

**Figure 5.** Calculated SVM distances of healthy controls (black columns) and pancreatic cancer patients (gray columns) in the training (A) and first validation (B) cohorts. Cases separated into the positive direction from the SVM hyperplane were classified as having "cancer" and those separated into the negative direction were classified as being "healthy."

of the pancreatic cancer patients and 80% (4 of 5) of the healthy controls, whereas the CA19-9 level correctly identified 66.7% (6 of 9) of the pancreatic cancer patients and 100% (5 of 5) of the healthy controls (Fig. 6). Again, in all the pancreatic cancer patients (9 of 9), the SELDI classifier and the CA19-9 level provided complementary results, even in this second validation cohort.

**Discussion**

Comparative proteomic profiling coupled with a computerized machine learning approach may revolutionize medical practice and cancer diagnosis. We compared the plasma protein profiles of a large number of pancreatic cancer patients and healthy controls with identical age and gender distributions (Table 1) to identify a biomarker for detecting pancreatic cancer patients in a large

**Table 4** Diagnostic accuracy of the SELDI classifier

	Training cohort		Validation cohort (NCCH)		Validation cohort (TMUH)	
	No. cases	No. correctly classified samples* (%)	No. cases	No. correctly classified samples* (%)	No. cases	No. correctly classified samples* (%)
Healthy	71	67 (94.4)	45	41 (91.1)	5	4 (80)
Pancreatitis					5	4 (80)
Tumor/cyst <sup>†</sup>					6	4 (66.6)
Cancer	71	69 (97.2)	33	30 (90.9)	9	8 (88.9)
Cancer location						
Head	34	33 (97.1)	17	14 (82.4)	7	7 (100)
Body or tail	37	36 (97.3)	10	10 (100)	2	1 (50)
Unknown	0	0	6	10 (100)		
Clinical stage						
I	1	0 (0)	1	1 (100)	0	0
II	6	6 (100)	4	3 (75)	0	0
III	10	9 (90)	1	1 (100)	3	3 (100)
IV	54	54 (100)	27	25 (92.6)	6	5 (83.3)

\*Number of healthy and chronic pancreatitis cases, considered to be "healthy," and number of pancreatic tumor/cyst and cancer cases given a diagnosis of "cancer."  
<sup>†</sup>Pathologically unproven pancreatic tumor and/or cyst.

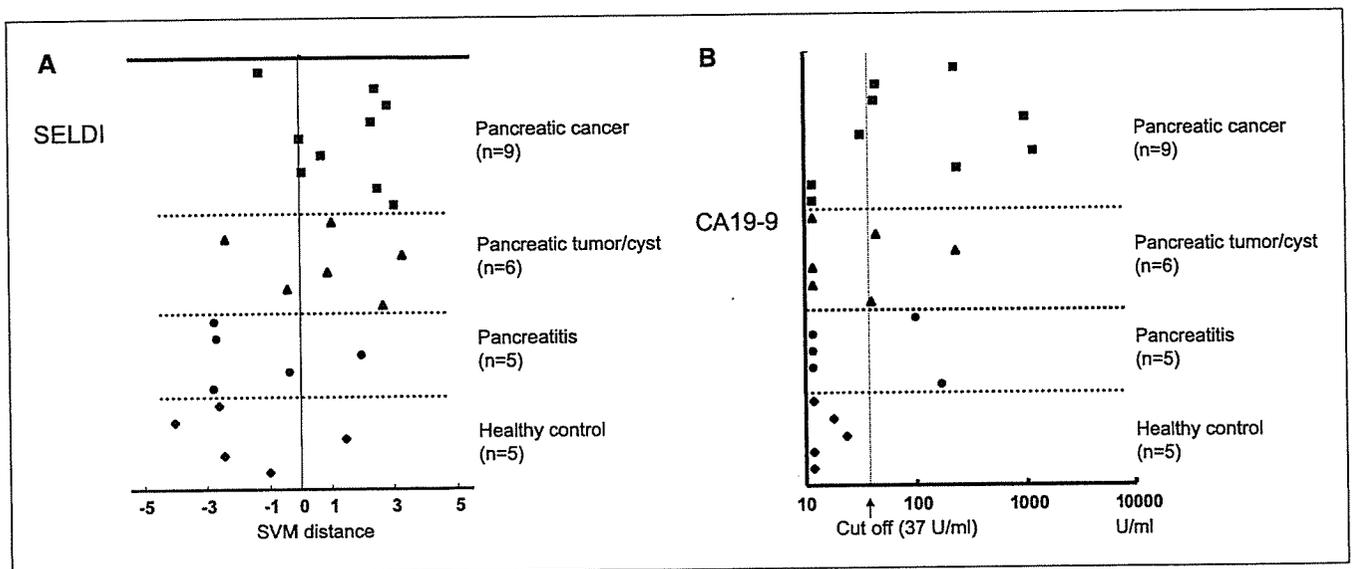
**Table 5. Detection rates with CA19-9, the SELDI classifier, and their combination**

	No. cases*	CA19-9, n (%)	SELDI, n (%)	Combination, n (%)
Healthy	39	3 (7.7)	4 (10.3)	6 (15.4)
Cancer	29	25 (86.2)	26 (89.7)	29 (100)
Clinical stage				
I	1	1 (100)	1 (100)	1 (100)
II	3	3 (100)	2 (66.6)	3 (100)
III	1	0 (0)	1 (100)	1 (100)
IV	24	21 (87.5)	22 (91.7)	24 (100)

\*Cases whose plasma samples were available for CA19-9 measurement in the NCCH validation cohort.

population composed mainly of healthy individuals. The reproducibility of data obtained using the low-resolution instrument of the ProteinChip system has been a concern, but employing a high-resolution QqTOF instrument was found to significantly improve mass accuracy and minimize day-to-day variations (Fig. 1B). The high reproducibility of measurements was confirmed not by using a few high-intensity peaks selected intentionally but rather by using all the peaks detectable in the entire range (intensity and *m/z*) of mass spectra (Fig. 2). We also eliminated fractionation procedures, which increased the number of detectable peaks but significantly decreased reproducibility (Table 2; Fig. 2). A minimal set of four low-molecular weight proteins (Fig. 3) was found to be sufficient for discriminating pancreatic cancer patients with a sensitivity of 97.2% (69 of 71) and a specificity of 94.4% (67 of 71; Fig. 5A). This high discriminating capacity was confirmed by LOO cross-validation and ROC analysis (Fig. 4). We confirmed the discriminating capacity of our classifier in two independent validation cohorts (Figs. 5B and 6) to eliminate accidental identification of nonbiological/mathematical multivariate classifiers within a closed cohort by overfitting.

We noticed that a peak at 11,516 *m/z* (H50) was detected in 19.4% of the pancreatic cancer patients in the training cohort but in only 1.4% of the healthy controls (the peaks are indicated by a red arrowhead in Fig. 3). Tolson et al. (26) reported that an 11.5-kDa protein was detected in 32% of renal cell carcinoma patients but in none of the normal controls. Howard et al. (27) identified 11,682 *m/z* proteins in the sera of lung cancer patients as a diagnostic biomarker using matrix-assisted laser desorption/ionization (MALDI)-TOF-MS. Both groups identified the proteins as fragments of serum amyloid A. Serum amyloid A is an acute-phase reactant and a biomarker for inflammatory disease. The serum amyloid A level is elevated up to 1,000-fold during tissue damage and inflammation and is also increased in patients with various solid tumors and hematopoietic malignancies. However, serum amyloid A has not been recognized as a tumor marker because of its low positive rate (28, 29). Consistently, the 11,516 *m/z* peak was not incorporated into our classifier. The discovery of a single biomarker differing markedly between cancer patients and controls as well as having a high positive rate in cancer patients would be ideal but is perhaps not realistic. Since the discovery of CA19-9 in



**Figure 6.** Confirmation in a second cohort treated at a different institution. *A*, calculated SVM distances of nine pancreatic cancer patients, six individuals with pancreatic tumors and/or cysts, five chronic pancreatitis patients, and five healthy controls seen at TMUH. *B*, plasma CA19-9 levels in nine pancreatic cancer patients, six individuals with pancreatic tumors and/or cysts, five chronic pancreatitis patients, and five healthy controls seen at TMUH. The cutoff value was set at 37 units/mL.

1982 (30), no single tumor marker applicable to the clinical diagnosis of pancreatic cancer has been identified. The carcinogenesis of pancreatic cancer is probably mediated via a variety of molecular pathways (2, 31), and multimarker analysis of proteins with different specificities is a realistic alternative to a conventional single biomarker assay.

There are pros and cons to SELDI-MS with high-resolution instruments. Although the primary goal of our study was the development of a bioassay applicable to the detection of pancreatic cancer, attempts to purify proteins from these four low-intensity peaks without contamination by neighboring high-intensity peaks have not been successful to date. However, the high reproducibility of QqTOF-MS warrants direct clinical application of its measurements and does not necessitate the actual protein identification of these peaks. Zhang et al. (12) reported that a set of three peaks, at 3,272, 12,828, and 28,043  $m/z$ , could be used to detect early-stage ovarian cancer. The 28,043  $m/z$  peak was down-regulated in ovarian cancer patients and was found to be derived from apolipoprotein A1. The relatively abundant 28,080  $m/z$  protein identified as one of the peaks down-regulated in pancreatic cancer patients in this study (Table 3; Fig. 3) may be related to apolipoprotein A1. The mass deviation of 0.3% seen in the low-resolution TOF-MS may represent a drift in this region as large as 84  $m/z$  ( $28,080 \times 0.003 = 84$ ). At least four peaks were detected between 28,000 and 28,100  $m/z$  using the high-resolution QqTOF-MS instrument (Fig. 3). These peaks merged and were detected as a single peak with the low-resolution instrument (data not shown). The intensities of the 8,766, 17,272, and 14,779  $m/z$  peaks were one magnitude smaller than that of the 28,080  $m/z$  peak (Table 3) and were apparently below the sensitivity of tandem MS. So-called top-down proteomics using Fourier transform (FT)-MS (32) may be necessary to identify the proteins indicated by the low-intensity peaks of our classifier. However, an interface to the SELDI arrays is currently not available for FT-MS.

No significant differences in the detection rates for our classifier were observed among different stages of pancreatic cancer (Table 4). Koomen et al. (33) did plasma protein profiling of pancreatic cancer patients using MALDI-MS and identified a set of eight peaks distinguishing pancreatic cancer patients from controls with a sensitivity of 88% and a specificity of 75%. Protein identification revealed these peaks to be derived mainly from host response proteins. Many low molecular weight proteins detected by SELDI-MS in serum or plasma samples have also been reported to be metabolic products, proteolytic fragments, or peptide hormones. These proteins may not always be attributable to direct secretion or production by cancer cells, instead being the results of host responses in the microenvironment of the tumor (7, 18, 34), such as stromal desmoplastic reactions, inflammation, and angiogenesis. Two of eight pancreatic cancer patients who were classified as having "cancer," but none of normal controls in the TMUH validation cohort, had diabetes (data not shown). This raises the possibility that diabetic conditions, which are often

associated with pancreatic cancer patients, also may influence the classifier.

All the pancreatic cancers were detected by complementary use of CA19-9 and/or the SELDI classifier (Table 5). CA19-9 is a tumor marker widely used for the evaluation of therapeutic effects and the detection of pancreatic cancer recurrence but is not considered to be applicable to mass screening (35–38). Ten percent to 15% of humans do not secrete CA19-9 because of their genetic Lewis antigen status (39). The CA19-9 level is often within reference range when pancreatic cancer is still at an early stage and is often elevated in benign biliary and pancreatic diseases. When the cutoff value for CA19-9 was set at 37 units/mL, which is widely used for clinical purposes, the false-positive rate of the combined CA19-9 and SELDI strategy reached 15.4% (Table 5). To increase diagnostic accuracy, the CA19-9 cutoff value may need to be adjusted and the selection of SELDI peaks may need to be further refined.

Early detection seems to be essential for improving the outcomes of pancreatic cancer patients. The SELDI classifier identified in this study has high potential for detecting pancreatic cancers (Tables 4 and 5), but one of the five pancreatitis patients in the TMUH validation cohort was classified into the pancreatic cancer category (Fig. 6). This pancreatitis patient may have a premalignant or preclinical condition and is currently being followed. Alternatively, because inflammatory conditions were not used in training, it is also possible that the classifier may not be entirely specific for the cancer phenotype. Machine learning was done with the training cohort, in which there were no cases with benign pancreatic diseases, because the discovery of biomarkers useful for pancreatic cancer screening in a large population made up mostly of healthy individuals was a primary goal of this study. The final diagnosis of pancreatic cancer is not made solely based on plasma protein profiling. CT, MRI, PET, ultrasound, and endoscopic and/or surgical approaches are employed as well. To evaluate the clinical significance of the biomarkers identified in this study and to refine the selection of biomarkers using a large number of subjects, including patients with pancreatic cancer and other pancreatic diseases, we need to undertake a prospective multi-institutional study.

## Acknowledgments

Received 5/27/2005; revised 8/5/2005; accepted 9/9/2005.

**Grant support:** "Third Term Comprehensive Control Research for Cancer" from the Ministry of Health, Labor and Welfare; "Program for Promotion of Fundamental Studies in Health Sciences" of the National Institute of Biomedical Innovation of Japan; and Foundation for the Promotion of Cancer Research resident fellowship to Y. Hayashida (patent pending in Japan, no. 2005-070512).

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We thank Drs. T. Kakizoe, N. Moriyama, and T. Yoshida (National Cancer Center) for helpful discussions and encouragement, Y. Ishiyama for her secretarial assistance, and Dr. K. Aoshima, H. Kuwabara, T. Isobe, and H. Matsuzuki (Mitsui Knowledge Industry) for the statistical analyses.

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*Reprinted from*

*Jpn J Clin Oncol 2005;35(12):733-738*

*doi:10.1093/jjcol/hyi190*

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## **Phase I Study of Fixed Dose Rate Infusion of Gemcitabine in Patients with Unresectable Pancreatic Cancer**

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Received July 20, 2005; accepted September 24, 2005; published online November 22, 2005

**Objective:** The purpose of this study was to determine the feasible dose of gemcitabine when administered as a fixed dose rate infusion (10 mg/m<sup>2</sup>/min) on a weekly schedule to Japanese patients with unresectable advanced pancreatic cancer.

**Methods:** Patients were required to have histologically or cytologically proven locally advanced or metastatic pancreatic cancer for which they had received no previous chemotherapy. Gemcitabine was administered intravenously weekly for three consecutive weeks every 4 weeks. Patients at three dose levels were scheduled to receive escalating doses of gemcitabine: 1000 mg/m<sup>2</sup> over 100 min (Level 1), 1200 mg/m<sup>2</sup> over 120 min (Level 2) and 1500 mg/m<sup>2</sup> over 150 min (Level 3).

**Results:** A total of 16 patients were enrolled in this study between December 2003 and September 2004. Maximum-tolerated dose was not reached during the first course. Dose-limiting toxicity was Grade 4 neutropenia. Grade 3 or 4 neutropenia was observed at Level 3 in all six patients in the first course, and administration of gemcitabine on Day 8 or 15 was skipped in all six patients. Non-hematologic toxicity was mild and the most common symptoms were anorexia, nausea and vomiting. Partial response was achieved in 1 of the 17 patients (7%). Median overall survival was 7.3 months.

**Conclusions:** Gemcitabine administered at a rate of 10 mg/m<sup>2</sup>/min was tolerated up to 1500 mg/m<sup>2</sup>, but 1200 mg/m<sup>2</sup> represented a more appropriate recommended dose in further studies owing to neutropenia in Japanese patients with advanced pancreatic cancer.

*Key words: advanced pancreatic cancer – systemic chemotherapy – gemcitabine – fixed dose rate infusion*

### INTRODUCTION

Pancreatic cancer is the fifth most common cause of cancer death in Japan, with an estimated 19 000 deaths annually (1). Early-stage diagnosis of pancreatic cancer is difficult because of the lack of specific early symptoms, and surgery with curative intent can be performed in only 5–20% of patients (2). The prognosis for unresectable pancreatic cancer remains extremely poor.

Gemcitabine (2',2'-difluorodeoxycytidine) is a novel pyrimidine antimetabolite with a broad spectrum of antitumor activity against various solid tumors, such as pancreatic and lung cancer (3). This prodrug is initially phosphorylated by deoxycytidine kinase to gemcitabine monophosphate, with subsequent phosphorylation steps yielding gemcitabine di- and

triphosphate (4). Gemcitabine triphosphate inhibits DNA synthesis by competing with deoxycytidine triphosphate for incorporation into DNA by DNA polymerase (5). A dose of 790 mg/m<sup>2</sup> gemcitabine weekly for 3 weeks every 28 days was recommended for Phase II studies on the basis of a Phase I study in which gemcitabine was administered as a once-weekly 30 min bolus infusion (6). This dosing schedule was used in subsequent Phases II and III studies, and once-weekly 30 min infusion of the 1000 mg/m<sup>2</sup> dose was subsequently selected as the standard schedule (7,8). In a randomized clinical trial, gemcitabine was confirmed to provide a survival advantage over 5-FU in addition to symptom-relieving benefits in patients with advanced pancreatic cancer (8). Based on these results, gemcitabine has generally been accepted as the standard chemotherapeutic agent for advanced pancreatic cancer. However, the advantages in terms of survival rate are inadequate, and various chemotherapeutic regimens have been investigated in clinical studies in efforts to prolong survival.

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The cellular pharmacokinetics of the active metabolite, gemcitabine triphosphate, in mononuclear cells have been examined in previous studies, and the rate of gemcitabine triphosphate accumulation and peak intracellular concentration were highest at a dose rate of 350 mg/m<sup>2</sup> over 30 min, during which steady-state gemcitabine levels of 15–20 µmol/l were achieved in plasma (6,9). A dose ~10 mg/m<sup>2</sup>/min that achieves plasma gemcitabine concentrations of 15–20 µmol/l would thus maximize the intracellular rate of accumulation for gemcitabine triphosphate. This schedule of gemcitabine administration, with fixed dose rate (FDR) infusion of 10 mg/m<sup>2</sup>/min, would enable exposure to higher concentrations of gemcitabine, and should improve clinical efficacy.

Phase I studies of FDR infusion of gemcitabine in the United States recommended a Phase II dose of 1500 mg/m<sup>2</sup> (10,11). A subsequent randomized Phase II trial comparing this FDR gemcitabine infusion schedule and high-dose gemcitabine (2200 mg/m<sup>2</sup>) using a standard 30 min infusion showed improved median survival time for the FDR arm (12). The FDR infusion schedule is expected to become the optimal method of gemcitabine administration, but has not previously been assessed in Japan. We, therefore, conducted a Phase I study to determine whether FDR infusion of gemcitabine would be tolerated in Japanese patients with unresectable advanced pancreatic cancer. The primary objectives of this study were to confirm whether the recommended dose in the United States, 1500 mg/m<sup>2</sup> over 150 min, would be feasible in Japanese patients and to determine the relationship between dose and toxicity for gemcitabine administered using the FDR infusion schedule. The secondary objective was to evaluate antitumor activity of the schedule.

## PATIENTS AND METHODS

### PATIENTS ELIGIBILITY

Eligibility criteria for enrollment in the study were as follows: (i) histologically confirmed pancreatic ductal adenocarcinoma; (ii) unresectable locally advanced or metastatic disease; (iii) no previous treatment for pancreatic cancer except surgery; (iv) age ≥20 and ≤74 years old; (v) Eastern Cooperative Oncology Group (ECOG) performance status of 0–2; (vi) adequate bone marrow (leukocyte count ≥4000 cells/mm<sup>3</sup>, platelet count ≥100 000 cells/mm<sup>3</sup> and hemoglobin ≥9.0 g/dl), renal function (serum creatinine concentration ≤upper limit of normal) and hepatic function (serum bilirubin level ≤2.0 mg/dl, serum albumin level ≥3.0 g/dl, serum aspartate and alanine transaminase (AST and ALT) levels ≤2.5 times upper limit of normal); (vii) life expectancy ≥8 weeks; and (viii) written informed consent from the patient. Percutaneous biliary drainage was performed in patients with obstructive jaundice, and these patients were required to have serum bilirubin levels of ≤2.0 mg/dl and serum AST and ALT levels ≤5 times the upper limit of normal before enrollment. Exclusion criteria comprised serious complications such as active infection, active gastrointestinal ulcer, cardiac disease or renal

disease; central nervous system metastasis; marked pleural effusion or ascites; symptomatic interstitial pneumonitis; and pregnancy or lactation for women. This protocol was approved by the National Cancer Center's institutional review board for clinical investigation.

### TREATMENT METHODS

Gemcitabine (Eli Lilly Japan K.K., Kobe, Japan) was administered intravenously at 10 mg/m<sup>2</sup>/min, weekly, for three consecutive weeks, followed by a week of rest. This cycle was continued until disease progression or serious adverse effects developed or until the patient requested discontinuation. When patients developed leukopenia of <2000/mm<sup>3</sup>, neutropenia of <1000/mm<sup>3</sup>, thrombocytopenia of <70 000/mm<sup>3</sup>, total bilirubin >2.0 mg/dl or AST and ALT levels >5 times the upper limit of normal, gemcitabine administration was suspended until the patient recovered. If a rest period of >4 weeks was required owing to toxicity, the patient was withdrawn from the study.

### STUDY DESIGNS

Patients at three dose levels were scheduled to receive escalating dose of gemcitabine. At the first dose level (Level 1), gemcitabine was administered at a dose of 1000 mg/m<sup>2</sup>. The dose level was increased to 1200 mg/m<sup>2</sup> for Level 2 and 1500 mg/m<sup>2</sup> for Level 3. Patient cohorts had a minimum of three patients at each level. If no dose-limiting toxicity (DLT) was observed in the initial three patients during the first cycle of treatment, the dose was advanced to the next level. If DLT occurred in the initial three patients, three additional patients were studied at the same dose level. If two or more of these six patients experienced DLT at that level, the dose was escalated to the next level. The maximum-tolerated dose (MTD) was defined as the highest dose level at which more than two of the six patients experienced DLT during the first cycle of treatment. If DLT occurred in three patients at Level 1, the dose was reduced to 800 mg/m<sup>2</sup> (Level 0). DLT was defined as follows: (i) Grade 4 leukopenia or neutropenia; (ii) febrile neutropenia; (iii) Grade 4 thrombocytopenia or Grade 3 thrombocytopenia requiring transfusion; (iv) ≥Grade 3 non-hematological toxicity with the exception of nausea, vomiting, anorexia, fatigue and constipation; and (v) any toxicity requiring two consecutive skips of administration or a >4 week delay in treatment. Toxicity was graded according to the National Cancer Institute common toxicity criteria version 2.0.

### CLINICAL ASSESSMENTS

Physical examination, complete blood cell counts, serum chemistries and urinalysis were performed at baseline and at least once weekly after initiating treatment. Patients underwent dynamic computed tomography (CT) to evaluate response at 4–8 week intervals after start of treatment. CT was performed by obtaining contiguous transverse sections using the helical scanning method at a section thickness of 5 mm. Tumor response was assessed according to the World Health Organization criteria (13). Serum carbohydrate antigen (CA)19-9

levels were measured monthly by immunoradiometric assay. Progression-free survival was calculated from the first day of treatment until evidence of tumor progression, clinical progression or death owing to any cause. Overall survival was calculated from the first day of treatment until death owing to any cause. Survival data were analysed using the Kaplan–Meier method.

**RESULTS**

**PATIENT CHARACTERISTICS**

Between December 2003 and September 2004, a total of 16 patients were enrolled in this study. Dose escalation schedule and the number of patients at each level are shown in Table 1. The first administration of 1200 mg/m<sup>2</sup> of gemcitabine in one patient receiving Level 2 was later found to have been infused over 90 min, departing from the FDR of 10 mg/m<sup>2</sup>/min. As a result, an additional patient was added to Level 2 and ultimately seven patients were treated at Level 2. Patient characteristics are shown in Table 2. The 16 patients received 60 courses of gemcitabine. Median number of courses administered per patient was 3 (range 1–9 courses). All 16 patients were evaluable for toxicity, but the Level 2 patient not infused with gemcitabine at a rate of 10 mg/m<sup>2</sup>/min was excluded from the evaluation of DLT.

**TOXICITY**

Toxicities of the 15 patients evaluated for DLT during the first course are shown in Table 3. The first three patients enrolled on Levels 1 and 2 did not experience any DLT, but one of the six patients at Level 3 experienced DLT. MTD was not reached in this study. However, since all six patients at Level 3 (1500 mg/m<sup>2</sup> over 150 min) experienced Grade 3 or 4 neutropenia after Day 1 or 8 of the first course and did not receive the second or third dose of gemcitabine, an additional three patients were entered at Level 2 to accurately determine the recommended FDR for gemcitabine. Finally, no Grade 4 hematological toxicity was observed in any of the six patients at Level 2, and Grade 3 neutropenia developed in three of these patients. While five of the six patients received the full three doses of gemcitabine in the first course, the remaining patient did not receive the third dose owing to Grade 3 neutropenia. Level 2 (1200 mg/m<sup>2</sup>) was therefore selected as the recommended dose for further studies of this FDR gemcitabine regimen in Japan.

**Table 1.** Dose escalation scheme

Dose levels	Gemcitabine (mg/m <sup>2</sup> /wk)	Infusion time (min)	n
1	1000	100	3
2	1200	120	7
3	1500	150	6

**Table 2.** Patient characteristics

Variable	No. of patients (n = 16)
Gender	
Male	7
Female	9
Median age (range)	62 (47–74) years
ECOG performance status	
0	11
1	4
2	1
Disease stage	
Locally advanced	3
Metastatic	13
Site of metastatic disease	
Liver	10
Lung	3
Distant lymph nodes	2
CA19-9 before treatment (U/ml)	
≤37	4
>37, ≤1000	6
>1000	6

ECOG, Eastern Cooperative Oncology Group; CA19-9, carbohydrate antigen 19–9.

**Table 3.** Toxicities across first course by patient

	Dose levels											
	Level 1 (n = 3)				Level 2 (n = 6)				Level 3 (n = 6)			
	Grades				Grades				Grades			
	1	2	3	4	1	2	3	4	1	2	3	4
Leukopenia	0	0	2	0	3	1	2	0	0	2	4	0
Neutropenia	0	0	2	0	1	2	3	0	0	0	5	1
Anemia	1	1	2	0	2	3	0	0	4	2	0	0
Thrombocytopenia	1	2	0	0	2	0	1	0	0	2	1	0
Anorexia	1	1	0	0	2	0	1	0	2	2	0	0
Nausea	1	1	0	0	1	0	1	0	4	1	0	0
Vomiting	0	1	0	0	0	0	1	0	1	1	0	0
Rash	0	0	0	0	2	2	0	0	1	3	0	0
Fatigue	2	0	0	0	2	0	0	0	0	1	0	0
Fever	0	1	0	0	0	0	0	0	1	0	0	0
Mucositis	0	1	0	0	0	0	0	0	0	0	0	0
Alopecia	0	0	0	0	0	0	0	0	1	0	0	0
AST, ALT elevation	0	1	0	0	1	1	0	0	0	1	0	0

AST, serum aspartate transaminase; ALT, serum alanine transaminase.

Toxicities throughout the entire period of this protocol were assessed in all 16 patients enrolled in this study (Table 4). The most common toxicity was leukopenia, particularly neutropenia, with 13 of the 16 patients (81%) developing Grade 3 or 4

Table 4. Toxicities during entire course by patient

	Dose levels											
	Level 1 (n = 3)				Level 2 (n = 7)				Level 3 (n = 6)			
	Grades				Grades				Grades			
	1	2	3	4	1	2	3	4	1	2	3	4
Leukopenia	0	0	2	0	2	2	3	0	0	1	4	1
Neutropenia	0	0	2	0	1	1	5	0	0	0	3	3
Anemia	1	0	2	0	1	5	1	0	3	2	1	0
Thrombocytopenia	1	2	0	0	2	1	1	0	0	2	2	0
Anorexia	1	0	1	0	4	0	1	0	4	2	0	0
Nausea	1	0	1	0	4	0	1	0	5	1	0	0
Vomiting	0	0	1	0	2	0	1	0	1	1	0	0
Constipation	0	0	0	0	0	1	0	0	1	0	0	0
Diarrhea	0	0	0	0	1	0	0	0	0	0	0	0
Rash	0	0	0	0	3	2	0	0	1	2	0	0
Fatigue	1	1	0	0	2	0	0	0	0	1	0	0
Fever	0	1	0	0	0	0	0	0	2	0	0	0
Mucositis	0	1	0	0	0	0	0	0	0	0	0	0
Alopecia	0	0	0	0	1	1	0	0	3	0	0	0
AST, ALT elevation	0	1	0	0	1	1	0	0	0	1	0	0

AST, serum aspartate transaminase; ALT, serum alanine transaminase.

neutropenia during treatment. Non-hematological toxicities were generally mild at all levels, and one patient developed Grade 3 nausea, vomiting, and anorexia at Level 1 and Level 2, respectively. Skin rashes were mild, but tended to occur in a larger number of patients as the dose was escalated.

#### TUMOR RESPONSE AND SURVIVAL

Partial response was achieved in 1 of the 16 patients (6.3%), but no complete responses were observed. Overall response rate was thus 6.3% (95% confidence interval = 0.2–30.2%). No change was noted in 12 patients (75.0%), and progressive disease was in two patients (12.5%). The patient with DLT was not evaluated for tumor response because she received standard gemcitabine chemotherapy as second-line chemotherapy before the evaluation. Serum CA19-9 levels were reduced to >50% in 2 of the 12 patients (16.7%) in whom pretreatment level was elevated to above the upper limit of normal.

Disease progression was finally observed in all patients and 12 of the 16 patients died of disease progression. Median progression-free survival was 3.2 months, and overall median survival time (MST) was 7.3 months (Figs 1 and 2).

#### DISCUSSION

Gemcitabine is a prodrug that requires initial intracellular phosphorylation by deoxycytidine kinase, ultimately undergoing phosphorylation to the active gemcitabine triphosphate,

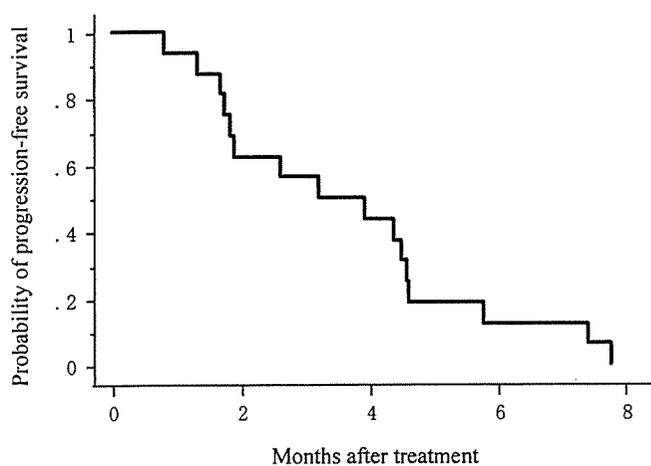


Figure 1. Progression-free survival of all 16 patients.

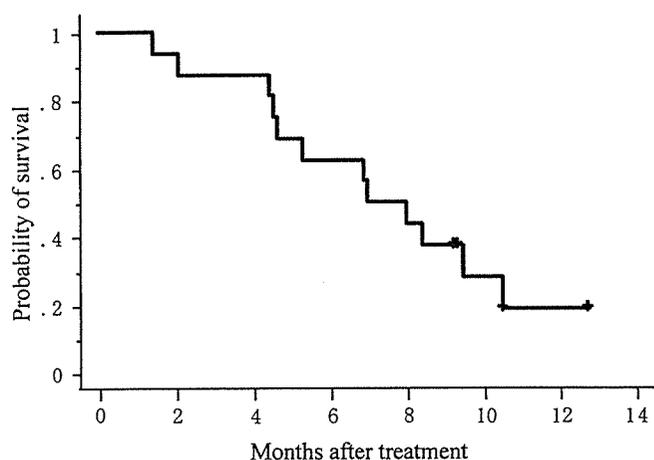


Figure 2. Overall survival of all 16 patients.

a cytotoxic agent that inhibits DNA synthesis. Tempero et al. (12) reported on intracellular concentrations of gemcitabine triphosphate in peripheral blood mononuclear cells in a randomized trial comparing FDR infusion over 150 min and high-dose gemcitabine (2200 mg/m<sup>2</sup>) using a standard 30 min infusion. The rate of gemcitabine triphosphate accumulation in patients who received conventional infusion decreased markedly after the end of infusion (30 min), whereas patients who received gemcitabine as FDR infusion exhibited linear accumulation of the triphosphate throughout the infusion. Intracellular gemcitabine triphosphate concentration in the FDR arm was 2-fold higher than that in the conventional infusion arm.

In the United States, two Phase I studies have been performed to determine the recommended dose for FDR infusion of gemcitabine (10,11). Brand et al. (11) conducted a Phase I study at dose levels of 1200 mg/m<sup>2</sup>, 1500 mg/m<sup>2</sup> and 1800 mg/m<sup>2</sup>, administered on Days 1, 8 and 15 of a 28 day cycle. MTD was defined as 1500 mg/m<sup>2</sup>, with granulocytopenia and thrombocytopenia representing the DLTs. Brand et al. concluded that myelosuppression was more severe than

anticipated based on previous reports regarding standard gemcitabine administration. Touroutoglou et al. (10) conducted the other Phase I study of FDR infusion of gemcitabine in which the weekly dose was escalated from 1200 to 2800 mg/m<sup>2</sup> for 3 weeks every 4 weeks. They reported that MTD was 1800 mg/m<sup>2</sup>, and recommended a Phase II starting dose of 1500 mg/m<sup>2</sup> owing to myelosuppressive effects.

The present study evaluated the safety of FDR infusion of gemcitabine and identified the feasible dose for Japanese patients with unresectable advanced pancreatic cancer. This Phase I study was conducted using dose levels of 1000, 1200 and 1500 mg/m<sup>2</sup>, administered on Days 1, 8 and 15 of the 28 day cycle. DLT was observed in only one patient at Level 3, and MTD was not reached in this study. However, all six patients displayed Grade 3 or 4 neutropenia during the first course at Level 3, and no patient received all three doses of gemcitabine during the first course. In contrast, three patients at Level 2 experienced Grade 3 neutropenia, and only one patient had to skip the dose of gemcitabine on Day 15. Based on these results, the recommended dose should be 1200 mg/m<sup>2</sup> in further studies of FDR infusion of gemcitabine in Japan from the perspective of dose intensity for gemcitabine.

Preclinical data, using primary human tumor cell lines including pancreatic carcinoma, have suggested a possible dose-response relationship, and exposure to high concentrations of gemcitabine, independent of infusion duration, might correlate with improved cytotoxicity and enhanced clinical effectiveness (12). Thus, a randomized trial of gemcitabine comparing high-dose gemcitabine (2200 mg/m<sup>2</sup>) administered using a standard 30 min infusion to FDR infusion of 1500 mg/m<sup>2</sup> over 150 min was conducted in patients with locally advanced or metastatic pancreatic cancer according to the results of two Phase I studies in the United States (10–12). Although no difference in tumor response was noted between the 30 min infusion and FDR arms, MST was reported as 5.0 months in the 30 min infusion arm and 8.0 months in the FDR arm ( $P = 0.013$ ), and 1 and 2 year survival rates were 9.0 and 2.2%, respectively, in the 30 min infusion arm, and 28.8 and 18.3%, respectively, in the FDR arm. In the study conducted by Burris et al. (8), MST for gemcitabine using the standard 30 min infusion of 1000 mg/m<sup>2</sup> was 5.7 months, and 1 and 2 year survival rates were 18 and 0%, respectively. A retrospective analysis reported that the MST of patients in Japan treated with gemcitabine by standard infusion of 1000 mg/m<sup>2</sup> was 5.7 months (14). In comparison, survival outcomes of patients treated using the standard 30 min infusion are similar, and MST is <6 months. In contrast, in a study with a limited number of patients using FDR infusion, MST was 7.3 months and similar to MST in the FDR arm of the randomized trial in the United States (12).

The most common toxicity for FDR infusion was myelosuppression, particularly neutropenia, as noted in a randomized trial by Tempero et al. (12). In our study, Grade 3 or 4 neutropenia developed in 81.3% of patients, and Grade 3 or 4 leukopenia and thrombocytopenia were observed in 62.5

and 18.8%, respectively. By contrast, a Phase I study for the standard infusion of gemcitabine in Japan reported rates of Grade 3 or 4 neutropenia, leukopenia and thrombocytopenia of 36.4, 27.3 and 0%, respectively (15). The FDR infusion schedule thus seems more hematologically toxic. Conversely, the non-hematological toxicity of FDR infusion was relatively mild. Grade 3 nausea and vomiting that occurred in 12.5% of patients on FDR infusion resembled the results obtained with standard infusion in the Japanese Phase I study, in which 9.1% of patients developed Grade 3 nausea and vomiting. Skin rashes were more frequent with FDR infusion, with 50% of patients developing Grade 1 or 2 skin rashes, than with standard infusion, in which 27.3% of patients developed Grade 1 or 2 skin rashes.

Various regimens of gemcitabine in combination with potentially synergistic drugs have been trialed to improve prognosis in patients with unresectable pancreatic cancer (16–22), and FDR infusion of gemcitabine has also been applied to combination chemotherapy with other anticancer drugs (20–22). A Phase III study comparing standard infusion of gemcitabine, FDR infusion of gemcitabine and combined FDR infusion of gemcitabine and oxaliplatin is under way as an ECOG study in the United States. The results of that study should be awaited before deciding whether FDR infusion of gemcitabine alone can be used as the standard treatment for unresectable pancreatic cancer. However, data from the present study confirm that FDR infusion of gemcitabine is tolerated by Japanese patients, and continued evaluation of FDR infusion, alone or in combination with other agents, is warranted in Japan.

## Acknowledgment

This study was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan.

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# Randomized, double-blind, placebo-controlled trial of bovine lactoferrin in patients with chronic hepatitis C

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(Received April 26, 2006/Revised June 6, 2006/Accepted June 8, 2006/Online publication July 27, 2006)

Several studies have suggested that lactoferrin administration may decrease the serum level of hepatitis C virus (HCV) RNA in patients with chronic hepatitis C. The aim of the present study was to confirm the efficacy of orally administered bovine lactoferrin (bLF) in patients with chronic hepatitis C. The patients with chronic hepatitis C randomly received either oral bLF at a dose of 1.8 g daily for 12 weeks, or an oral placebo. The primary endpoint was the virologic response, defined as a 50% or greater decrease in serum HCV RNA level at 12 weeks compared with the baseline. The secondary endpoint was the biochemical response, which was defined as a 50% or greater decrease in the serum alanine aminotransferase (ALT) level at 12 weeks compared with the baseline. One hundred and ninety-eight of 199 patients were evaluable for efficacy and safety. bLF treatment was well tolerated and no serious toxicities were observed. A virologic response was achieved in 14 of 97 patients (14.4%) in the bLF group, and 19 of 101 (18.8%) in the placebo group. There was no significant difference in virologic response rates between the two groups (-4.4%, 95% confidence interval -14.8, 6.1). In addition, bLF intake did not have any favorable effect on the serum ALT level. The virologic responses were not different between two groups in any subgroup analysis. In conclusion, orally administered bLF does not demonstrate any significant efficacy in patients with chronic hepatitis C. (*Cancer Sci* 2006; 97: 1105-1110)

Hepatitis C virus is a leading cause of chronic liver disease in Japan, and nearly two million people are estimated to be infected.<sup>(1)</sup> It is well known that HCV infection frequently causes chronic hepatitis, and that chronic hepatitis eventually progresses to liver cirrhosis and HCC approximately 30 years after HCV infection.<sup>(2)</sup> In Japan, more than 30 000 people die of HCC annually, and approximately 80% of HCC patients are infected with HCV.<sup>(3)</sup> Therefore, effective anti-HCV therapy is necessary to reduce the number of patients suffering from cirrhosis or HCC. To date, interferon-based therapy is the only effective treatment used clinically for chronic hepatitis C. A sustained complete virologic response (loss of detectable serum HCV RNA) occurs in 15-20% of patients with chronic hepatitis C after interferon therapy.<sup>(4)</sup> Moreover, recent studies have demonstrated that interferon with ribavirin or peginterferon with ribavirin improves the sustained complete virologic response rate by up to 40-50%.<sup>(5,6)</sup> However, because more than half of patients do not respond to interferon therapy, and because interferon therapy sometimes induces strong adverse effects, further developments in the treatment of chronic hepatitis C are required.

Lactoferrin, a member of the transferrin family of iron-binding glycoproteins, is present mainly in breast milk and other exocrine secretions. Several biological activities of lactoferrin have been demonstrated, including regulation of iron absorption in the intestine and modulation of immunoreactions.<sup>(7)</sup> Lactoferrin also plays an important role in human innate defense mechanisms against bacteria, fungi and viruses.<sup>(8)</sup> *In vitro* studies to date have shown that lactoferrin has antiviral effects against human immunodeficiency virus-1 and human cytomegalovirus.<sup>(9)</sup> Recent experimental studies have suggested that lactoferrin has antiviral effect against HCV.<sup>(10-12)</sup> Yi *et al.* have reported that lactoferrin binds to HCV envelope proteins *in vitro*.<sup>(10)</sup> Ikeda *et al.* have reported that lactoferrin prevents HCV infection in cultured human hepatocytes, and suggested that the anti-HCV activity of lactoferrin might be related to its direct binding to viral surfaces.<sup>(11,12)</sup> In addition, recent clinical studies have demonstrated the potential efficacy of lactoferrin against chronic hepatitis C.<sup>(13,14)</sup> Tanaka *et al.* reported that 8-week oral administration of bLF at a dose of 1.8 or 3.6 g/day decreased the serum level of HCV RNA markedly in three of four patients with a low pre-treatment HCV RNA level (<100 Kcopy/mL).<sup>(13)</sup> Iwasa *et al.* administered bLF (3.6 g/day) orally to 15 patients with high viral loads ( $\geq 100$  KIU/mL), and reported that the mean serum HCV RNA level decreased significantly from 1106 KIU/mL at entry to 612 KIU/mL after 6 months of treatment ( $P < 0.01$ ).<sup>(14)</sup> Based on these promising findings, we planned to investigate the efficacy of orally administered bLF in patients with chronic hepatitis C. First, we conducted a dose-finding study in 45 patients with chronic hepatitis C.<sup>(15)</sup> In that study, three dose levels of bLF (1.8, 3.6 and 7.2 g/day) were scheduled, and 15 patients at each dose level received the determined dose of bLF for 8 weeks. bLF treatment was well tolerated up to 7.2 g/day, and no serious adverse events were observed. Although no relationship between bLF dose and efficacy was recognized, a 50% or greater decrease in the serum HCV RNA level was seen in four of 45 patients (8.9%). Furthermore, the HCV RNA level was decreased by 50% or more in eight patients (17.8%) at week 8 after the end of treatment. These results encouraged us to conduct further investigations, and the present randomized

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Abbreviations: ALT, alanine aminotransferase; bLF, bovine lactoferrin; CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IL, interleukin; NK, natural killer.

trial was designed to clarify the anti-HCV activity of bLF in patients with chronic hepatitis C.

## Patients and Methods

**Patients.** Each patient was required to meet the following eligibility criteria: 20–74 years of age; positivity for anti-HCV antibody; an HCV RNA level of 0.5–850 KIU/mL evaluated within 1 month before entry; a sustained elevation of serum ALT level for at least 6 months; a serum ALT level of at least twice the upper normal limit evaluated within 1 month before entry; no evidence of HCC on the basis of ultrasonography or computed tomography carried out within 3 months before entry; and adequate bone marrow function (white blood cell count  $\geq 4000/\text{mm}^3$ , platelet count  $\geq 100\,000/\text{mm}^3$ , and hemoglobin level  $\geq 11\text{ g/dL}$ ), liver function (total bilirubin level  $\geq 2.0\text{ mg/dL}$ , serum albumin level  $\geq 3.5\text{ g/dL}$ , and serum aspartate aminotransferase and ALT level  $\geq 200\text{ IU/L}$ ) and renal function (normal serum creatinine and blood urea nitrogen levels).

The exclusion criteria were: positivity for hepatitis B surface antigen; interferon therapy within 6 months before entry; immunomodulatory or corticosteroid therapy within 3 months before entry; intravenous glycyrrhizin therapy within 1 month before entry; past or present history of bLF tablet intake; pregnant or lactating females; severe hepatic disease (e.g. autoimmune hepatitis and primary biliary cirrhosis); other serious medical conditions (e.g. gastrointestinal bleeding, active infection, severe pulmonary disease and psychiatric disorders).

**Methods.** This double-blind, placebo-controlled phase III trial was conducted at 11 centers in Japan. The study was approved by the institutional review board at each center, and all the participants provided written informed consent. Eligible participants were assigned randomly to one of two treatment groups in equal proportions using permutation blocks stratified by centers. A randomization list was drawn up using the SAS random number generator at the data center (Quintiles Transnational Japan K. K. Tokyo, Japan). The treatments consisted of bLF at a dose of 1.8 g/day or a placebo, administered orally twice daily for 12 weeks. In the current study, bLF at 1.8 g/day was selected on the basis of the previous dose-finding study, which indicated that there was no significant relationship between bLF dose (range, 1.8–7.2 g/day) and anti-HCV activity.<sup>(15)</sup> After the treatment allocation, the data center sent a numbered container of bLF or placebo tablets to a participant. During treatment, combined use of interferon, immunomodulatory therapy, corticosteroid and intravenous glycyrrhizin was prohibited. bLF (450 mg/tablet) and placebo tablets were provided by Morinaga Milk Industries (Tokyo, Japan).

In the current study, we tested the hypothesis that oral administration of bLF would: (1) reduce the serum HCV RNA level; and (2) reduce the serum ALT level in patients with chronic hepatitis C. In addition, we investigated the influence of orally administered bLF on systemic immune response in a small group of participants. The participants were evaluated every 4 weeks as outpatients until 4 weeks after completion of treatment. Serum HCV RNA level and serum ALT level were measured before treatment, during treatment at weeks 4, 8 and 12, and at 4 weeks after treatment. Serum HCV RNA level was determined by reverse transcription–polymerase chain reaction using the Amplicor-HCV monitor V 2.0 kit with a sensitivity of 0.5 KIU/mL (Roche Diagnostics, Tokyo, Japan). Anti-HCV antibody was determined by chemiluminescent enzyme immunoassay (Ortho-Clinical Diagnostics, Tokyo, Japan). HCV serotyping was carried out as described previously.<sup>(16)</sup> HCV serotype 1 corresponds to genotypes 1a and 1b of the Simmonds classification, and HCV serotype 2 corresponds to genotypes 2a and 2b.<sup>(17)</sup> Serum concentration of IL-18 was measured in participants at two institutions (National Cancer Center Hospital and Osaka Red Cross Hospital), and the percentage of CD4<sup>+</sup>, CD8<sup>+</sup>,

CD16<sup>+</sup> and CD56<sup>+</sup> peripheral blood lymphocytes was measured in participants at the National Cancer Center Hospital. IL-18 and all lymphocytes were measured before treatment, during treatment at weeks 4, 8 and 12, and at 4 weeks after completion of treatment. Serum concentration of IL-18 was assayed with a human IL-18 enzyme-linked immunosorbent assay kit (Medical and Biological Laboratories, Nagoya, Japan). Lymphocyte surface phenotypes of CD4, CD8, CD16 and CD56 were determined by flow cytometry.

Adverse events were graded for severity according to the Japan Society for Cancer Therapy criteria,<sup>(18)</sup> which are similar to the National Cancer Institute Common Toxicity criteria. During treatment, participants were asked to record in a daily journal both compliance and any adverse events they experienced.

**Assessment of efficacy and statistical analysis.** Analyses were carried out on an intention to treat basis. The primary endpoint was a virologic response. In the current study, we defined a virologic response as a 50% or greater decrease in the serum HCV RNA level at 12 weeks compared with the baseline. Secondary endpoints were a biochemical response, as were changes in serum HCV RNA level and serum ALT level. If the serum ALT level at 12 weeks showed both a  $\geq 50\%$  decrease compared with the baseline and was  $\leq$  twice the upper normal limit, we considered it a biochemical response. Response rate was calculated as the number of responders divided by the total number in each group. Participants whose HCV RNA (or ALT) data at 12 weeks were missing were included only in the denominator. Change in HCV RNA level (or ALT level) at 12 weeks minus the logarithm of these at the baseline. Differences in the virologic or biochemical response rates between two groups were analyzed using a test for the difference between two proportions. Differences in the change in HCV RNA level or ALT level between two groups were analyzed using a test for the difference between two means. In addition to the above planned analyses, subgroup analyses for virologic response were carried out based on pretreatment variables including age, serum HCV RNA level and HCV serotype. In a small group of participants, change in the serum concentration of IL-18 and changes in the percentage of CD4<sup>+</sup>, CD8<sup>+</sup>, CD16<sup>+</sup> and CD56<sup>+</sup> peripheral blood lymphocytes during the study period were investigated. Analyses were carried out using JMP4.0 and PC SAS Release v.8.02 (SAS Institute Japan Ltd, Tokyo, Japan). All *P*-values are two-tailed, and differences at *P* < 0.05 were regarded as statistically significant.

We estimated that a total of 250 participants would be the maximum to enroll for a 2-year enrollment period. Subsequent power analysis revealed that 125 participants per group would have 75% power to detect a 10% difference in the virologic response rate (15 vs 5%) at the 5% level of significance. An interim analysis by the independent data monitoring committee was planned after the first 125 participants had been enrolled. All trial personnel and participants were blinded to treatment assignment for the duration of the trial. Only the trial statistician and the independent data monitoring committee saw unblinded data. In the interim analysis of the primary endpoint, the O'Brien-Fleming method was used.<sup>(19)</sup>

## Results

**Patients.** Enrollment began at seven institutions in April 2001. Because 250 participants were not enrolled for the 2 years planned originally, we extended the registration period for one more year and increased the number of participating institutions from seven to 11. An interim analysis was carried out in March 2004 with the data from the first 125 participants. Because the results of the interim analysis indicated that it was highly unlikely that a significant difference in treatment efficacy between

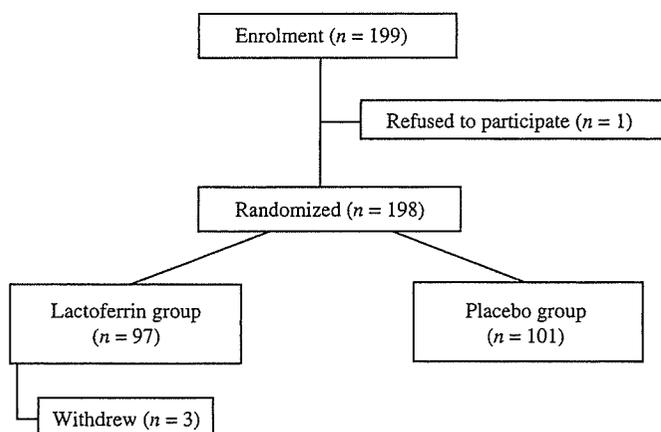


Fig. 1. Flow diagram of participant enrolment.

Table 1. Baseline characteristics of the patients

Characteristic	Bovine lactoferrin	Placebo
No. patients	97	101
Age (years)*	61 (29–74)	58 (31–74)
Sex (male/female)	53/44	55/46
History of interferon therapy	25	29
ALT level (IU/L)*	91 (41–340)	98 (27–250)
HCV RNA level (KIU/mL)*	378 (8.8–960)	452 (8.0–1560)
HCV serotype (1/2/ND)	78/17/1	76/22/3

\*Median (range). ALT, alanine aminotransferase; HCV, hepatitis C virus; ND, not determined.

the two groups would be observed with the planned full enrollment of 250 participants, the data monitoring committee recommended discontinuation of further enrollment. Therefore, enrollment was stopped on 31 March 2004, at which point 199 participants had been enrolled. Because one patient refused to participate in the study before randomization, efficacy and safety were analyzed in the remaining 198 participants (97 bLF and 101 placebo) (Fig. 1). Although three participants in the bLF group discontinued treatment for reasons other than an adverse event, the remaining 195 participants completed the scheduled 12 weeks of treatment. The baseline characteristics of the 198 participants are shown in Table 1. There was no significant difference between the bLF and placebo groups regarding the pretreatment characteristics including age, sex, serum ALT level and serum HCV RNA level.

**Virologic efficacy.** Virologic response, the primary endpoint, was assessed in all 198 participants who received at least one dose of treatment. Virologic response was observed in 14 of 97 participants (14.4%) in the bLF group, and in 19 of 101 (18.8%) in the placebo group (Table 2). No complete virologic response (loss of detectable serum HCV RNA) was seen in either of the groups. There was no significant difference in the virologic response rate with bLF treatment in comparison with the placebo (–4.4%, 95% CI –14.8, 6.1). Change in the HCV RNA level at 12 weeks compared with the baseline was assessed in 190 participants (93 bLF group, 97 placebo group), excluding eight participants for whom HCV RNA data at 12 weeks were lacking. The change in the mean logarithm of the HCV RNA level was –0.09 in the bLF group and –0.09 in the placebo group, indicating no significant difference between the groups ( $P = 1.00$ ).

**Biochemical efficacy.** Biochemical response was assessed in 198 participants. Biochemical response was seen in six of 97 participants (6.2%) in the bLF group, and in four of 101

participants (4.0%) in the placebo group (Table 2). No significant difference in the biochemical response rate was seen between the groups (2.2%, 95% CI –3.9, 8.3). Change in the serum AST level was assessed in 192 participants (93 bLF group, 99 placebo group), excluding six participants for whom ALT data at 12 weeks were lacking. The change in the mean logarithm of the ALT level was –0.085 in the bLF group and –0.080 in the placebo group, indicating no significant difference ( $P = 0.93$ ).

**Subgroup analysis.** The rates of virologic response with respect to pretreatment variables are presented in Table 3. Among participants with a low HCV RNA level (<100 KIU/mL), the virologic response rate was 29.4% in the bLF group and 15.4% in the placebo group, indicating no significant difference between the groups (14.0%, 95% CI –15.2, 43.2). The virologic responses were also not different between two groups in other subgroup analyses such as age, sex and HCV serotype.

**Analysis of IL-18 and lymphocytes.** The serum concentration of IL-18 was measured in 73 participants enrolled at the National Cancer Center Hospital and Osaka Red Cross Hospital (36 bLF, 37 placebo). Figure 2 shows the changes in the mean IL-18 levels in the bLF group and placebo group. The mean IL-18 levels in the bLF and placebo groups were 293.9 pg/mL and 309.9 pg/dL at the baseline and 280.7 pg/mL and 291.5 pg/mL at 12 weeks, respectively. The corresponding changes in the mean IL-18 level at 12 weeks were –14.5 pg/mL and –15.9 pg/mL, respectively, indicating no significant difference between the groups ( $P = 0.91$ ). Similarly, there were no significant differences between the groups at any other points during the study period. The percentage of lymphocyte was measured in 46 participants at the National Cancer Center Hospital (bLF 23, placebo 23), and the results are shown in Fig. 3. The percentage of CD4<sup>+</sup>, CD8<sup>+</sup>, CD16<sup>+</sup> and CD56<sup>+</sup> peripheral blood lymphocytes remained almost unchanged throughout the study in both groups, and the differences between them were not significant.

**Safety.** Safety was assessed in 198 participants who received at least one dose of bLF or placebo during the study. The bLF treatment was well tolerated, and no serious complications occurred during the treatment. Although minor adverse events including neutropenia,  $\gamma$ -GTP elevation and hyperglycemia were observed in participants treated with bLF, their frequency and intensity did not differ from those in the placebo group. HCC was detected in one participant in the bLF group and in one participant in the placebo group during the study period.

## Discussion

The present study was carried out to confirm the anti-HCV activity of orally administered bLF in patients with chronic hepatitis C. A virologic response (a 50% or greater decrease in the serum level of HCV RNA at 12 weeks compared with the baseline) was observed in 14 of 97 participants (14.4%) in the bLF group, and 19 of 101 (18.8%) in the placebo group, the difference between the groups being non-significant. The virologic responses were not different between two groups in any subgroup analysis. Furthermore, bLF intake did not have any favorable effect on the serum ALT level. On the basis of these results, we concluded that orally administered bLF did not have any efficacy, including anti-HCV activity, in patients with chronic hepatitis C.

The virologic response rate of 14.4% observed in the bLF group was somewhat higher than that reported in the previous dose-finding study,<sup>(15)</sup> in which four of 45 patients (8.9%) showed a virologic response at the end of bLF treatment. Nevertheless, the current study failed to demonstrate any anti-HCV activity of bLF, because a similar virologic response rate to that in the bLF group was seen in the placebo group. Having designed this randomized study, we assumed that a virologic

Table 2. Virologic and biochemical efficacy

Characteristic	Bovine Lactoferrin	Placebo	Difference (95% CI)	P-value
Virologic efficacy				
Response rate (%)	14.4	18.8	-4.4 (-14.8, 6.1)	
Change in HCV RNA level <sup>†</sup>	-0.09	-0.09		1.00
Biochemical efficacy				
Response rate (%)	6.2	4.0	2.2 (-3.9, 8.3)	
Change in ALT level <sup>†</sup>	-0.085	-0.080		0.93

<sup>†</sup>Mean logarithm. ALT, alanine aminotransferase; CI, confidence interval; HCV, hepatitis C virus.

Table 3. Virologic response rate as a function of baseline variables

Variable	Bovine lactoferrin (n = 97)		Placebo (n = 101)		Difference	
	Response/total	%	Response/total	%	%	95% CI
Age						
<65 years	12/62	19.4	14/77	18.2	1.2	-11.9, 14.2
≥65 years	2/35	5.7	5/24	20.8	-15.1	-33.1, 2.9
Sex						
Male	10/53	18.9	10/55	18.2	0.7	-14.0, 15.3
Female	4/44	9.1	9/46	19.6	-10.5	-24.7, 3.8
ALT level						
<100 IU/L	6/57	10.5	7/51	13.7	-3.2	-15.6, 9.2
≥100 IU/L	8/40	20.0	12/50	24.0	-4.0	-21.1, 13.1
HCV RNA level						
<100 KIU/mL	5/17	29.4	2/13	15.4	14.0	-15.2, 43.2
≥100 KIU/mL	9/80	11.3	17/88	19.3	-8.0	-18.8, 2.7
HCV serotype <sup>†</sup>						
1	11/78	14.1	16/76	21.1	-7.0	-18.9, 5.0
2	3/18	16.7	2/22	9.1	7.6	-31.4, 28.6

<sup>†</sup>Hepatitis C virus serotype was not measured in four patients. ALT, alanine aminotransferase; CI, confidence interval; HCV, hepatitis C virus.

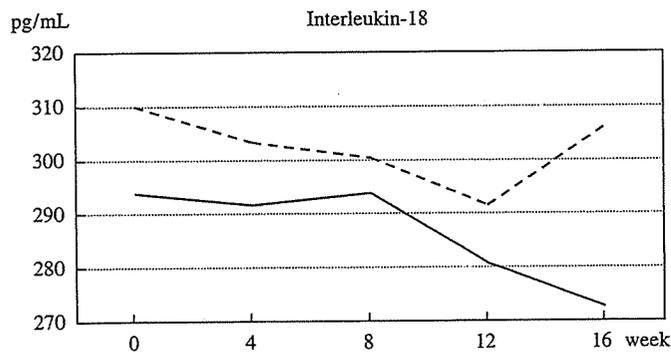
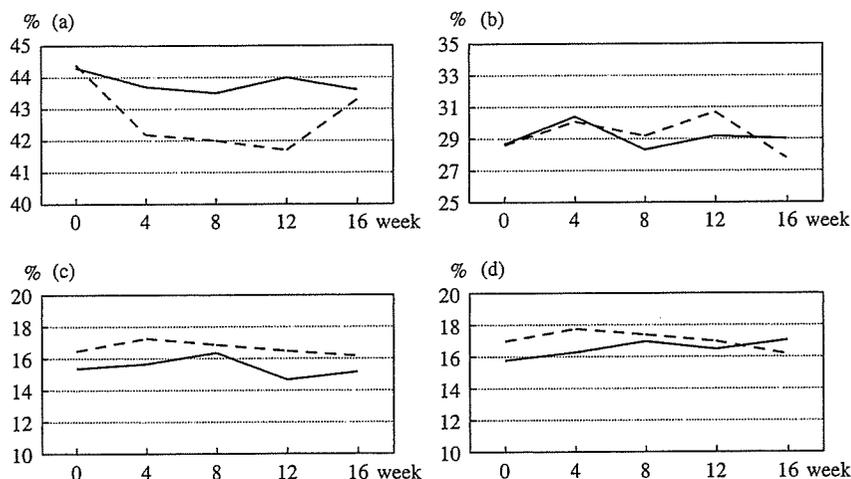


Fig. 2. Changes in the mean serum concentration of interleukin-18 in the bovine lactoferrin group (straight line, n = 36) and the placebo group (dotted line, n = 37).

response rate of around 5% would be seen in the placebo group due to spontaneous remission of viral activity. However, contrary to our expectation, 19 of 101 participants (18.8%) in the placebo group showed a ≥50% decrease in the HCV RNA level at 12 weeks, indicating that our assumption was inappropriate. Our results suggested that in order to assess the reduction of the HCV RNA level, periodic evaluation would be necessary to exclude the influence of spontaneous fluctuation of HCV RNA.

Several experimental studies have suggested that lactoferrin has some activity against HCV. Yi *et al.*<sup>(10)</sup> reported that lactoferrin binds to the HCV E1 and E2 envelope proteins *in vitro*, and Ikeda *et al.*<sup>(11,12)</sup> reported that lactoferrin prevents HCV

infection in cultured human hepatocytes. They suggested that the anti-HCV activity of lactoferrin might be due to a neutralizing efficacy, in which the administered lactoferrin became bound directly to the HCV virion, thus inhibiting adsorption of the HCV-lactoferrin complex into human hepatocytes. Therefore, intravenous administration of lactoferrin might improve the viremic state in patients with chronic hepatitis C. However, for practical application, administration of lactoferrin directly into blood does not seem to be a suitable approach because lactoferrin is a large glycoprotein molecule (80 kDa) that may cause allergic reactions. Therefore, oral administration of bLF was selected for the present study, even though the metabolism and mechanism of ingested lactoferrin are yet to be clarified. As to absorption, it has been reported that intact lactoferrin and its fragments are present in the urine of human milk-fed preterm infants.<sup>(20)</sup> However, in adult rats, lactoferrin and its fragments are not detectable in portal blood after bLF ingestion,<sup>(21)</sup> and in adult humans, the serum lactoferrin level does not increase after oral administration of recombinant human lactoferrin.<sup>(22)</sup> However, several studies have suggested that orally administered lactoferrin might enhance immune responses via cytokine production.<sup>(23,24)</sup> It has been reported that oral administration of bLF to mice enhances the production of IL-18 and interferon- $\gamma$  in the mucosa of the small intestine, and increases the number of CD4<sup>+</sup>, CD8<sup>+</sup> and NK cells in the small-intestinal epithelium.<sup>(25,26)</sup> Varadhachary *et al.* reported that oral administration of recombinant human lactoferrin to mice stimulates IL-18 production from gut enterocytes, and augments the NK activity of spleen cells and production of blood CD8<sup>+</sup> cells.<sup>(27)</sup> Furthermore, a recent clinical study has demonstrated that oral administration of bLF (0.6 g/day) for 3 months in 36 patients with chronic hepatitis C increased the serum IL-18 level significantly compared with the



**Fig. 3.** Changes in the mean percentages of (a) CD4<sup>+</sup>, (b) CD8<sup>+</sup>, (c) CD16<sup>+</sup> and (d) CD56<sup>+</sup> peripheral blood lymphocytes in the bovine lactoferrin group (straight line,  $n = 23$ ) and the placebo group (dotted line,  $n = 23$ ).

baseline.<sup>(28)</sup> However, our study found no evidence that oral administration of bLF influences the serum concentration of IL-18 or the percentage of CD4<sup>+</sup>, CD8<sup>+</sup>, CD16<sup>+</sup> and CD56<sup>+</sup> lymphocytes. Further investigations are required to clarify the peripheral and systemic effects of orally administered lactoferrin. In addition, as many *in vitro* studies have suggested that lactoferrin has direct binding neutralizing efficacy against HCV,<sup>(29-31)</sup> further investigations are needed to devise a means of delivering lactoferrin or its fragment into the bloodstream safely and effectively.

Recently, several studies have investigated the value of adding lactoferrin to interferon therapy for chronic hepatitis C. Hirashima *et al.* randomly assigned 21 patients with chronic hepatitis C to either a consensus interferon plus oral lactoferrin (3.0 g/day) group or a consensus interferon monotherapy group.<sup>(32)</sup> Three of 10 patients in the consensus interferon plus lactoferrin group showed a sustained complete virologic response, as did four of 11 patients in the consensus interferon group, indicating no statistically significant difference between the groups. Ishibashi *et al.* conducted a randomized controlled trial to investigate the efficacy of interferon  $\alpha$ -2b and ribavirin plus oral lactoferrin (0.6 g/day) compared with interferon  $\alpha$ -2b and ribavirin plus placebo in 36 patients with chronic hepatitis C.<sup>(33)</sup> A sustained complete virologic response was seen in six of 18 patients in the lactoferrin group and in five of 18 patients in the placebo group, there being no statistically significant difference between the groups

( $P = 0.7$ ). Although the numbers of patients recruited in the two randomized trials were small, these results suggested that the additional value of oral lactoferrin combined with interferon therapy would be negative for the treatment of chronic hepatitis C.

In summary, oral administration of bLF at a dose of 1.8 g/day for 12 weeks showed an acceptable safety profile in patients with chronic hepatitis C. However, there was no significant difference in the virologic responses between patients who received oral bLF and those receiving placebo. In addition, bLF intake did not have any favorable effect on the serum ALT level. These findings do not support the practical use of oral bLF in patients with chronic hepatitis C.

## Acknowledgments

This article is dedicated to the memory of the late Dr S. Okada, a principal investigator, and the late Dr N. Okazaki, the chairman of the data monitoring committee. We thank Drs M. Kato, Y. Inaba and K. Hayashi as members of the data monitoring committee. We are grateful to Dr H. Tsuda as a scientific contributor. We also thank Ms K. Kondo and Dr H. Nakajima for assistance in data management, and Morinaga Milk Industries for providing the bLF tablets and placebo. This work was supported by Grants-in-Aid for Cancer Research for the Second- and Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan.

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## Phase II trial of intra-arterial chemotherapy using a novel lipophilic platinum derivative (SM-11355) in patients with hepatocellular carcinoma

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**Key words:** intra-arterial chemotherapy, SM-11355, hepatocellular carcinoma (HCC)

### Summary

SM-11355, a lipophilic platinum derivative, is a novel intra-arterial chemotherapeutic agent for hepatocellular carcinoma (HCC). A phase II study of SM-11355 was conducted to evaluate the antitumor activities and the toxicity in chemotherapy-naïve patients with HCC. Sixteen patients were treated with transcatheter arterial injection of SM-11355–lipiodol emulsion (20–120 mg/body). The responses were evaluated by computed tomography 3 months after treatment. Complete response (CR) was defined as disappearance or 100% necrosis of all tumors, and lipiodol accumulation in tumors was regarded as indicating necrosis. Nine patients achieved CR (56%; 95% confidence interval, 29.9–80.2%). The grade 3 toxicities were neutropenia (19%), total bilirubin elevation (19%), AST elevation (44%), and ALT elevation (19%). None of the patients showed grade 4 toxicities or episodes of renal dysfunction. Other common adverse effects were eosinophilia (100%) and pyrexia (94%). Intra-arterial chemotherapy with SM-11355, which was well tolerated, showed promising antitumor activity in patients with HCC.

### Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors throughout the world. Although screening of high-risk populations for HCC using ultrasonography and tests of serum  $\alpha$ -fetoprotein (AFP) levels has recently caused an increase in the number of candidates for effective local treatments such as hepatic resection and local ablation therapy, many patients exhibit multiple HCC at the time of initial diagnosis or at the time of recurrence after the local treatment. In these patients, various anticancer agents have been employed as intra-arterial chemotherapy alone [1–9] or in combination with transcatheter arterial embolization (TAE) [10–14]. However, it has not yet been proven which

anticancer agent is most effective, and the impact of intra-arterial chemotherapy on survival is still uncertain.

SM-11355 (*cis*-[*(((1R,2R)-1,2-cyclohexanediamine-N,N'*) bis(myristato)]-platinum (II) monohydrate, Sumitomo Pharmaceuticals Co., Osaka, Japan; Figure 1) is a novel lipophilic cisplatin derivative that can be suspended in lipiodol, a lipid lymphographic agent [15]. When lipiodol is injected into an artery feeding HCC nodules, it selectively accumulates in the tumor [16]. Accordingly, a SM-11355–lipiodol emulsion is deposited within the HCC nodules and releases active platinum compounds into tumor tissues gradually [17]. In a phase I study, a concentration escalation study for this agent, the recommended concentration was determined to be 20 mg/ml when the maximum

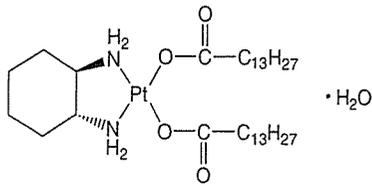


Figure 1. Chemical structure of SM-11355 (*cis*-[(1*R*,2*R*)-1,2-cyclohexanediamine-*N,N'*]bis(myristato)]-platinum (II) monohydrate).

volume was fixed at 6 ml [18]. To evaluate the anti-tumor effect and the toxicity of SM-11355, a phase II study of this agent was conducted in patients with HCC. The pharmacokinetics of SM-11355 was also investigated in this study.

### Patients and methods

TAE-naive and chemotherapy-naive patients with unresectable HCC were eligible if they had no indication for local ablation therapy. Diagnosis was confirmed histologically and/or clinically by angiography and computed tomography (CT). Each patient was required to meet the following criteria: at least one measurable intrahepatic lesion that showed tumor staining by angiography, adequate hematological function (white blood cells  $\geq 3000/\text{mm}^3$ , platelets  $\geq 50,000/\text{mm}^3$ , hemoglobin  $\geq 9.5$  g/dl), adequate hepatic function (serum total bilirubin  $\leq 3.0$  mg/dl, serum albumin  $\geq 3.0$  g/dl, serum aspartate aminotransferase (AST)  $\leq 200$  U/L, serum alanine aminotransferase (ALT)  $\leq 200$  U/L), adequate renal function (serum creatinine  $\leq$  the upper limit of normal value), Eastern Cooperative Oncology Group [19] performance status of 0–2, 20–74 years of age, a minimum life expectancy of more than 2 months, and written informed consent. Eligible patients were also with clinical stage I or II in accordance with the classification of underlying liver cirrhosis by the Liver Cancer Study Group of Japan [20], defined as ICG retention at 15 min (ICG R-15)  $\leq 40\%$ , prothrombin time  $\geq 50\%$ , and no refractory ascites. Patients who had previous hepatic resection and/or local ablation therapy were eligible if they had no evidence of local tumor recurrence in the treated lesions. The previous anticancer treatment had to have been discontinued for at least 4 weeks before enrollment in this study. Patients were excluded if they met the following criteria: a history of allergy to iodine-containing agent and/or contrast material, concomitant malignancy, extrahepatic metastasis or tumor thrombus in

the portal vein and/or the hepatic vein, intrahepatic arteriovenous shunting, pregnant or lactating women and patients of reproductive potential, or other serious medical conditions. The study was approved by the ethics committee of each participating center.

SM-11355/lipiodol emulsion (20 mg/ml) was injected slowly under fluoroscopic monitoring into the artery feeding the HCC by use of a catheter. The emulsion was prepared by suspending SM-11355 (120 mg) in lipiodol (6 ml), which was shaken just before injection. The volume of the emulsion, up to a maximum of 6 ml (containing 120 mg of SM-11355), was adjusted according to the tumor size and tumor distribution, that is, the injection was discontinued when full accumulation of the emulsion in the tumor vessels was obtained, as defined in the protocol. When tumor staining was noted in enhanced CT or angiography after the initial treatment, the second treatment was performed within 3 months after the initial treatment. Patients who showed no tumor staining after the initial treatment did not undergo a second treatment.

The antitumor effect was evaluated by CT, which was performed 3 months after the second treatment. In patients who did not receive a second treatment, CT was performed 3 months after the initial treatment to evaluate the response. Tumor size was measured by the sum of the products of the perpendicular longest diameters of all measurable lesions. Lipiodol accumulation in tumors was regarded as an indication of necrosis [21,22]. We defined complete response (CR) as disappearance or 100% necrosis of all tumors, and partial response (PR) as more than 50% reduction and/or more than 50% necrosis. Progressive disease (PD) was defined as more than 25% enlargement of the tumor. No change (NC) was considered as disease not qualifying for classification as CR, PR, or PD. Survival curves were calculated from the day of initiation of this treatment using the Kaplan–Meier method. Toxicity was assessed according to the criteria of the Japanese Society for Cancer Therapy [23], which are fundamentally similar to the World Health Organization (WHO) criteria [24] and National Cancer Institute (NCI) Common Toxicity Criteria.

Pharmacokinetic evaluation was performed in patients who gave written informed consent. Peripheral blood samples (5 ml) were collected in a heparinized tube before initiation of the treatment, and 7 days, 3 weeks, 4–6 weeks, 12–15 weeks, 6–8 months, and 10–14 months after the initial treatment. In patients who underwent a second treatment, the

scheduled blood samples collections after the initial treatment were discontinued. Instead, blood samples were drawn 7 days, 3 weeks, 4–6 weeks, 12–15 weeks, 6–8 months, and 10–14 months after the second treatment. All samples were centrifuged immediately after sampling and stored below  $-25^{\circ}\text{C}$ . Platinum concentrations in the treated tumor and nontumorous tissues were examined in patients who underwent hepatic resection after administration of SM-11355. The plasma and the tissue total platinum concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS). The maximum plasma concentration ( $C_{\text{max}}$ ) and the maximum plasma concentration time ( $T_{\text{max}}$ ) were analyzed.

The sample-size goal of this study was set at 15 eligible patients. This number of patients was planned using a design based on the assumptions that the CR rate in conventional TAE was 9–11% [25], and the expected CR rate in this trial were 40%. If the CR rate in this trial were 40% in 15 patients, the lower limit of the value of 95% confidence interval in the CR rate would be more than 15%.

## Results

A total of 17 patients were enrolled in this study between July 1998 and April 1999. Of these 17 patients, 16 were eligible and one was ineligible and was not treated because of the presence of intrahepatic arteriovenous shunting. The patient characteristics in the 16 eligible patients before treatment are summarized in Table 1. Of the 16 patients, 12 underwent a second treatment, while the remaining four who showed no viable lesions after the initial treatment did not receive a second treatment.

All of the 16 patients were assessed for toxicity. Response could be assessed in 15 of the 16 patients. One patient was not evaluated for response because he underwent TAE with gelatin sponge before the response evaluation for SM-11355, at his request.

### Antitumor effect

Nine patients (56%) achieved CR, three patients (19%) showed NC, three patients (19%) had PD, and one patient (6%) was not evaluated. The CR rate was 56% (9/16) with a range of 30–80% within the 95% confidence limits. In nine CR patients, three had local recurrence of the treated tumor, four showed recurrence in the lesion isolated from the treated tumors, and one showed no recurrence at the time of analysis

Table 1. Patient characteristics

Total patients	16	
Gender (men : women)	15 : 1	(94% : 6%)
Age (years)	Median 65.5 (range)	(49–74)
PS* <sup>1</sup> (0 : 1 : 2)	16 : 0 : 0	
HBs Ag* <sup>2</sup> positive	2	(13%)
HCV Ab* <sup>3</sup> positive	12	(75%)
Albumin (g/dl)	Median 3.6 (range)	(3.2–4.1)
Total bilirubin (mg/dl)	Median 0.95 (range)	(0.4–2.4)
Clinical stage* <sup>4</sup> (I : II : III)	8 : 8 : 0	
History of PEI* <sup>5</sup> and/or resection	4	(25%)
Intrahepatic nodules	1 8 2–4 7 5– 1	(50%) (44%) (6%)
Maximum tumor diameter (mm)	Median 20 (range)	(14–50)
Tumor stage* <sup>6</sup> (I : II : III : IV–a)	5 : 5 : 5 : 1	
$\alpha$ -fetoprotein > 100 ng/ml	4	(25%)

\*<sup>1</sup>PS: performance status.

\*<sup>2</sup>HBs Ag: hepatitis B surface antigen.

\*<sup>3</sup>HCV Ab: hepatitis C virus antibody.

\*<sup>4</sup>Grading of underlying liver cirrhosis by the Liver Cancer Study Group of Japan [18].

\*<sup>5</sup>PEI: percutaneous ethanol injection.

\*<sup>6</sup>Tumor stage according to the criteria of the Liver Cancer Study Group of Japan [20].

(23.6 months after the achievement of CR). In one CR patient, hepatic resection was carried out 3 months after the second treatment at his request. The pathological examination showed more than 95% necrosis in this tumor. In CR patients except for the patient who underwent the resection, the median duration of response (from the date CR was first recorded to the date on which progressive disease was first noted) was 7.1 months (range, 2.5–23.6 + months). Regarding the additional assessment of tumor response in terms of the WHO criteria, seven patients (44%) showed 50% or greater decrease in total tumor size of the treated lesions, as determined by two observations not less than 4 weeks apart.

### Change in serum $\alpha$ -fetoprotein levels

Serum AFP levels were measured serially after treatment. Of the four patients whose serum AFP levels