image-guided HAIC. Kerr et al (10) reported an HAIC initiation rate of 68% following the surgical procedure used in their randomized study comparing HAIC with systemic chemotherapy. In the present small, prospective study, the HAIC initiation rate was 96%. This suggests that image-guided catheter placement is suitable for the initiation of HAIC.

In the present study, one patient developed cerebral infarction after catheter implantation. There have been several reports of cerebral ischemia as a complication of catheter implantation via the subclavian artery, and the incidence of this complication is approximately 5% (28,29). This complication should therefore be recognized as a severe AE caused by radiologic catheter placement via the subclavian artery, and other access routes such as the femoral or hypoepigastric artery should be considered.

The liver response rate of 72% we observed is similar to those of other studies of image-guided deliver of fluorouracil with the same infusion protocol through HAIC (17,21,22). This indicates that the addition of systemic irinotecan might not affect tumor response in the liver. However, previous studies have demonstrated an incidence of extrahepatic metastases of approximately 70% when patients were treated with HAIC alone. In the present study, no extrahepatic metastases were observed during the study. Therefore, systemic irinotecan may have reduced the occurrence of extrahepatic metastases. Because more than 90% of the fluorouracil administered via the hepatic artery is reported to pass through the liver and enter systemic circulation (30), irinotecan combined with fluorouracil may have prevented extrahepatic metastases in the present study.

It is notable that the median OS of the present study exceeded 4 years. The large proportion of patients with good PS may be a prominent factor in this result. Standard first- and second-line systemic chemotherapies have demonstrated a median survival of 18–24 months (31–34). Concerning the combination of HAIC with systemic chemotherapy, Kemeny et al (35) reported an OS of 36 months with fluorodeoxyuridine HAIC plus systemic oxaliplatin and irinotecan, and an OS of 22 months with fluorodeoxyuridine HAIC plus systemic oxaliplatin, fluorouracil, and leucovorin. Ducreux et al (36) reported an OS of 27 months with HAIC of oxaliplatin plus systemic irinotecan and fluorouracil. Therefore, HAIC combined with systemic chemotherapy may prolong the survival of patients with unresectable liver metastases from CRC.

The present study has several limitations. First, it was a phase I/II study involving a small number of patients. Second, we did not record postprotocol treatment. Therefore, OS may have been influenced by modern systemic chemotherapy with fluorouracil, leucovorin, oxaliplatin, irinotecan, and molecular-targeting agents. However, the OS observed in the present study is still a promising result. Thus, accurate HAIC that uses CT angiography for appropriate drug distribution in combination with systemic chemotherapy may lead to improved patient outcomes.

The present study demonstrates the feasibility of HAIC as an interventional procedure and that HAIC of fluorouracil combined with systemic irinotecan at 150 mg/m^2 is well tolerated. Also, the OS exceeding 4 years was a promising result, although it may have been affected by the treatments after the protocol. In conclusion, this combination chemotherapy should be evaluated in a larger study.

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IGFBP7 downregulation is associated with tumor progression and clinical outcome in hepatocellular carcinoma

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Insulin-like growth factor-binding protein 7 (IGFBP7) functions in several cellular processes including proliferation, senescence and apoptosis. This study analyzed IGFBP7 function in hepatocellular carcinoma (HCC) cells by gene manipulation and investigated the prognostic significance of IGFBP7 expression in clinical HCC samples. In this study, we investigated changes in malignant potential such as cell growth and invasiveness in an HCC cell line, PLC/PRF/5, after transfection with shRNA against *IGFBP7*. The extent of apoptosis and cell cycle progression were examined after the transfection. The correlation between immunohistochemically determined IGFBP7 expression and long-term postoperative prognosis after curative resection was also investigated in clinical HCC specimens obtained from 104 patients. PLC/PRF/5 cells transfected with shRNA against *IGFBP7* showed significantly more rapid growth and stronger invasiveness than control cells. Annexin V assays showed that the IGFBP7-depleted cells were significantly more resistant to apoptosis than the control cells, and showed decreased expression of cleaved caspase-3 and PARP. Cell cycle progression was more rapid in the IGFBP7-suppressed cells. In clinical HCC specimens, IGFBP7 expression was judged as positive in 67 patients (64.4%) and negative in the remaining 37 patients (35.6%). The IGFBP7 downregulation correlated significantly with poor postoperative prognosis, and IGFBP7 status was identified as an independent significant prognostic factor. Our results indicated that IGFBP7 expression correlated significantly with the malignant potential in HCC cells, suggesting that the expression could be a useful prognostic marker for HCC.

Hepatocellular carcinoma (HCC) is a common malignancy worldwide, but especially in Japan and other East Asian countries.[1,2] Although surgery plays a major role in the treatment of HCC, less than 30% of patients with HCC are surgical candidates owing to limiting factors such as severe impairment of reserve hepatic function, bilobar tumor distribution and extrahepatic metastasis. Additionally, no effective chemotherapy regimens have been established for treating HCC.[3] Thus, no effective therapy can be offered in many cases of HCC. Such dismal prognosis is not always predicted by conventional prognostic indicators such as vascular invasion, tumor multiplicity and tumor size.[4–6] New indicators are thus clearly needed.

Insulin-like growth factor binding protein 7 (IGFBP7), which is also known as IGFBP-rP1 and MAC25, has been implicated in several cellular processes such as proliferation, senescence and apoptosis. IGFBP7 also shows tumor suppressive activity through the induction of apoptosis and it is downregulated in some cancers.[7–13] In addition, several studies found a significant association between IGFBP7 and not only apoptosis, but also prognosis, in some kinds of cancers including colorectal and breast cancer.[14,15] However, the functional significance of IGFBP7 in HCC remains unclear.

This study analyzed the function of IGFBP7 in HCC cells in gene manipulation experiments, and investigated the prognostic significance of IGFBP7 expression in clinical HCC samples by immunohistochemical analysis of resected specimens.

Key words: hepatocellular carcinoma (HCC), insulin-like growth factor binding protein 7 (IGFBP7), apoptosis

Abbreviations: 95% CI: 95% confidence interval; AFP: alpha-fetoprotein; Anti-HCV Ab: anti-hepatic C virus antibody; DFS: disease-free survival; HBs-Ag: hepatitis B surface antigen; HCC: hepatocellular carcinoma; IGFBP7: insulin-like growth factor binding protein 7; OR: odds ratio; OS: overall survival; PBGD: porphobilinogen deaminase; pERK: phoshorylated ERK; PI: propidium iodide; PIVKA-II: protein induced by vitamin K absence or antagonists-II; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; shRNA: short hairpin RNA

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Material and Methods

HCC cell lines and clinical tissue specimens

Four human HCC cell lines, PLC/PRF/5, HuH7, HLE and HepG2 were obtained from the Japan Cancer Research

Resources Bank (Tokyo, Japan). These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2 in air.

Surgical specimens were obtained from 104 patients with HCC who underwent curative hepatic resection in the Osaka University Hospital from 2000 to 2007 after informed consent in accordance with the institutional ethical guidelines of Osaka University. Curative resection was defined as complete removal of all macroscopically evident tumors. Patients who underwent transarterial chemoembolization preoperatively were excluded from this study. After hepatic resection, the patients were followed up at regular intervals of 3–4 months with physical examination, assaying of tumor markers including alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonists-II (PIVKA-II), liver biochemistry testing, abdominal ultrasonography and abdominal computed tomography. The median duration of clinical follow-up after the initial hepatectomy was 3.5 ± 2.3 years.

Drugs and reagents

A polyclonal goat anti-human IGFBP7 antibody and polyclonal rabbit anti-human IGFBP7 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used for immunohistochemistry and western blot analysis, respectively. Antibodies to caspase-3, cleaved caspase-3, PARP, cleaved PARP, ERK, phoshorylated ERK (pERK), cyclin D1 and p27 were purchased from Cell Signaling Technology (Beverly, MA), antibodies to cyclin E and p21 were purchased from Santa Cruz Biotechnology, and an antibody to actin was purchased from Sigma-Aldrich Co. (Louis, MO).

Plasmids and transfection

Plasmid coding for short hairpin RNA (shRNA) against *IGFBP7* and *IGFBP7* expression plasmid were purchased from OriGene Technologies (Rockville, MD) and used to transfect HCC cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the instructions provided by the manufacturer. After transfection of the shRNA plasmid and the *IGFBP7* expression plasmid into the HCC cells for 24 hr, stable transfectants were selected and maintained in 1.0 μg/ml of puromycin (Sigma-Aldrich, St. Louis, MO) and 600 μg/ml of G418 (Gibco-BRL, Grand Island, NY), respectively. The control vector plasmid expressing non-effective shRNA was similarly introduced into cells to establish negative control cells for the shRNA plasmid experiments. Empty vector plasmid was also similarly used to establish negative control cells for the *IGFBP7* expression plasmid for the *IGFBP7* expression plasmid experiments.

Cell proliferation assay

Cells were uniformly seeded (4×10^4/well for PLC/PRF/5 and 2×10^4/well for HuH7) in triplicates into 24-well dishes (Day 0). Cells were counted using a CellTac kit (Nihon Koden, Tokyo, Japan) on Days 1–5.

Real-time quantitative reverse transcription-polymerase chain reaction

Total RNA isolated from cells was prepared using TRIzol reagent (Invitrogen), and reverse transcription was performed with SuperScript II (Invitrogen) based on the protocols supplied by the manufacturer. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using the Light Cycler (Roche Diagnostics, Mannheim, Germany), and the amount of target gene expression was calculated. The expression of the target gene was normalized 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′; *SMARCB1* forward primer; 5′-TCTGGATTTGAACCCGCTGA-3′; *SMARCB1* reverse primer; 5′-TGCTGTATGCGATGGTGGTG-3′; *BNIP3L* forward primer; 5′-CGGACTCGGCTTGTTGTGTT-3′; *BNIP3L* reverse primer; 5′-ATGGGTAGCTCCACCCA GGA-3′; *PBGD* forward primer; 5′-TGTCTGGTAACGGC AATGCGGCTGCAAC-3′; *PBGD* reverse primer; 5′-TCAA TGTTGCCACCACACTGTCCGTCT-3′

Western blot analysis

Cells grown to semiconfluence were washed and collected with a rubber scraper. After centrifugation, the cell pellets were resuspended, and the extracts were centrifuged and the supernatant fraction was collected. Western blot analysis was carried out as described previously.[16,17] The expression of the target protein was evaluated by comparison to the expression of actin.

Annexin V assay

The binding of annexin V was used here as a sensitive assessment of apoptosis, as described previously.[17,18] Cells were stained by Annexin V-APC and propidium iodide (PI) (BD Biosciences, Franklin Lakes, NJ), and analyzed on a FACS Aria (BD Biosciences).

Invasion assay

The invasion assay was performed using transwell culture chambers (BD Biosciences) according to the instructions provided by the manufacturer. The upper chamber was loaded with cell suspension and the lower chamber was loaded with 10% FBS. After incubation (48 hr for PLC/PRF/5 and 24 hr for HuH7), cells that had invaded the undersurface of the membrane were counted under a microscope. Four microscopic fields were randomly selected for cell counting.

Cell cycle analysis

Cell cycle analysis was performed based on flow cytometric analysis, as described previously.[19] Briefly, cells were washed and fixed. PI and RNase (Sigma-Aldrich) were then added, and data were acquired on the FACS Calibur (BD Biosciences). The cell cycle analysis was carried out using ModFIT software (BD Biosciences).