options for further anticancer treatment are limited. S-1 is an orally administered anticancer drug that consists of a combination of tegafur, 5-chloro-2,4-dihydroxypyridine and oteracil potassium in a 1 : 0.4 : 1 molar ratio [27]. The antitumor effect of S-1 has already been demonstrated in a variety of solid tumors including pancreatic cancer [7, 11, 12, 14, 20, 21, 25, 26, 32, 33]. In patients with chemo-naïve pancreatic cancer, an overall response rate of 21.1% was achieved, and the median time-to-progression and median overall survival period were 3.7 and 8.3 months, respectively [32]. In gemcitabine-refractory metastatic pancreatic cancer, our recent phase II study of S-1 yielded results that demonstrated marginal activity including a response rate of 15%, a median progression-free survival time of 2.0 months and a median overall survival time of 4.5 months, with a favorable toxicity profile [17]. In addition, other reports also demonstrated marginal antitumor activity [1, 28]. Gemcitabine administration via infusion at a fixed dose rate of 10 mg/m^2/min (FDR-Gem) has been found to increase the intracellular drug concentrations, compared with gemcitabine at a standard dose rate infusion over a period of 30 min. A recent phase II study of combination therapy consisting of FDR-Gem and oxaliplatin (GEMOX) yielded results that demonstrated activity in gemcitabine-refractory advanced pancreatic cancer [5], although oxaliplatin is inactive against pancreatic cancer when used as a single agent [6]. The increased intracellular concentrations of gemcitabine as a result of FDR infusion and/or the synergistic effect of gemcitabine and oxaliplatin may play an important role in the antitumor effect of GEMOX. This finding is of interest when considering the effect of combination therapy consisting of FDR-Gem and some other agent that exhibits a synergistic effect with gemcitabine in patients with metastatic pancreatic cancer who failed standard dose rate gemcitabine.

The inhibition of ribonucleotide reductase by gemcitabine is considered to enhance the effect of the 5-FU metabolite 5-FdUMP by reducing the concentration of its physiological competitor [10]. Preclinical studies have demonstrated a synergy between gemcitabine and 5-FU in tumor cell lines, including pancreatic cancer cells [3, 23]. S-1 is a fluoropyrimidine, and several phase II studies of S-1 and gemcitabine combination therapy have yielded results that demonstrated a promising activity in chemo-naïve advanced pancreatic cancer patients, including a response rate of 32–48% and a median survival times of 7.89–12.5 months [16, 18, 19, 31].

Therefore, we conducted the present phase I/II study to determine the recommended doses of FDR-Gem and S-1 (FGS) to use for combination therapy and to evaluate the toxicity and efficacy at the recommended doses in patients with gemcitabine-refractory pancreatic cancer.

Materials and methods

Eligibility criteria

The eligibility criteria were histologically proven pancreatic adenocarcinoma with measurable metastatic lesions, disease progression during gemcitabine-based first-line chemotherapy, age 20 years or over, ECOG performance status of 0–2 points, more than 2-week interval between the final dose of the prior chemotherapy regimen and study entry, adequate bone marrow function (leukocyte count $\geq$ 3,500/mm^3, neutrophil count $\geq$ 1,500/mm^3, platelet count $\geq$ 100,000/mm^3, hemoglobin concentration $\geq$ 9.0 g/dL), adequate renal function (serum creatinine level $\leq$ 1.1 mg/dL) and adequate liver function (serum total bilirubin level $\leq$ 2.0 mg/dL, transaminase levels $\leq$ 100 U/L). Patients with obstructive jaundice or liver metastasis were considered eligible if their total bilirubin level $\leq$ 3.0 mg/dL and transaminase levels could be reduced to 150 U/L by biliary drainage. The exclusion criteria were regular use of phenytoin, warfarin or flucytosine, history of fluorinated pyrimidine use, severe mental disorder, active infection, ileus, watery diarrhea, interstitial pneumonitis or pulmonary fibrosis, refractory diabetes mellitus, heart failure, renal failure, active gastric or duodenal ulcer, massive pleural or abdominal effusion, brain metastasis, and active concomitant malignancy. Pregnant or lactating women were also excluded. Written informed consent was obtained from all patients. This study was approved by the institutional review board of the National Cancer Center of Japan.

Treatment

Considering the patients' quality of life, we adopted biweekly schedule. Gemcitabine (Eli Lilly Japan K.K., Kobe, Japan) was administered by FDR intravenous infusion of 10 mg/m^2/min on day 1. S-1 (Taiho Pharmaceutical Co., Ltd., Tokyo, Japan) was administered orally twice daily on day 1 to day 7, followed by a 1-week rest. Treatment cycles were repeated every 2 weeks until disease progression or unacceptable toxicity occurred. If blood examination revealed leukocytopenia $<$ 2,000/mm^3, thrombocytopenia $<$ 75,000/mm^3, total bilirubin $>$ 3.0 mg/dL, aspartate aminotransferase or alanine aminotransferase level $>$ 150 U/L, or creatinine $>$ 1.5 mg/dL, both gemcitabine and S-1 were withheld until recovery. If a patient experienced dose-limiting toxicity (DLT), the dose of gemcitabine and S-1 was reduced by one level in the subsequent cycle. If a rest period of more than 15 days was required because of toxicity, the patient was withdrawn from the study. Patients were scheduled to receive gemcitabine and S-1 at four dosage levels (Table 1). Two dosage levels of S-1 were established according to the body

Table 1 Dosage levels of gemcitabine and S-1

Dosage level	Gemcitabine	S-1
Level 0	600 mg/m^2/60 min	Dosage A
Level 1[a]	800 mg/m^2/80 min	Dosage A
Level 2	1,000 mg/m^2/100 min	Dosage A
Level 3	1,200 mg/m^2/120 min	Dosage A
Level 4	1,200 mg/m^2/120 min	Dosage B

[a] Starting dosage

surface area as dosage A, about 80 mg/m^2/day, and dosage B, about 100 mg/m^2/day (Table 2). At the first dose level (level 1), gemcitabine was administered at a dosage of 800 mg/m^2 administered as a 80-min infusion, and S-1 was administered at dosage A. At the next dose level (level 2), the gemcitabine dosage was increased to 1,000 mg/m^2 administered as a 100-min infusion, and S-1 was administered at the same dosage. At the next dose level (level 3), the gemcitabine dosage was increased to 1,200 mg/m^2 administered as a 120-min infusion, and S-1 was administered at the same dosage. At the final dosage level (level 4), gemcitabine administered at the same dosage, and S-1 was administered at dosage B.

Study design

This study was an open-label, four-center, single-arm phase I/II study performed in two steps. The objective of step 1 (phase I) was to evaluate the frequency of DLT during first 2 cycles (4 weeks) and then use the frequency of DLT to determine which of the four dosages tested to recommend (Table 1). At least 3 patients were enrolled at each dosage level. If DLT was observed in the initial three patients, up to three additional patients were entered at the same dosage level. The highest dosage level that did not cause DLT in 3 of the 3 or $\geq$3 of the 6 patients treated at that level during the first two cycles of treatment was considered the maximum-tolerated dosage (MTD). DLT was defined as (1) grade 4 leucopenia or grade 4 neutropenia or febrile neutropenia, (2) grade 4 thrombocytopenia or thrombocytopenia requiring transfusion, (3) grade 3 or 4 non-hematological toxicity excluding hyperglycemia and electrolyte disturbances, (4) serum transaminases levels, γ-glutamyl transpeptidase level and alkaline phosphatase level $\geq$10 times UNL, (5) serum creatinine level $\geq$ 2.0 mg/dL and (6) any toxicity that necessitated a treatment delay of more than 15 days. Toxicity was graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. In step 2, the recommended dosages (RD) of FGS were then administered, and the effect of this combination therapy on objective tumor response was evaluated in patients who were given the RD (phase II). The number of patients to be enrolled in phase II was determined by using a SWOG's standard design (attained design) [8, 9]. The phase II included the patients who received the RD in the step 1. The null hypothesis was that the overall response rate would be $\leq$5%, and the alternative hypothesis was that the overall response rate would be $\geq$20%. The α error was 5% (one-tailed), and the β error was 10% (one-tailed). The alternative hypothesis was established based on the preferable data in previous reports [5, 15, 24, 30, 34]. Interim analysis was planned when 20 patients were enrolled. If none of the first 20 patients had a partial response or complete response, the study was to be ended. If a response was detected in any of the first 20 patients, an additional 20 patients were to be included in a second stage of accrual to more precisely estimate the actual response rate. If the number of objective responses after completing the trial was 5 or more among the 40 patients, then we would reject the null hypothesis and conclude that FGS was effective, and we would proceed to the next large-scale study. The severity of adverse events and progression-free survival and overall survival were investigated as secondary objectives in phase II.

Table 2 Dosage of S-1 (tegafur equivalent)

Body surface area (m^2)	Dosage A ($\fallingdotseq$80 mg/m^2/day)	Dosage B ($\fallingdotseq$100 mg/m^2/day)
<1.25	40 mg × 2/day	50 mg × 2/day
1.25–<1.5	50 mg × 2/day	60 mg × 2/day
$\geq$1.5	60 mg × 2/day	75 mg × 2/day

Results

Patient characteristics

Between June 2006 and March 2009, 49 patients were enrolled in this study. Fifteen patients (level 1: 3 patients, level 2: 3 patients, level 3: 6 patients, level 4: 3 patients) were enrolled into the phase I (STEP 1), and an additional 34 patients were enrolled into the phase II (STEP2) at dose level 3. Table 3 shows the baseline characteristics of the patients in step 1 and step 2. A total of the 40 patients who were given the recommended dose, 6 patients and 34 patients who entered into the study at phase I and phase II, respectively, were evaluated for efficacy and detailed safety profile.

Phase I (STEP 1)

No DLT occurred during the first 2 cycles (4 weeks) at level 1 or level 2. At dose level 3, three patients were

Table 3 Patient characteristics

Characteristic	Step 1				Step 2	Total at the recommended dose (level 3)
	Level 1	Level 2	Level 3	Level 4	Level 3	
No. of patients	3	3	6	3	34	40
Age, years						
Median	66	58	64	62	63.5	64
Range	55–69	51–58	48–71	52–70	40–80	40–80
Sex, *n* (%)						
Male	1 (33)	3 (100)	4 (67)	1 (33)	19 (56)	23 (58)
Female	2 (67)	0	2 (33)	2 (67)	15 (44)	17 (48)
ECOG performance status, *n* (%)						
0	2 (67)	2 (67)	5 (83)	2 (67)	22 (65)	27 (68)
1	1 (33)	1 (33)	1 (17)	1 (33)	12 (35)	13 (33)
Primary tumor, *n* (%)						
Head	1 (33)	2 (67)	2 (33)	2 (67)	17 (50)	19 (48)
Body/tail	2 (67)	1 (33)	4 (67)	1 (33)	17 (50)	21 (53)
Metastatic site, *n* (%)						
Liver	3 (100)	3 (100)	6 (100)	1 (33)	25 (74)	31 (78)
Lung	1 (33)	0	0	2 (67)	7 (21)	7 (18)
Peritoneum	1 (33)	1 (33)	0	1 (33)	11 (32)	11 (28)
Lymph node	0	2 (67)	0	0	11 (32)	11 (28)
Tumor stage at the start of prior treatment, *n* (%)						
Locally advanced	0	0	0	1 (33)	7 (21)	7 (18)
Metastatic	3 (100)	3 (100)	6 (100)	2 (67)	27 (79)	33 (83)
Prior treatment, *n* (%)						
Gemcitabine alone	3 (100)	3 (100)	5 (83)	3 (100)	26 (76)	31 (78)
Gem + Axitinib	0	0	0	0	2 (6)	2 (5)
Gem + Erlotinib	0	0	1 (17)	0	6 (18)	7 (18)

evaluated first, and none developed DLT. Since all 3 patients experienced DLT at dose level 4 (grade 4 neutropenia in two patients, grade 3 stomatitis in one patient), 3 additional patients were evaluated at dose level 3. A DLT (grade 4 neutropenia) was experienced by 2 of the 3 patients in this additional cohort in dose level 3, and dose level 3 was determined to be the MTD. Based on these results, the RD was determined to be level 3.

Phase II (efficacy and safety profile in the 40 patients treated at dose level 3)

In step 2, the RD of FDR-Gem and S-1 was administered to an additional 34 patients, and a total 40 patients were treated at dose level 3 to evaluate the objective tumor response to this combination therapy. As of the date of the analysis, the protocol treatment had been concluded in 39 of the 40 patients, and a total of 286 courses (median: 5 courses; range 1–31 courses) had been administered at level 3. The actual mean weekly dose administered were gemcitabine 545 mg/m^2/week (90.8% of planned dosage) and 90.1% of planned dosage of S-1. Dose reduction was required in 10 patients because of grade 4 neutropenia (five patients), grade 3 fatigue (1 patient), grade 2 fatigue with grade 2 appetite loss (one patient), grade 2 nausea (two patients) and grade 3 rash (1). The reasons for treatment discontinuation in phase II were radiological disease progression (33 patients), clinical disease progression (two patients), recurrent grade 4 neutropenia despite dose reduction due to grade 4 neutropenia (two patients), grade 4 myocardial infarction (one patients) and patient request to return to his distant hometown (one patient). All patients who discontinued treatment because of adverse events recovered from the toxicities after discontinuation. Twelve patients received third-line chemotherapy after discontinuation of FGS: S-1 monotherapy in four patients, gemcitabine + S-1 combination therapy on another treatment schedule in three patients, chemoradiotherapy with S-1 in one patient and new molecularly targeted agents in four patients who participated in a different clinical trial. Twenty-two patients received best supportive care, the other five patients transferred to another hospital, and no

information is available about their treatment after discontinuation of FGS.

Toxicity

All patients in steps 1 and 2 were evaluated for toxicity. In step 1, grade 3/4 non-hematological toxicity was observed in two patients (grade 3 fatigue during the third course in one patient, grade 3 stomatitis during the second course in one patient). No grade 4 leukocytopenia was observed at any dose level, but grade 4 neutropenia was observed in one out of three patients at dose level 1, none of the three patients at dose level 2, two of the six patients at dose level 3 and all three of the patients at dose level 4. Grade 3 thrombocytopenia was observed in one patient at dose level 2.

Table 4 summarizes the toxicities in the 40 patients who received the RD (level 3). All 40 eligible patients were assessable for toxicities, and FGS combination therapy at the RD was generally well tolerated. The most common toxicities were leukocytopenia (60%) and neutropenia (60%), but most of these toxicities were tolerable and reversible. Grade 4 neutropenia was noted as hematological toxicity in five patients (13%). Grade 3 non-hematological toxicities consisted of fatigue (one patient), vomiting (one patient), rash (one patient) and liver abscess (one patient). The patient who developed the grade 3 liver abscesses recovered after appropriate treatment with intravenous antibiotic alone. One female patient, who had hypercholesterolemia and history of smoking of 30 cigarettes/day, experienced a grade 4 acute myocardial infarction on day 1 of the third course of treatment, after gemcitabine had been administered but before the start of oral S-1. Emergency coronary angiography showed total occlusion of the left anterior descending coronary artery. The patient recovered from the cardiogenic shock due to myocardial infarction after coronary stent implantation and appropriate supportive treatment. S-1 monotherapy for the pancreatic cancer was started about 1 month after the infarction. No other severe or unexpected toxicities were noted in any of the patients.

Table 4 Treatment-related adverse events among the 40 patients who received the recommended dosages: highest grade reported during the treatment period

	Grade				Grade 1–4	Grade 3–4
	n					
	1	2	3	4	*n* (%)	*n* (%)
Hematological toxicities						
Leukocytes	11	4	9	0	24 (60)	9 (23)
Neutrophils	10	1	8	5	24 (60)	13 (33)
Hemoglobin	5	11	1	0	17 (43)	1 (3)
Platelets	11	2	1	0	14 (35)	1 (3)
Non-hematological toxicities					(0)	
Aspartate aminotransferase	8	1	0	0	9 (23)	0 (0)
Alanine aminotransferase	8	3	0	0	11 (28)	0 (0)
Alkaline phosphatase	5	2	0	0	7 (18)	0 (0)
Total bilirubin	3	0	0	0	3 (8)	0 (0)
Fatigue	15	2	1	0	18 (45)	1 (3)
Nausea	13	4	0	0	17 (43)	0 (0)
Vomiting	8	1	1	0	10 (25)	1 (3)
Anorexia	19	6	0	0	27 (68)	0 (0)
Stomatitis	4	0	0	0	4 (10)	0 (0)
Alopecia	8	0	–	–	8 (20)	–
Diarrhea	7	2	0	0	9 (23)	0 (0)
Rash	3	4	1	0	8 (20)	1 (3)
Hyperpigmentation	9	1	–	–	10 (25)	–
Hand-foot skin reaction	1	2	0	0	3 (8)	0 (0)
Watery eye	2	0	0	–	2 (5)	0 (0)
Hoarseness	1	0	0	0	1 (3)	0 (0)
Infection liver abscess	0	0	1	0	1 (3)	1 (3)
Myocardial infarction	0	0	0	1	1 (3)	1 (3)

Three patients died within 30 days after the final dose of the study drug. All 3 of the deaths were attributed to disease progression, and there were no treatment-related deaths.

Efficacy

It was possible to assess all 40 eligible patients who received the RD for response. Thirty-four patients had died by the completion of the follow-up period. There were no complete responses, but a partial response was achieved in seven patients (18, 95% confidence interval, 7.3–32.8%). Stable disease was noted in 19 patients (48%) and progressive disease in 14 patients (35%). Tumor responses to second-line FGS therapy are classified according to the tumor responses to first-line gemcitabine in Table 5. Three of 10 patients whose best response was progression disease in first-line chemotherapy achieved partial response in FGS therapy. The median progression-free survival time was 2.8 months. The median overall survival time after the start of second-line therapy was 7.0 months (range 1.3–18.9+), and the 1-year survival rate was 18% (Fig. 1). The median overall survival time after the start of first-line therapy was 13.9 months (range 5.2–31.4).

Table 5 Objective tumor response

Response (2nd line)	*n* (%)	Response (1st line)		
		PR	SD	PD
PR	7 (18)	1	3	3
SD	19 (48)	3	12	4
PD	14 (35)	2	9	3
Total	40 (100)	6	24	10

Response rate: 18% (95% CI: 7.3–32.8)

RECIST criteria

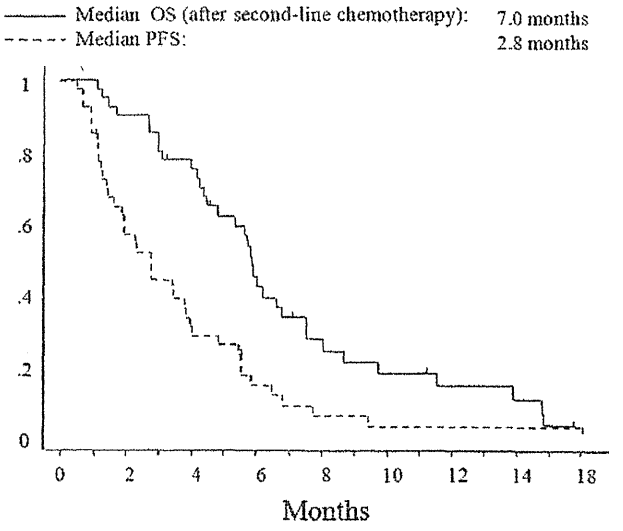

Fig. 1 Survival curves. Survival ($n = 40$). Progression-free survival (*dashed line*) and overall survival time (*solid line*) curves of patients with gemcitabine-refractory pancreatic cancer receiving systemic chemotherapy with FGS

Discussion

In the last decade, several clinical trials (mainly phase II) have been conducted in patients with advanced pancreatic cancer after failure of first-line gemcitabine or a gemcitabine-based combination regimen. The results of a randomized trial ($n = 168$) comparing fluorouracil and folinic acid versus oxaliplatin, fluorouracil and folinic acid (OFF) indicated that OFF improved progression-free survival and overall survival as a second-line chemotherapy. The median progression-free survival time and median survival time of OFF were 3 and 6 months, respectively [22]. In the present study, FGS yielded a median progression-free survival time of 2.8 months and a median overall survival time of 7.0 months, similar to the data mentioned above. Furthermore, the response rate of 18% in the present study was above the pre-established boundary (objective response in five or more of the 40 patients) required for the regimen to be considered effective. However, the gap between the median overall survival time and the median progression-free survival time in the present study was relatively large. Although the reason for this gap is unknown, a bias arising from the selection of patients with a good general condition or with a small tumor burden may explain these findings.

Whether gemcitabine as an FDR infusion is active even after progression during treatment with the standard 30-min administration of gemcitabine was the critical clinical question examined in this study. Differentiating between the relative roles of gemcitabine and S-1 in overcoming tumor resistance is difficult. The efficacy and survival data obtained in the present study seem to be better than those of previous studies for oral fluoropyrimidine monotherapy as a salvage chemotherapy for advanced pancreatic carcinoma (Table 6) [1, 2, 17, 28, 29]. However, since all the data were obtained in single-arm studies, a randomized study is needed to make these suggestions reliable. Furthermore, whether the combined regimen in the present study is superior to other regimens, such as the OFF regimen, remains an essential clinical question.

Safety and convenience as well as antitumor efficacy are critically important issues with regard to second-line chemotherapy. One patient experienced an acute myocardial infarction. Although she had other risk factors, such as a smoking habit and hyperlipidemia, a relation between gemcitabine and the acute myocardial infarction cannot be ruled out because gemcitabine had been administered on the day of the infarction. The toxicity profile of FGS

Table 6 Comparison between the current study and previous studies of oral fluoropyrimidine monotherapy as salvage chemotherapy for advanced pancreatic carcinoma

Study	References	Phase	Regimen	*n*	PR + CR (%)	Median PFS (months)	Median OS (months)
Morizane et al.	[12]	II	S-1	40	15	2.0	4.5
Abbruzzese et al.	[29]	II	S-1	45	0	1.4	3.1
Sudo et al.	[31]	II	S-1	21	9.5	4.1	6.3
Todaka et al.	[32]	Retrospective	S-1	52	4	2.1	5.8
Boeck et al.	[30]	II	Capecitabine	39	0	2.3	7.6
Morizane et al.	Current study	II	FGS	40	18	2.8	7.0

therapy in the other patients was acceptable, and the most common grade 1–4 adverse reactions were anorexia (68%), leukocytopenia (60%) and neutropenia (60%), although most episodes were tolerable and reversible. The safety profile in this study suggests that FGS can be safely administered to pancreatic cancer patients even in a second-line setting, at least in select populations. The biweekly schedule allows enough time to recover from myelosuppression and non-hematological toxicities before the following cycle, enabling patients to receive treatment as scheduled. Actually, the relative dose intensities of gemcitabine and S-1 in our study were high (90.8 and 90.1%, respectively). Furthermore, because of the biweekly schedule, patients do not need to come to the hospital for treatment as often compared with the first-line standard schedule of gemcitabine therapy. Our new treatment schedule may therefore improve the patients' quality of life during anticancer treatment.

We concluded that combination therapy consisting of gemcitabine as a fixed dose rate infusion and S-1 (FGS) provided a promising antitumor activity and tolerable toxicity in patients with gemcitabine-refractory metastatic pancreatic cancer. A larger randomized controlled trial is needed to confirm the clinical benefits of FGS following gemcitabine failure.

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ORIGINAL ARTICLE

Clinical impact of c-Met expression and its gene amplification in hepatocellular carcinoma

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Abstract

Background *c-Met* is an oncogene encoding a receptor for hepatocyte growth factor and, as such, plays a key role in hepatocellular carcinomas (HCC). We evaluated c-Met protein expression and its gene amplification in order to assess whether they were related to tumor recurrence and survival rates among patients who had undergone tumor resection.

Methods We used the polymer-based method to perform an immunohistochemistry analysis of c-Met expression on 59 formalin-fixed, paraffin-embedded sections of surgical specimens. *c-Met* gene amplification was investigated with fluorescence in-situ hybridization. Kaplan–Meier methods and Cox proportional hazards models were used to investigate relationships between c-Met expression, patient characteristics, tumor recurrence, and survival.

Results c-Met expression was associated with portal vein invasion ($p = 0.006$). Recurrence-free survival rates were significantly lower in patients with high levels of c-Met expression ($p < 0.001$). However, c-Met expression levels did not significantly affect overall survival rates ($p = 0.12$). Only 1 patient was found to have *c-Met* gene amplification; 22 patients were found to have aneuploidy of chromosome 7, on which the *c-Met* gene is located. Tumors with chromosome 7 polysomy tended to have higher levels of c-Met expression than those with chromosome 7 monosomy or disomy, but this difference was not statistically significant.

Conclusion Although c-Met expression was not significantly associated with *c-Met* gene amplification, it may be a useful predictive marker of recurrence in resected HCC patients.

Keywords c-Met · Hepatocellular carcinoma · Recurrence · Amplification

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Introduction

Liver cancer is the sixth most common cancer worldwide, with an estimated 626,000 new cases occurring each year. It causes 598,000 deaths annually, making it the third most deadly cancer [1]. The most common type of primary liver cancer is hepatocellular carcinoma (HCC), and is often secondary to hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, both of which increase the risk of liver cancer 20-fold [2].

c-Met was discovered as an oncogene and encodes a tyrosine kinase-type growth factor receptor with an affinity for hepatocyte growth factor (HGF). The HGF–c-Met signaling pathway regulates multiple cellular functions,

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including proliferation, motility, differentiation, tubulogenesis, and angiogenesis [3]. c-Met signaling controls cell invasion and metastasis in a variety of cancers, including HCC [4]. Downregulation of c-Met expression by RNA interference inhibits human HCC cell growth and invasion. A variety of selective c-Met inhibitors have thus been developed as cancer therapies.

In clinical practice, a number of systems have been proposed for predicting the prognosis of HCC patients; however, none of these systems has been universally adopted. These prognostic classifications variably incorporate four features that have been recognized as important determinants of survival: the severity of the underlying liver disease, the size of the tumor, extension of the tumor into adjacent structures, and the presence of metastasis. The three most commonly used systems are the TNM and Okuda staging systems and the CLIP score [5, 6]. Moreover, several molecules have been proposed as predictive markers for HCC, but these factors have not received adequate clinical testing [7, 8].

In this study, immunohistochemical (IHC) analysis was used to investigate c-Met protein expression and its gene amplification in HCC. We hypothesized that c-Met expression would be correlated with tumor invasion and that this correlation would predict recurrence and overall survival (OS).

Materials and methods

Patients

Patients qualified for enrollment in this study if they had undergone tumor resection at the National Cancer Center Hospital in Tokyo between April 2001 and May 2005, did not display apparent distant metastasis, and had not previously received any other treatment. A total of 59 consecutive patients were included in the study.

All patients were followed up every 3–6 months after tumor resection, and patients underwent follow-up examinations to identify possible tumor recurrence. Exam methods included computed tomography, magnetic resonance imaging, abdominal ultrasonography, and measurement of serum alpha-fetoprotein (AFP) levels. Although recurrence could be diagnosed by clinical, radiological, or pathological methods, the main evaluation technique was radiological (e.g., computed tomography and ultrasonography). Clinical and pathological profiles were obtained from a database of liver tumors based on the medical records of the patients.

This study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan, and written informed consent was obtained from all patients.

Immunohistochemistry

A polymer-based method (Envision + Dual Link System-HRP; Dako, Glostrup, Denmark) was used to perform IHC analysis of c-Met expression on formalin-fixed, paraffin-embedded sections of surgical specimens, according to the manufacturer's instructions. For antigen retrieval, sections were autoclaved in 10 mM citrate buffer (pH 6.0) at 121°C for 10 min. A rabbit anti-c-Met polyclonal antibody (Immuno-Biological Laboratories Co., Ltd., Gumma, Japan) was used at a dilution of 1:500. Two pathologists (SK and HO) independently performed blind evaluations of the staining intensity. c-Met overexpression in each tumor was assessed using a 4-point scoring system of epidermal growth factor receptor 2 testing in breast cancer and was performed as described previously [9], as follows: 0 = no staining observed in invasive tumor cells; 1+ = weak, incomplete membrane staining in any proportion of the invasive tumor cells, or weak, complete circumferential membrane staining in fewer than 10% of cells; 2+ = weak but complete membrane staining in at least 10% of cells, or intense complete circumferential membrane staining in 30% or fewer of tumor cells; 3+ = intense complete circumferential membrane staining in more than 30% of tumor cells. We defined scores 0 and 1+ as c-Met^{low}, and scores 2+ and 3+ as c-Met^{high}.

Fluorescence in-situ hybridization for the *c-Met* gene locus

Fluorescence in-situ hybridization (FISH) was also performed for 44 tumor samples with IHC scores of 3+, 2+, and 1+. *c-Met* gene amplification was investigated with FISH using a *c-Met/CEP7* probe cocktail prepared with *c-Met* DNA developed in-house (RP 11-95I20 BAC clone) labeled with SpectrumRed, and *CEP7* DNA labeled with SpectrumGreen (Abbott Molecular, Abbott Park, IL, USA). The FISH assays were carried out as previously described [10].

Tissue sections, 5 μm thick, were subjected to pretreatment with 2× SSC at 75°C and digestion with proteinase K for 7–15 min each, co-denaturation with probe DNA at 85°C for 15 min, hybridization for 12–18 h, and rapid post-hybridization washes with 2× SCC/0.4 NP40.

Signals were enumerated in 20 tumor nuclei per specimen, using a fluorescence microscope with single-interference filter sets for green (FITC), red (Texas red), and blue (DAPI), as well as dual (red/green) band-pass filters. For these 20 tumor cell nuclei, the total number of *c-Met* and *CEP7* was counted, and the ratio of *c-Met* and *CEP7* (*c-MET/CEP7*) calculated. Positivity for gene amplification was defined as *c-Met/CEP7* = 2.0 or higher. In parallel, chromosome 7 aneusomies were defined as mean *CEP7* signals of 2.5 or higher per nucleus.

Statistical analyses

Differences in response rate and association with clinical characteristics were compared by Fisher's exact tests or chi-squared tests. We monitored for relapse every 3–6 months after resection with image testing and AFP. For recurrence-free survival (RFS; calculated from the time of surgical resection to the time of disease progression or last disease assessment), OS (calculated from the time of surgical resection to patient death or last contact) and survival curves were drawn using the Kaplan–Meier method. Group RFS and OS were compared using the log-rank test. Multivariate analysis was performed with a Cox proportional hazards regression model. For all analyses, statistical significance was defined as $p < 0.05$. All statistical analyses were conducted using SPSS version 18 (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

A total of 59 HCC specimens surgically resected from 59 patients were included in this analysis. These patients were mainly males (83%), and their tumors were classified as Child–Pugh A (93%), uninodular (70%), possessing high AFP (35%), and displaying histologically moderate differentiation (59%) (Table 1). At the analysis, 46 patients had recurrence (intrahepatic recurrence 34 patients and extrahepatic recurrence 12 patients).

c-Met protein expression results

Levels of nonspecific background staining were very low. There was also some evidence of c-Met staining within most tumor samples, predominantly localized to the cell membrane in non-neoplastic cells including endothelial cells and fibroblasts. c-Met staining was 3+ for 3 (5%) tumors, 2+ for 12 (20%) tumors, 1+ for 29 (49%) tumors, and negative for 15 (25.3%) tumors (Fig. 1). Low c-Met protein expression (c-Met^{low}) was defined as an IHC score of 0 or 1+, and high c-Met expression (c-Met^{high}) as an IHC score 2+ or 3+. Portal vein invasion was seen in 15 (34%) of the c-Met^{low} patients and 13 (87%) of the c-Met^{high} patients ($p < 0.001$). The difference between the two groups was statistically significant in AFP level ($p = 0.02$). However, the two groups were not statistically different in terms of tumor morphology, type of infection (HBV vs. HCV), size of tumor, or histology (Table 1).

Throughout the median follow-up period of 1266 days (range 84–3251 days), the median RFS across the whole population was 300 days. The median RFS time became significantly shorter as the intensity of c-Met expression increased (0: 1647 days; 1+: 493 days; 2+: 133 days; 3+: 166 days). The median RFS time among patients with c-Met^{high} tumors (166 days; 95% confidence interval [CI] 99.0–232.9) was significantly shorter than the median RFS time among those with c-Met^{low} tumors (748 days; 95% CI 355.2–1140.8) ($p < 0.001$; Fig. 2a).

There were no clear relationships between c-Met expression level and median OS time when each level of expression was considered individually (0: 1450 days, 95% CI 595.6–2304.4; 1+: 1760 days, 95% CI 473.4–2687.8;

Table 1 Association of c-Met protein expression with patient characteristics

Parameter	c-Met IHC		Total	p
	c-Met^{low}	c-Met^{high}		
Total	44	15	59	
Age (years, mean ± SD)	62 ± 11	59 ± 11		
Sex				
Male	36	13	49 (83%)	0.67
Female	8	2	10 (17%)	
Hepatitis				
HCV	7	2	9 (15%)	0.21
HBV	17	8	25 (42%)	
HBV + HCV	10	0	10 (17%)	
Others	10	5	15 (25%)	
Child–Pugh				
A	40	15	55 (93%)	0.22
B	4	0	4 (7%)	
UICC TNM stage				
I	25	2	27 (46%)	0.01
II	10	8	18 (30%)	
III	9	5	14 (24%)	
Tumor morphology				
Multinodular	15	3	18 (30%)	0.31
Uninodular	29	12	41 (69%)	
Portal vein invasion				
Positive	10	13	23 (39%)	<0.001
Negative	34	2	36 (61%)	
Size				
Over 5 cm	17	10	27 (46%)	0.06
Below 5 cm	27	5	32 (54%)	
AFP (IU/L)				
≥400	12	9	21 (36%)	0.02
<400	32	6	38 (64%)	
Histology				
Poorly differentiated	14	7	21 (36%)	0.40
Moderately differentiated	27	8	35 (59%)	
Well differentiation	3	0	3 (5%)	

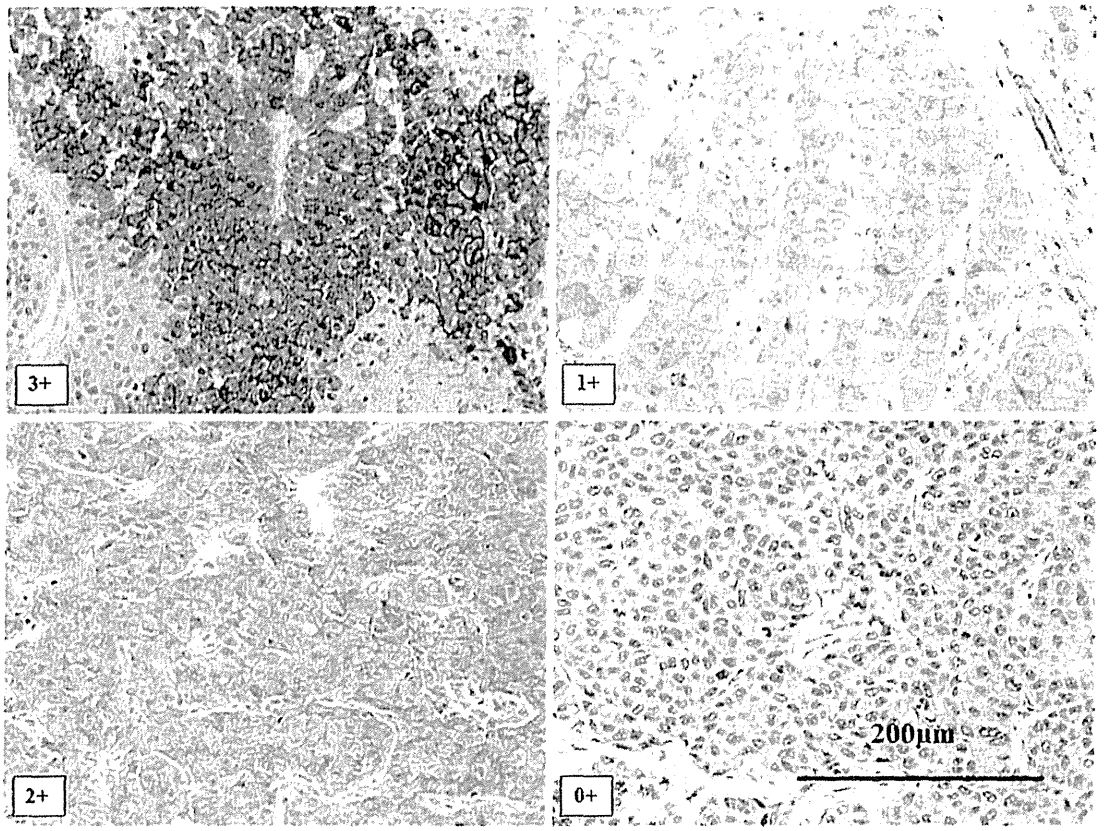

Fig. 1 Representative immunohistochemical staining scores for c-Met expression. 3+, complete membrane staining in almost all tumor cells. 2+, weak complete membrane staining in ≤20% of the cells. Although complete, membrane staining is non-uniform and weak. 1+, weak incomplete membrane staining in invasive tumor cells. 0, no staining observed in invasive tumor cells. *Scale bar* 200 μm

2+: 456 days, 95% CI 0–982.2; 3+: 784 days, 95% CI 371.1–1196.8). The median OS time was not significantly different between the c-Met^{low} (1754 days, 95% CI 1130.1–2377.9) and c-Met^{high} (740 days, 95% CI 325.9–1154.0) groups ($p = 0.12$; Fig. 2b).

Multivariate analysis indicated that recurrence was more likely in patients classified as c-Met^{high}, those who showed multinodular tumor morphology, or those who experienced portal vein invasion, than in patients classified as c-Met^{low} (HR 3.10; $p = 0.002$), or those who experienced no portal vein invasion (HR 3.21; $p = 0.006$). c-Met expression did not significantly affect OS (HR 0.96; $p = 0.91$) (Table 2).

c-Met FISH results

FISH analysis revealed *c-Met* gene amplification (4.09 copies per cell) in only 1 tumor (Fig. 3). Chromosome 7 aneusomy was detected in 25 of 44 tumors (57%) analyzed by FISH. Polysomy was detected in a total of 18 tumors (41%), 1 of which also displayed *c-Met* amplification.

Chromosome 7 monosomy without *c-Met* gene amplification was seen in 7 (16%) tumors. Among c-Met IHC-positive patients, there was only 1 patient (2% of the total) in whom *c-Met* gene amplification was detected (Table 3). IHC analysis revealed that 9 (50%) of 18 tumors from patients with chromosome 7 polysomy, with or without *c-Met* gene amplification, were c-Met^{high}. On the other hand, IHC analysis revealed that only 6 (23%) of 26 tumors from patients with chromosome 7 monosomy or disomy were c-Met^{high}. However, this difference was not statistically significant ($p = 0.17$). Moreover, chromosome 7 aneusomy was not significantly related to either RFS ($p = 0.77$) or OS ($p = 0.99$).

Discussion

In the present study, IHC analysis showed that c-Met^{high} expression in HCC was significantly correlated with pathological vascular invasion and shorter RFS, indicating that this characteristic could serve as a predictive factor for recurrence of HCC after resection.

The HGF/c-Met pathway promotes cell proliferation and inhibits apoptosis in tumor cells [11, 12] and, additionally,

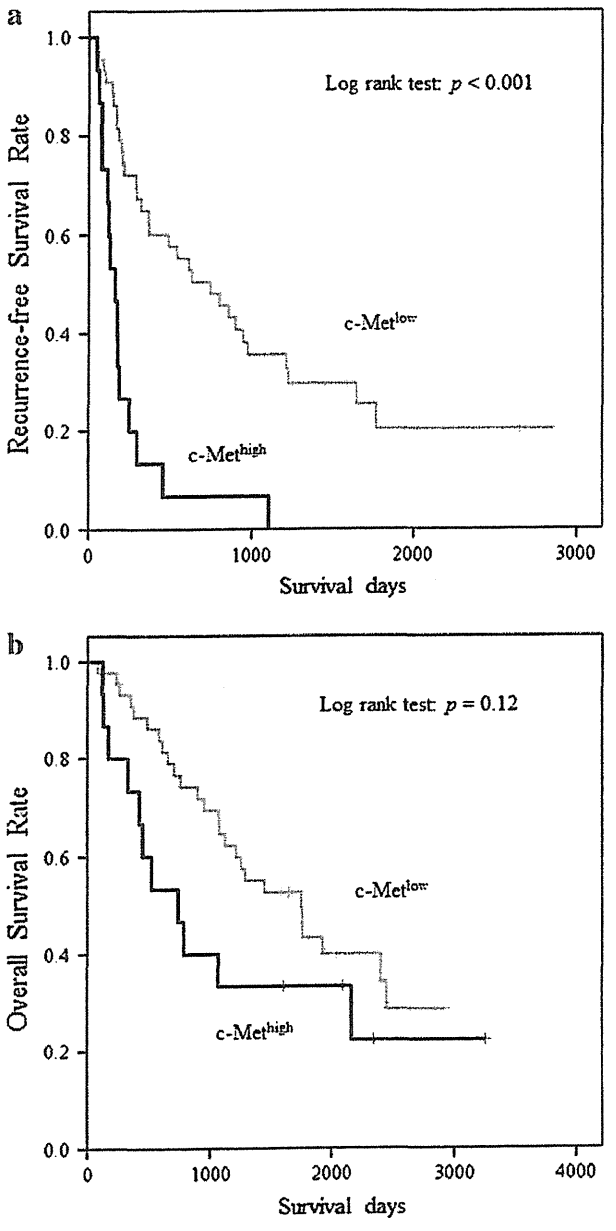

Fig. 2 **a** Kaplan–Meier estimates of recurrence-free survival (RFS) rates of patients with hepatocellular carcinoma (HCC). Median RFS time was 166 days for patients with c-Met^{high} tumor (*black line*; $n = 15$) and 748 days for patients with c-Met^{low} tumor (*gray line*; $n = 44$). The survival curves were significantly different ($p < 0.001$). **b** Kaplan–Meier estimates of overall survival (OS) rates of patients with HCC. Median OS time was 740 days for patients with c-Met^{high} tumor (*black line*; $n = 15$) and 1754 days for patients with c-Met^{low} tumor (*gray line*; $n = 44$). The curves were not significantly different ($p = 0.12$)

it has been reported to stimulate cell motility (e.g., cell dissociation, migration, and infiltration), promote changes in cell distribution, and affect morphogenesis [13]. In our study, the tendency of c-Met to promote HCC cells was poorly differentiated, and a statistical association between c-Met expression level and pathological differentiation was not shown. In HCC patients, the extracellular stromas, hypoxia and inflammatory cytokines or pro-angiogenic factors are activated by c-Met, which aggravates the intrinsic malignant properties of transformed cells by proliferative, anti-apoptotic, and migratory signals. Moreover, increases in c-Met expression have been associated with both decreased survival and increased proliferative activity of HCC cells, suggesting that c-Met could be used as a prognostic factor [14]. Previously, Ke et al. [15] reported that poor prognosis of patients with HCC resulted from an interaction between tetraspanin CD151 and c-Met, and that c-Met protein expression alone did not predict low survival rates. However, we observed high levels of c-Met expression in 25% of cases, which is similar to the rate reported by Ke et al. However, in contrast to the results of their study, we found that c-Met overexpression alone was capable of serving as a predictive factor for recurrence of HCC after surgery.

Patients with intrahepatic recurrence after surgery received additional local treatments, including surgical resection, local necrosis therapy via radiofrequency ablation, ethanol injection therapy, and hepatic arterial embolization therapy. Therefore, even if recurrence was promoted by c-Met, the use of multimodality therapy for lesion control made it difficult to determine whether c-Met expression could accurately predict prognosis. The high frequency of intrahepatic recurrence in this study may lend support to the view that c-Met expression cannot be used as a prognostic factor.

c-Met gene amplification has been reported in stomach [16], colorectal [17], and lung cancers [18], and it is thought to be correlated with metastasis and outcome in gastric and colorectal cancers. Amplification of the *c-MET* gene has been reported in lung cancer cases that were resistant to EGFR-TKI gefitinib [19]. Additionally, mRNA amplification has been reported in HCC patients [20]. However, we found that *c-Met* gene amplification was rare (1 in 44 patients), suggesting that it may not be involved in high c-Met expression in HCC.

Alterations in the *c-Met* gene were first reported in association with hereditary papillary renal carcinoma [21, 22], and were later found in children with HCC [23] and squamous cell carcinomas of the head and neck [24]. To date, there have been few reports of gene alterations in cases of adult HCC, and their frequency is not expected to be high. However, research on the *HER2* breast cancer gene showed correlations between non-*HER2*-amplification-related aberrations and a variety of breast cancer characteristics, such as nuclear histological grade and tumor stages. Furthermore, these aberrations have been associated with increased HER2 expression, as assessed by IHC analysis [25]. Thus, we examined correlations between

Table 2 Univariate and multivariate Cox regression analyses for RFS and OS

Parameter	RFS Hazard ratio (95% CI)	*p*	OS Hazard ratio (95% CI)	*p*
Univariate Cox regression analyses				
Age				
Over 70 versus under 70 years	1.50 (0.59–3.82)	0.39	1.60 (0.56–4.55)	0.38
Sex				
Male versus female	1.30 (0.64–2.63)	0.47	1.68 (0.65–4.35)	0.28
HCV versus non-HCV	1.51 (0.81–2.80)	0.19	1.61 (0.78–3.23)	0.20
HBV versus non-HBV	1.35 (0.75–2.42)	0.32	1.17 (0.60–2.27)	0.65
Child–Pugh				
B versus A	1.59 (0.38–6.58)	0.53	1.57 (0.47–5.20)	0.46
Portal vein invasion				
Positive versus negative	5.56 (2.89–10.68)	<0.001	3.78 (1.90–7.53)	<0.001
AFP				
Over 400 IU/L versus below 400 IU/L	4.59 (2.27–9.29)	<0.001	2.30 (1.19–4.47)	<0.01
Tumor morphology				
Multinodular versus uninodular	1.59 (0.86–2.93)	0.14	1.11 (0.56–2.19)	0.77
Size				
Over 5 cm versus 5 cm or less	4.85 (1.17–20.10)	0.03	1.47 (0.45–4.86)	0.53
Histology				
Poorly versus moderately + well	3.85 (2.03–7.31)	<0.001	2.66 (1.38–5.14)	<0.01
c-Met^{high} versus c-Met^{low}	4.11 (2.12–7.98)	<0.001	1.75 (0.86–3.54)	0.12
Multivariate Cox-regression analyses				
Portal vein invasion				
Positive versus negative	3.21 (1.40–10.56)	0.006	4.09 (1.56–10.76)	0.004
AFP				
Over 400 IU/L versus below 400 IU/L	2.15 (0.86–5.32)	0.10	0.82 (0.31–2.12)	0.68
Size				
Over 5 cm versus 5 cm or less	2.43 (0.56–10.56)	0.24	0.67 (1.81–2.49)	0.55
Histology				
Poorly versus moderately + well	0.98 (0.40–2.41)	0.97	1.55 (0.57–4.19)	0.39
c-Met^{high} versus c-Met^{low}	3.10 (1.49–6.46)	0.002	0.96 (0.44–2.07)	0.91

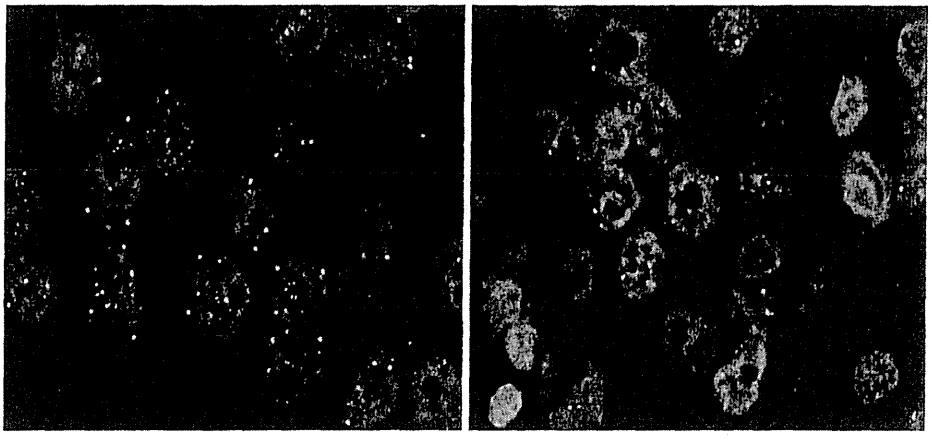

Fig. 3 Results of interphase FISH analysis of paraffin-embedded tumor tissue sections. Tumor cells with *c-Met* gene amplification (*left*), 20 or more *c-Met* gene signals (*red*), and 1–5 copies of *CEP7* (*green*) are present in each nucleus. Non-amplified tumor cells (*right*), 1–2 *c-Met* gene signals (*red*), and 1–2 *CEP7* signals (*green*) are present in each nucleus

Table 3 Association of c-Met protein expression with FISH analysis

FISH analysis	c-Met^{low}	c-Met^{high}	Total
Disomy	14	5	19/44 (43.2%)
Amplification with polysomy	0	1	1/44 (2.3%)
Polysomy without amplification	9	8	17/44 (38.6%)
Monosomy	6	1	7/44 (15.9%)

FISH fluorescence in-situ hybridization

overexpression of the c-Met protein and aneusomy (e.g., monosomy or polysomy) of chromosome 7, on which the *c-Met* gene is located. However, we found no correlation between chromosome 7 aneusomy and increased c-Met expression, although the incidence of high c-Met expression tended to be higher in tumors from patients with chromosome 7 polysomy than in those with chromosome 7 monosomy or disomy. Thus, an increased number of *c-Met* copies caused by gene amplification or chromosomal polysomies does not appear to be the mechanism behind increased levels of c-Met expression. It is possible that other mechanisms, such as autocrine or paracrine HGF, ligand-independent interactions with other receptors, and/or epigenetic expression regulation may play an important role in c-Met expression.

In conclusion, the present study demonstrates that c-Met can be used as a predictive factor for the recurrence of HCC after resection. It may also provide a useful target for future HCC treatments.

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

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Circulating endothelial cells and other angiogenesis factors in pancreatic carcinoma patients receiving gemcitabine chemotherapy

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Abstract

Background: Pancreatic carcinoma is a significant cause of cancer-related death in developed countries. As the level of circulating endothelial cells (CECs) is known to increase in response to various cancers, we investigated the predictive potential of CEC levels and the association of these levels with the expression of proangiogenic factors in pancreatic carcinoma patients.

Methods: Pancreatic carcinoma patients receiving gemcitabine chemotherapy were prospectively assigned to this study. CEC levels were measured using the CellTracks system, and the plasma levels of several angiogenesis factors were measured using multiplex immunoassay. Associations between clinical outcomes and the levels of these factors were evaluated.

Results: Baseline CEC levels were markedly higher in pancreatic carcinoma patients (n = 37) than in healthy volunteers (n = 53). Moreover, these high CEC levels were associated with decreased overall survival (median, 297 days versus 143 days, $P < 0.001$) and progression-free survival (median, 150 days versus 64 days, $P = 0.008$), as well as with high vascular endothelial growth factor, interleukin (IL)-8, and IL-10 expression in the pancreatic carcinoma patients.

Conclusions: Several chemokines and proangiogenic factors correlate with the release of CECs, and the number of CECs detected may be a useful prognostic marker in pancreatic carcinoma patients undergoing gemcitabine chemotherapy.

Trial registration: UMIN000002323

Keywords: Pancreatic carcinoma, Circulating endothelial cells, Angiogenesis factors

Background

Pancreatic carcinoma is one of the most lethal tumors and is the fourth leading cause of cancer-related death in developed nations [1]. As pancreatic carcinoma has a high propensity for both local invasion and distant metastasis, surgery is precluded as a treatment for most patients who present with advanced-stage disease. These patients have a median survival of only 6 months and an overall 5-year survival of less than 5%. The prognosis for advanced pancreatic carcinoma patients is therefore extremely poor, and the impact of standard therapy is only modest, despite many advances that have improved the outcome of this disease.

Pancreatic carcinoma is not a grossly vascular tumor; however, it overexpresses multiple mitogenic growth factors that are also angiogenic, such as epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), platelet-derived growth factor B chain (PDGF-BB), and vascular endothelial growth factor (VEGF). Angiogenesis often occurs in response to an imbalance in which proangiogenic factors predominate over antiangiogenic factors. For instance, VEGF expression has been shown to promote tumor growth in pancreatic carcinomas [2]. High VEGF expression is also

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associated with increased microvessel density [3] and is a predictor of poor outcomes and early tumor recurrence after curative resection [4]. Although agents that target the VEGF signaling pathway have been shown to inhibit tumor growth, metastasis, and angiogenesis [5], treating advanced pancreatic carcinoma patients with axitinib—a selective inhibitor of VEGF receptors 1, 2, and 3—in combination with gemcitabine was not found to improve overall survival in a phase 3 trial [6]. Despite this finding, proangiogenic factors remain an important therapeutic target for the treatment of pancreatic carcinoma.

Circulating endothelial cells (CECs) are mature cells that are not associated with vessel walls but are detached from the endothelium and circulate within peripheral blood. The number of CECs present in the blood has been found to increase in response to cardiovascular disease, vasculitis, infectious disease, and various cancers [7,8]. Indeed, the level of CECs has been recognized as a useful biomarker for vascular damage. It has also been reported that the number of CECs found in non-small cell lung cancer patients treated with carboplatin plus paclitaxel is a promising predictive marker of the clinical efficacy of these drugs [9]. We believe that CEC levels may also be a potential biomarker for pancreatic carcinoma; therefore, we investigated the levels of CECs found in patients with different severities of pancreatic carcinoma, as well as the effects of gemcitabine treatment on CEC levels. Furthermore, the associations between CEC levels and the expression levels of several factors involved in angiogenesis and neovascularization were also examined in this study.

Methods

Study approval

This prospective study was approved by the Institutional Review Board of the National Cancer Center, and written informed consent was obtained from all patients. This study is registered with the University Hospital Medical Information Network in Japan (UMIN; number UMIN000002323) and has been completed.

Patients and blood sample collection

A total of 37 chemotherapy-naïve patients with histologically or cytologically confirmed invasive ductal pancreatic carcinoma were prospectively enrolled in this study between April 2009 and March 2010 and received gemcitabine chemotherapy. Patients with coexisting infections and/or cardiovascular illness were excluded. The detailed history of all the patients was obtained and a physical examination was performed before beginning gemcitabine treatment. Pretreatment baseline laboratory parameters were also assessed for all patients. The baseline tumor status of each patient was evaluated using computed tomography (CT) scans of the chest, abdomen, and pelvis, while peripheral blood sampling was performed both prior to treatment initiation (baseline) and at day 28 ± 7 after starting chemotherapy. A dose of 1000 mg/m^2 gemcitabine was administered intravenously for 30 min on days 1, 8, and 15 of a 28-day cycle until disease progression, unacceptable toxicity, or patient refusal occurred. The data collected included those pertaining to standard demographics and disease characteristics, the date of initial treatment, the best response to treatment, date of progression, and the date of death or last follow-up. The tumors were evaluated every 6–8 weeks after starting each course of gemcitabine, and best responses were documented according to the Response Evaluation Criteria in Solid Tumors (RECIST).

CEC enumeration

Blood samples from advanced pancreatic carcinoma patients were drawn into 10 mL CellSave Preservative Tubes (Immunicon Corp. Huntingdon Valley, PA) for CEC enumeration. Samples were obtained both before starting chemotherapy (baseline) and at 28 ± 7 days after starting chemotherapy. Samples were kept at room temperature and processed within 42 h of collection. All of the evaluations were performed without knowledge of the clinical status of the patients. The CellTracks system (Veridex, LLC), which consists of the CellTracks AutoPrep system and the CellSpotter Analyzer system, was used for endothelial cell enumeration. In this system, CECs are defined as CD146$^+$/DAPI$^+$/CD105-PE$^+$/CD45APC$^-$ cells. Briefly, CD146$^+$ cells were captured immunomagnetically by using ferrofluids coated with CD146 antibodies. The enriched cells were then labeled with the nuclear dye 4 V, 6-diamidino-2-phenylindole (DAPI), CD105 antibodies were conjugated to phycoerythrin (CD105-PE), and the pan-leukocyte antibody CD45 was conjugated to allophycocyanin (CD45-APC). Cells with the DAPI$^+$/CD105$^+$/CD45$^-$ phenotype were enumerated. We evaluated morphological cell viability and excluded dead cells from the cell count. The number of CECs in each sample was determined twice, and the mean value was calculated.

Antibody suspension bead array system

Peripheral blood was drawn into prechilled tubes containing ethylenediaminetetraacetic acid; was immediately subjected to centrifugation at 1000 g and 4°C for 15 min, plasma was transferred to microtubes and subjected to further centrifugation at 10,000 g and 4°C for 10 min to remove contaminating platelets. Plasma samples were collected from patients before gemcitabine treatment was initiated and were stored at −80°C until they were used for testing. The plasma concentrations of 7 biological markers (interleukin [IL]-6, IL-8, IL-10,

PDGF-BB, VEGF, HGF, and SDF-1 alpha) were assayed in a subgroup of patients and control individuals by using the Bio-Plex suspension array system (Bio-Rad, Hercules, CA), which allows the simultaneous identification of cytokines in a 96-well filter plate. In brief, the appropriate cytokine standards and diluted plasma samples were added to a 96-well filter plate and incubated at room temperature for 30 min with antibodies chemically attached to fluorescent-labeled micro beads. After 3 filter washes, premixed detection antibodies were added to each well and incubated for 30 min. After 3 more washes, premixed streptavidin-phycoerythrin was added to each well and incubated for 10 min, followed by 3 more washes. The beads were then resuspended in 125 μL of assay buffer and the reaction mixture was quantified using the Bio-Plex protein array reader. Data were automatically processed and analyzed with Bio-Plex Manager Software 4.1 by using the standard curve obtained using a recombinant cytokine standard.

Statistical analyses

The Mann–Whitney test was used to compare the distributions of clinical factors and marker concentrations between patients with progressive disease (PD) and those without PD, stages III and IV disease, or recurrence. The survival time (progression-free survival [PFS] and overall survival [OS]) and clinical factors (age, gender, and Eastern Cooperative Oncology Group [ECOG] performance status

Table 1 Patient characteristics and CEC detection

		Mean CEC level 166 cells/4 mL	Range (2–1195 cells/4 mL)	Total	P^a
		≥ 166 cells/4 mL	<166 cells/4 mL		
		CEC^{high}	CEC^{low}		
		12	25	37	
Age	Over 70	8	10	18 (49%)	0.17
	Below 70	4	15	19 (51%)	
Sex	Male	7	17	24 (65%)	0.72
	Female	5	8	13 (35%)	
Stage	III	3	11	14 (38%)	0.59
	IV	8	12	20 (54%)	
	Recurrence	1	2	3 (8%)	
ECOG PS	0	5	18	23 (62%)	0.09
	1	6	4	10 (27%)	
	2	1	3	4 (11%)	
Pancreatic tumor location	Head	5	12	17 (46%)	>0.9
	Body	5	9	14 (38%)	
	Tail	2	4	6 (16%)	
CA19-9 (U/mL)	≥10,000	3	5	8 (22%)	>0.9
	< 10,000	9	20	29 (78%)	
CRP (mg/dL)	≥1.0	7	3	10 (27%)	<0.01
	<1.0	5	22	27 (73%)	
Histology	Poorly differentiated	5	9	14 (38%)	0.62
	Moderately differentiated	4	10	14 (38%)	
	Adenosquamous	1	0	1 (2%)	
	N.E (cytology only)	2	6	8 (22%)	
Tumor response	Partial response	2	2	4 (11%)	<0.05
	Stable disease	4	18	22 (59%)	
	Progressive disease	6	5	11 (30%)	
Second line therapy	S-1	6	12	18 (49%)	1
	Oxaliplatin + S-1	0	2	2 (5%)	
	No	6	11	17 (46%)	

[a]*P* values were calculated for each variable using Fisher's exact test.
Abbreviations: CEC = circulating endothelial cell; ECOG = Eastern Cooperative Oncology Group; CA19-9 = carbohydrate antigen 19–9; CRP = C-reactive protein.

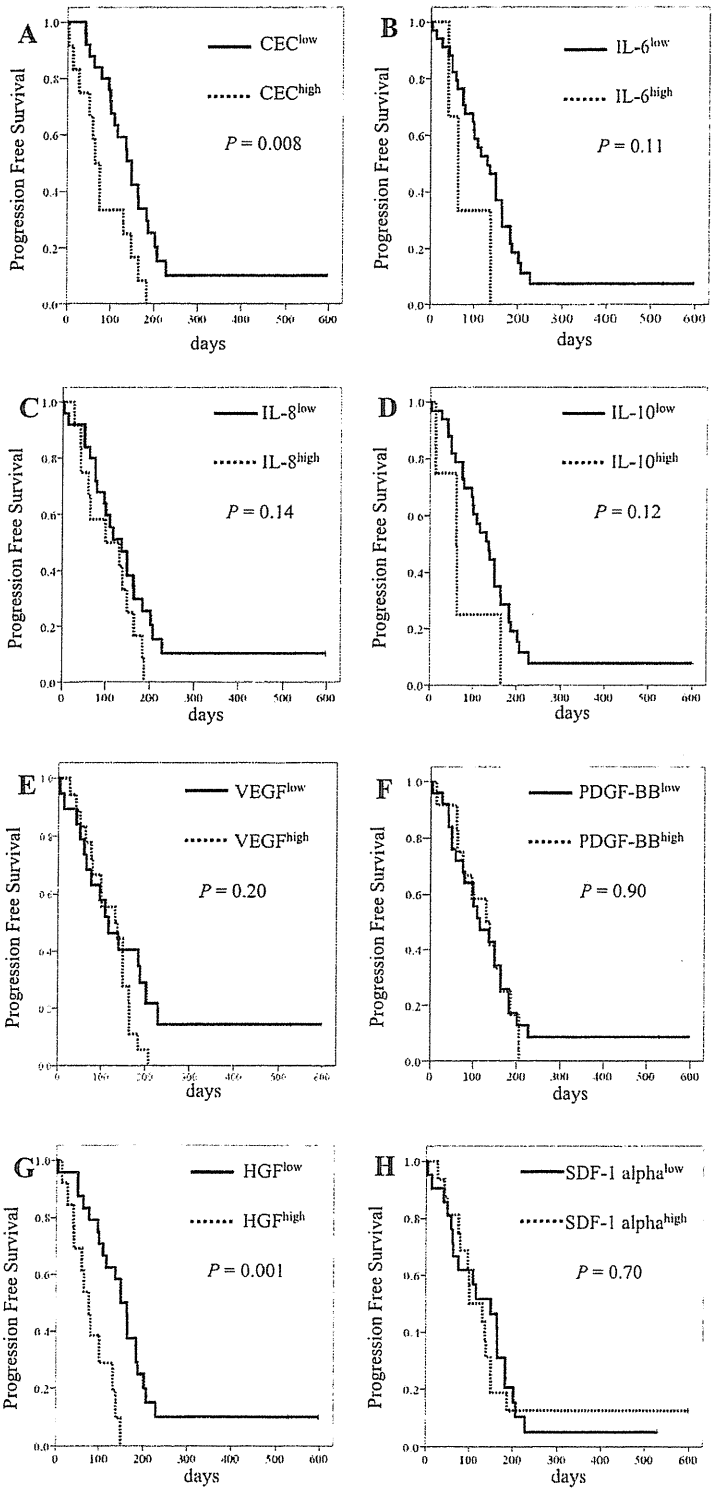

Figure 1 Kaplan-Meier curves for (A) progression-free survival with CEC counts, (B) progression-free survival with IL-6 levels, (C) progression-free survival with IL-8 levels, (D) progression-free survival with IL-10 levels, (E) progression-free survival with VEGF levels, (F) progression-free survival with PDGF-BB levels, (G) progression-free survival with HGF levels, and (H) progression-free survival with SDF-1 alpha levels. The cut-off points for the angiogenic factors were determined to be equal to or greater than these mean levels.

[PS], and clinical stage of the patients) were examined using the Cox proportional hazards model. The survival curves for PFS and OS were estimated using the Kaplan-Meier method. Kaplan-Meier curves were used only to determine the trends of the associations between the molecules and PFS/OS, as any determination of the optimal cutoff point for the molecules relative to PFS/OS was beyond the scope of the present study. All statistical analyses were performed using IBM SPSS Statistics 18 (IBM Corporation, Somers, NY, USA).

Results

Patient characteristics

A total of 37 patients with pancreatic carcinoma were prospectively enrolled in this study. Fourteen of these patients (38%) presented with locally advanced pancreatic carcinoma, 20 patients (54%) presented with metastases, and 3 patients (8%) were enrolled following recurrence after surgery. Twenty-three patients (62%) had ECOG PS0, 10 patients (27%) had ECOG PS1, and 4 patients (11%) had ECOG PS2. Histologically, 14 patients (38%) had poorly differentiated adenocarcinoma, 14 patients (38%) had moderately differentiated adenocarcinoma, 1 patient (2%) had an adenosquamous tumor, and 8 patients (22%) had cytological adenocarcinoma. No patient experienced a complete response to treatment. Four patients (11%) exhibited a partial response (PR) rate to treatment (11%), stable disease (SD) was observed in 22 patients (59%), and PD was observed in 11 patients (30%). Second-line therapy was administered to 20 patients (54%), whereby 18 patients (49%) received S-1 monotherapy and 2 patients (5%) received oxaliplatin and S-1 combination therapy (Table 1).

Baseline levels of CECs and angiogenic factors

The mean CEC level found in the pancreatic carcinoma patients was 166 cells/4 mL (range: 2–1195 cells/4 mL) while the median CEC level was 66 cells/4 mL. These CEC levels were higher than those of randomly-selected healthy volunteers ($P < 0.01$), as previously reported (n = 53, mean ± SD = 46.2 ± 86.3 cells/4 mL) [9]. In this study, the cut-off point of CEC^{high} was determined to be equal to or greater than 166 cells/4 mL while that of CEC^{low} was lower than 166 cells/4 mL. CEC^{high} was significantly associated with high levels of C-reactive protein (CRP) (over 1.0 mg/dL; $P < 0.01$). The median PFS was 64 days (95% confidence interval [CI], 45–83) in the CEC^{high} group, while that in the CEC^{low} group was 150 days (95% CI, 130–170; log-rank test; $P = 0.008$; Figure 1A). The median OS was 143 days (95% CI, 53–233) in the CEC^{high} group and 297 days (95% CI, 240–354) in the CEC^{low} group (log-rank test; $P < 0.001$; Figure 2A). Univariate analysis of CEC levels and clinical factors for OS was performed using the Cox proportional hazard model. The hazard ratio (HR) for CEC levels (CEC^{high} versus CEC^{low}) was 5.18 (95% CI, 2.23–12.03; $P < 0.001$).

The mean levels of IL-6, IL-8, IL-10, PDGF-BB, VEGF, HGF, and SDF-1 alpha were found to be 19.3 pg/mL, 11.3 pg/mL, 7.82 pg/mL, 1127.5 pg/mL, 44.1 pg/mL, 471.3 pg/mL, and 110.6 pg/mL, respectively. The cut-off points for the angiogenic factors were determined to be equal to or greater than these mean levels, and the median PFS in HGF^{low} was longer than the HGF^{high} group ($P = 0.001$; Figure 1G). However, other factors were not found to have statistical significance with regard to PFS. The median OS was longer in the case of IL-10 (112 days [95% CI, 50–173] in $IL\text{-}10^{high}$ vs. 264 days [95% CI, 204–324] $IL\text{-}10^{low}$, log-rank test: $P = 0.003$; Figure 2d) and HGF (150 days [95% CI, 65–234] in HGF^{high} vs. 291 days [95% CI, 223–359] in HGF^{low}, log-rank test: $P = 0.01$; Figure 2G).

Among the clinical factors that were examined in this study, a poor PS (PS 1 and 2), advanced stage (stage IV and recurrence), and high levels of IL-10, HGF, and CRP were significantly correlated with poor OS in univariate cox analysis, with HRs of 2.72 (95% CI, 1.29–5.70; $P = 0.008$), 2.21 (95% CI, 1.03–4.71; $P = 0.04$), 5.05 (95% CI, 1.55–16.39; $P = 0.007$), 2.52 (95% CI, 1.22–5.21; $P = 0.01$), and 2.49 (95% CI, 1.14–5.42; $P = 0.02$), respectively. In a multivariate Cox analysis model that included clinical stage, PS, CRP levels, CEC levels, IL-10 levels, and HGF levels, the number of CECs detected remained statistically stable at 0.05. The resulting HRs were 2.04 (95% CI, 0.78–5.35; $P = 0.15$), 2.58 (95% CI, 0.98–6.76; $P > 0.05$), 2.04 (95% CI, 0.62–6.76; $P = 0.24$), 5.14 (95% CI, 1.83–14.45, $P = 0.002$), 5.26 (95% CI, 1.26–22.22; $P = 0.02$) and 1.34 (95% CI, 0.46–3.91; $P = 0.59$), respectively (Table 2).

Changes in CEC number during treatment

The number of CECs was analyzed in 22 of the 37 patients at 28 ± 7 days after the start of gemcitabine therapy. The mean number of CECs detected in these patients after 28 ± 7 days was 133 cells/4 mL (range: 15–664 cells/4 mL), while the median number of CECs was 68 cells/4 mL. The absolute counts of CECs did not change significantly between day 1 and day 28 ± 7 of treatment (Mann–Whitney test, $P = 0.11$). Furthermore, a change in CEC counts from baseline to after 28 ± 7 days of treatment was not statistically associated with tumor response (Mann–Whitney test, $P > 0.05$, Figure 3).

Association between CEC number and blood angiogenic factors

The numbers of CECs were compared between non-PD (PR and SD, n = 26) and PD patients (n = 11) for

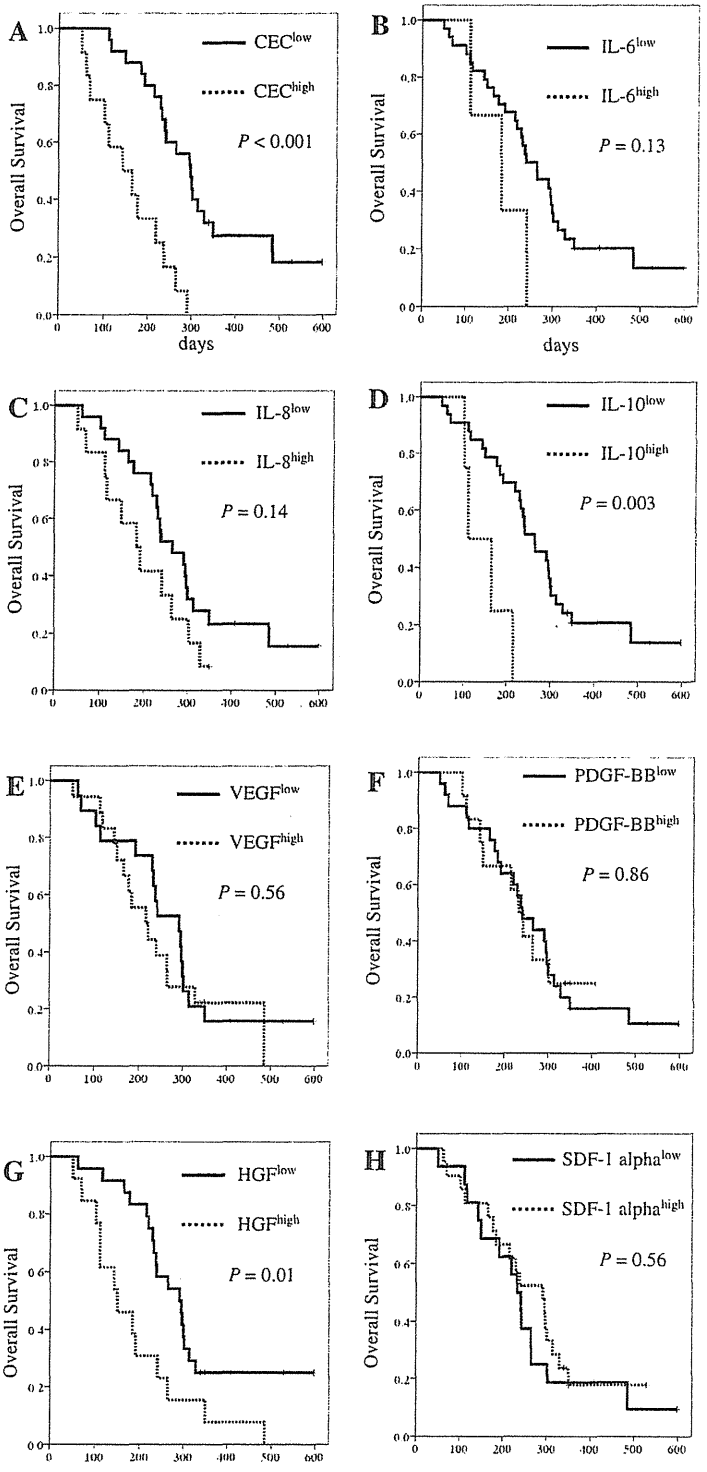

Figure 2 Kaplan-Meier curves for (A) overall survival with CEC counts, (B) overall survival with IL-6 levels, (C) overall survival with IL-8 levels, (D) overall survival with IL-10 levels, (E) overall survival with VEGF levels, (F) overall survival with PDGF-BB levels, (G) overall survival with HGF levels, and (H) overall survival with SDF-1 alpha levels. The cut-off points for the angiogenic factors were determined to be equal to or greater than these mean levels.