Table 1 Patient characteristics ($n = 40$)

Age	
Median (range)	62 (36–74)
Gender	
Male	21
Female	19
KPS	
100	17
90	19
80	4
Biliary drainage	
(+)	6
Prior pancreatectomy	
(+)	7
Location of primary tumor	
Head	17
Body	14
Tail	9
Sites of metastasis	
Liver	33
Lymph node	16
Lung	3
Peritoneum	4
Prior chemotherapy	
Gemcitabine[a]	36
FDR-GEM[b]	4
TTP of prior treatment (months)	
Median (range)	2.8 (0.7–13.5)
CEA (ng/ml)	
Median (range)	14.9 (1.1–1,187)
CA19-9 (U/ml)	
Median (range)	4,673 (0.1–2,960,000)

KPS Karnofsky performance status, *TTP* time to progression, *CEA* carcinoembryonic antigen, *CA19-9* carbohydrate antigen 19-9

[a] Gemcitabine as a standard 30-min infusion

[b] FDR-GEM: gemcitabine as a fixed dose rate infusion

treatment response could not be obtained. The median time to progression in the prior treatment was 2.8 months (range 0.7–13.5 months).

Treatments

A total of 94 courses were administered to the 40 patients with a median of two courses per patient (range 1–12). The initial administered dose of S-1 was 80 mg/day in 1 patient, 100 mg/day in 18 patients, and 120 mg/day in 21 patients. Treatment interruption was necessary in 18 patients, due to fatigue (grade 3: one patients, grade 2: one patient, grade 1: two patients), nausea (grade 2: three patients, grade 1: one patient), diarrhea (grade 3: two patients, grade 1: two patients), drainage tube related problem (two patients), grade 3 appetite loss (1), grade 1 leukocytopenia (1), grade 2 hand-foot skin reaction (1), and grade 1 pneumonitis (1). Dose reduction was required in three patients because of grade 3 diarrhea (1), grade 2 fatigue (1), and grade 1 nausea (1). The relative dose intensity was 94.7%. The reasons for discontinuation of treatment were radiologically confirmed progressive disease (PD) in 31 patients, clinical PD without radiological PD in 6 patients, at the patients request due to unacceptable toxicities in 2 patients (grade 2 fatigue and grade 3 anorexia), and loss to follow up in one patient.

Toxicity

All 40 eligible patients were assessable for adverse events. The treatment-related adverse reactions are listed in Table 2. One patient developed grade 3 pneumonitis without neutropenia and required hospitalization, but she recovered from the pneumonitis with antibiotic treatment. As to other grade 3 non-hematological toxicities, aspartate aminotransferase elevation (two patients), alanine aminotransferase elevation (2), fatigue (2), anorexia (2), diarrhea (2) were noted. Regarding hematological toxicities, grade 3 anemia was noted in one patient. No other severe or unexpected adverse reactions were noted. The most common adverse reactions were fatigue (78%) and anorexia (73%), although most of those adverse reactions were tolerable and reversible. Although five patients died within 4 weeks after discontinuation of treatment due to rapid disease progression, no treatment-related deaths were observed.

Efficacy

Out of the total of 40 eligible patients, 38 patients were assessable for response. Two patients discontinued chemotherapy at their request due to unacceptable toxicities (grade 2 fatigue and grade 3 anorexia) and moved to another hospital before tumor assessment. Although no complete response was seen, partial response was obtained in six patients (15, 95%, confidence interval 3.9–26%). Stable disease was noted in 17 patients (43%), and progressive disease in 15 patients (38%). Tumor responses to second-line S-1 therapy are classified according to tumor responses to first-line gemcitabine in Table 3. The serum CA 19-9 level was reduced to less than half in 8 (23%) of 35 patients with a pretreatment serum CA19-9 level of the upper limit of normal or greater. At the time of enrollment, nineteen of forty (47.5%) patients were eligible for the evaluation of clinical benefit response. Out of nineteen evaluable patients, a clinical benefit response was observed in four patients (21%). The median progression free survival time was 2.0 months, and the median survival time was 4.5 months (range 1.2–14.3+) with a 1-year survival rate of 14.1% (Fig. 1).

Table 2 Treatment-related adverse events ($n = 40$): worst grade reported during treatment period

	Grade				Grade 1–4	Grade 3–4
	1	2	3	4	*n* (%)	*n* (%)
Hematological toxicity						
Leukocytes	8	2	0	0	10 (25)	0 (0)
Neutrophils	3	2	0	0	5 (13)	0 (0)
Hemoglobin	5	13	1	0	19 (48)	1 (3)
Platelets	9	0	0	0	9 (23)	0 (0)
Non-hematological toxicity						
Aspartate aminotransferase elevation	13	1	2	0	16 (40)	2 (5)
Alanine aminotransferase elevation	8	1	2	0	11 (28)	2 (5)
Total bilirubin elevation	4	3	0	0	7 (18)	0 (0)
Fatigue	21	8	2	0	31 (78)	2 (5)
Nausea	18	6	0	0	24 (60)	0 (0)
Vomiting	5	1	0	0	6 (15)	0 (0)
Anorexia	22	5	2	0	29 (73)	2 (5)
Stomatitis	11	3	0	0	14 (35)	0 (0)
Diarrhea	8	4	2	0	14 (35)	2 (5)
Rash	3	0	0	0	3 (8)	0 (0)
Pigmentation	6	1	–	–	7 (18)	–
Hand-foot skin reaction	1	1	0	–	2 (5)	0 (0)
Pneumonitis without neutropenia	0	0	1	0	0 (0)	1 (3)

Table 3 Objective tumor response (RECIST criteria) ($n = 40$)

Response (2nd line)	*n* (%)	Response (1st line)			
		PR	SD	PD	NE
CR	0 (0%)	0	0	0	0
PR	6 (15%)	1	4	0	1[a]
SD	17 (43%)	2	9	5	1[a]
PD	15 (38%)	1	6	7	1[a]
NE	2 (5%)	0	2	0	0
Total	40 (100%)	4	21	12	3

Treatment response to second-line S-1 therapy is tabulated according to treatment response to first-line gemcitabine

[a] Three patients received first-line chemotherapy at another hospital and accurate data about treatment ressponse was unobtainable

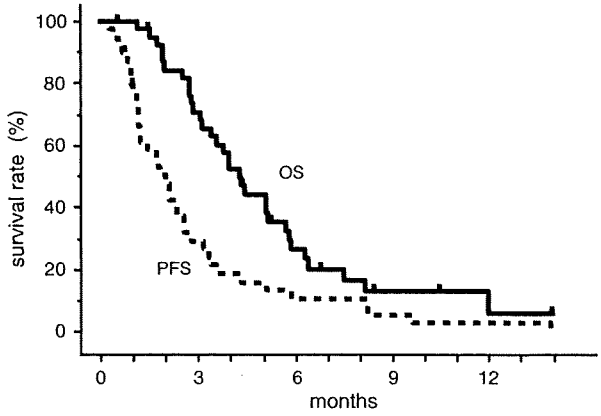

Fig. 1 Survival ($n = 40$). Progression free survival (*dashed line*), and overall survival time (*solid line*) curves of patients with gemcitabine refractory pancreatic cancer receiving systemic chemotherapy with S-1

Discussion

Over the last several years, many studies have been designed to establish effective treatment for gemcitabine-refractory pancreatic cancer patients. So far, the results of two randomized phase III studies had been reported. Jacobs et al. reported on a phase III study comparing Rubitecan, a new topoisomerase I inhibitor, versus "physicians' choice" in 409 pretreated patients. The study was unable to indicate any statistically significant survival benefit in the Rubitecan arm (3.7 months vs. 3.1 months, $P = 0.626$), although progression-free survival was significantly improved in Rubitecan arm (1.9 months vs. 1.6 months, $P = 0.001$) [12]. On the other hand, Oettle et al. [22] reported on phase III study comparing a combination of oxaliplatin, 5-FU and folinic acid with best supportive care (BSC). The BSC arm closed to accrual after 46 out of 165 planned patients were enrolled because physicians deemed it unethical. The median survival of second-line therapy was 21 weeks compared to 10 weeks for the BSC group ($P = 0.0077$). However, a worldwide consensus regarding this result has not been established because of the small number of patients in

this study. Other studies have investigated the feasibility and activity of second-line treatments in phase II studies [1, 4, 7, 17, 19, 26, 27, 33, 36, 38]. Compared with monotherapy, combination regimens exhibited superior activity in these studies. Fluoropyrimidine-, Irinotecan- or oxaliplatin-based combinations indicated relatively preferable activity with objective responses rate of about 20% and a median survival of 5–6 months in this setting [7, 17, 27, 36, 38]. The safety profiles of such combination regimens require further careful evaluation, and well-designed, larger randomized controlled studies are needed.

In the current study, S-1 produced a response rate of 15%, which was superior to the rates obtained for other reported single agents, including paclitaxel (5.5%) [21], raltitrexed (0%) [38], rubitecan (7%) [4]. However, this response rate failed to reach the pre-established boundary of 17.5% required for the agent to be considered effective. Furthermore, the progression-free survival (median 2 months) and the overall survival (median 4.5 months) were still extremely poor in this study. Although S-1 seems to have some degree of anti-tumor activity in patients with gemcitabine refractory metastatic pancreatic cancer, monotherapy may be insufficient to prolong survival. This limitation may be due to the strong chemo-resistance and heterogeneity of the tumors caused by the nature of the disease and acquired from previous chemotherapy regimens.

The toxicity of S-1 was acceptable and no life-threatening toxicities were observed. Although a population with an extremely poor prognosis was targeted in this study and the general condition of the participating patients was expected to be unstable, the toxicities were similar to the results of previous clinical studies for S-1 in chemo-naïve patients with pancreatic cancers [24, 37]. The safety profile of this study suggests that S-1 can be safely administered to pancreatic cancer patients even in a second-line setting, at least in selected populations.

We conclude that S-1 as monotherapy had marginal anti-tumor activity with tolerable toxicity in patients with gemcitabine refractory metastatic pancreatic cancer. In view of the favorable toxicity profile, its combination with other agents might have potential to improve therapeutic results.

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Do Recurrent and Metastatic Pancreatic Cancer Patients Have the Same Outcomes with Gemcitabine Treatment?

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Key Words
Gemcitabine · Metastatic · Outcomes · Pancreatic cancer · Recurrent · Survival

Abstract
Background: Whether recurrence after surgery and primary metastatic pancreatic cancer should be included in the same category when conducting gemcitabine-based clinical trials remains controversial. ***Objective:*** To clarify the outcomes of recurrent and metastatic pancreatic cancers. ***Methods:*** 326 patients who received gemcitabine monotherapy as a first-line treatment for advanced pancreatic cancer between 2001 and 2007 were reviewed. Multivariate analysis was performed to determine the prognostic relevance of recurrence or metastasis in relation to other factors possibly influencing treatment outcomes with respect to overall survival. Differences in response to chemotherapy, drug delivery and adverse events were also analyzed. ***Results:*** There were 65 recurrent and 261 metastatic cancer patients. Recurrent cancer patients had a significantly longer time to treatment failure and survival (respective medians 138 and 77 days, p = 0.017) than the metastatic patients (respective medians 270 and 185 days, p = 0.0003). Multivariate analysis revealed poor Karnofsky performance status (<80), presence of liver or peritoneal metastasis, elevated lactate dehydrogenase (>220 U/l), elevated alkaline phosphatase (>330 U/l) and elevated C-reactive protein (>1.0 mg/dl) to be significantly correlated with short survival, while neither recurrent nor metastatic status were related to survival (hazard ratio 0.76, 95% CI 0.53–1.09, p = 0.14). The response rates and dose intensities of gemcitabine were similar in these groups, although leukopenia was more frequently observed in the recurrence group (p = 0.008). ***Conclusion:*** When conducting clinical trials, it appears to be acceptable to treat recurrent pancreatic cancer after surgery and pancreatic cancer with primary metastasis under the same category.

Introduction

Pancreatic cancer is the fifth most common cause of cancer death in Western countries and Japan [1]. Advanced pancreatic cancer patients have a dismal prognosis, and the median survival time of symptomatic patients is no longer than 6 months. More than 80% of patients present at an advanced stage at diagnosis, and of those offered surgical resection, nearly 90% develop local and/or metastatic recurrence [2]. Gemcitabine is now considered to be the standard agent for advanced pancreatic cancer since it produced a modest increase in sur-

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vival as compared with fluorouracil (median 5.6 vs. 4.4 months, respectively) and has significant clinical benefit [3]. However, the efficacy of gemcitabine is limited because no more than 15% of patients can be expected to respond, and median survival is only approximately 6 months with gemcitabine treatment. Recently, various agents have been investigated in combination with gemcitabine but most have failed to improve survival as compared with gemcitabine monotherapy [4–16]. Erlotinib added to gemcitabine showed superiority in survival as compared with gemcitabine alone but the majority of oncologists still regard gemcitabine monotherapy as the standard approach because the survival benefit of adding erlotinib has little clinical significance [17].

Most clinical trials of chemotherapy for advanced pancreatic cancer involve locally advanced disease and recurrent disease after surgery in addition to primary metastatic disease. It has been recently recognized that the outcomes of locally advanced disease is different from that of metastatic disease, and in many randomized phase III studies, patients are stratified by 'locally advanced versus metastatic disease' [4–10, 17, 18]. Recurrent disease is not generally distinguished from primary metastatic disease, and these patients are generally categorized together (stage IV) when conducting clinical trials. However, it is still unclear whether the prognosis of recurrent disease is similar to that of primary metastasis. Therefore, we retrospectively investigated the treatment outcomes of patients with recurrent pancreatic cancer after surgery and of those with tumors with primary metastasis, who received gemcitabine monotherapy. The outcomes, response, adverse events and gemcitabine delivery were compared between the 2 groups.

Patients and Methods

Patient Population

We extracted cases with corresponding eligibility criteria from the database of the National Cancer Center Hospital in Tokyo. Patients with recurrent disease after pancreatic resection and those with primary metastatic disease were eligible for this study. All of the enrolled patients had been histologically or cytologically diagnosed as having pancreatic adenocarcinoma and had received gemcitabine monotherapy as first-line chemotherapy between January 1, 2001, and December 31, 2007. Locally advanced unresectable pancreatic cancer (stage III) patients were excluded from the study.

Gemcitabine Treatment

For all patients, an initial 1,000 mg/m^2 dose of gemcitabine was administered intravenously for 30 min on days 1, 8 and 15 of a 28-day cycle until disease progression, an unacceptable adverse event or patient refusal occurred. If patients experienced white blood cell count of <2,000/μl or platelet count of <70,000/μl, gemcitabine administration was omitted on that day and postponed to the next scheduled treatment day. Gemcitabine dose (mg/m^2), treatment durations and the number of injections were reviewed. Dose intensity was calculated by the following formula [19]:

Dose intensity (mg/m^2/week) = Total dose of gemcitabine (mg/m^2)/[(last date of injection – first date of injection) + 7]/7

Tumor responses were evaluated according to RECIST. The disease control rate includes complete response, partial response and stable disease. Adverse events were retrospectively ranked by the National Cancer Institute Common Toxicity Criteria version 3.0.

Statistical Analysis

Differences in baseline characteristics, response rate and adverse events between recurrent and metastatic status were detected by Fisher's exact test, and for the numeric data we used the Mann-Whitney U test. The differences in gemcitabine delivery among groups were assessed by t test. Time to treatment failure was defined as the time between the date of starting gemcitabine treatment and the date of recognition of progressive disease, discontinuation due to an adverse event or patient refusal. Overall survival was the duration from starting gemcitabine treatment to death or the last follow-up. Survival curves were estimated by the Kaplan-Meier method.

Univariate analysis was carried out using the Cox proportional hazard model to identify the factors correlating with survival. A significant relation with survival was detected by the multivariate stepwise modeling procedure. In the stepwise analysis, a 0.15 significance level was used for inclusion or exclusion of explanatory variables to determine factors independently affecting survival. For the final model for the Cox regression analysis, we chose a significance level of $p < 0.05$. All statistical analyses were performed using StatView (SAS Inc., Ver. 5.0, Tokyo, Japan).

Results

Patient Characteristics

In total, 452 patients who underwent gemcitabine-based treatment for advanced pancreatic cancer from our database were reviewed and 326 patients were found to be eligible for this analysis (fig. 1). There were 65 recurrent and 261 primary metastatic cancer patients. Baseline characteristics are shown in tables 1 and 2. Compared to metastatic cancer patients, the frequency of liver metastasis was lower in those with recurrences. In addition, patients with recurrences had better Karnofsky performance status (KPS), higher serum albumin, lower white blood cell counts, lower C-reactive protein (CRP) and lower carbohydrate antigen (CA) 19-9 levels at baseline.

Table 1. Baseline characteristics of recurrent and metastatic cancer patients

	Recurrence (n = 65)		Metastasis (n = 261)		p value
	n	%	n	%	
Age					
≤65	32	49.2	144	55.2	0.41
>65	33	50.8	117	44.8	
Gender					
Male	39	60	141	54	0.41
Female	26	40	120	46	
KPS					
70, 80	5	7.7	60	23	<0.005
90, 100	60	92.3	201	77	
Biliary drainage	7	10.8	56	21.5	0.054
Presence of liver metastasis	28	43.1	198	73.9	<0.001
Presence of peritoneal metastasis	13	20	54	20.3	0.99
Presence of lung metastasis	10	15.4	41	15.7	0.99

KPS = Karnofsky performance status.

Treatment Delivery and Response

There were 304 radiographically evaluable patients among the 326. Overall, 38 patients responded (12.5%; 1 complete response, 37 partial responses). In the recurrence group, the response rate was 12.7% (8 of 63 patients), similar to that in the metastasis group (12.0%; 29 of 241 patients, p = 0.83). However, the disease control ratio (complete response + partial response + stable disease) in the recurrence group was significantly higher than that in the metastasis group (82.5 vs. 68.9%, respectively, p = 0.04).

In the recurrence group, the number of gemcitabine administrations and total dose of gemcitabine were higher than in the metastasis group (administrations: median 15.9 vs. 11.8 times, p = 0.012; dosage: 15,318 vs. 11,297 mg, p = 0.021). The median dose intensity of gemcitabine was 576.9 mg/m^2/week in the recurrence group, similar to that in the metastasis group (655.7 mg/m^2/week, p = 0.09).

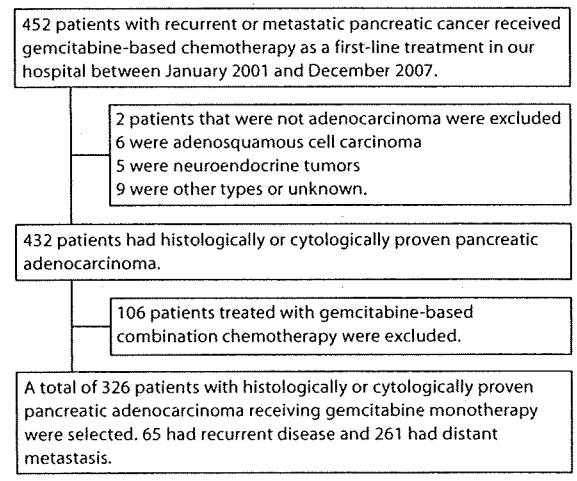

Fig. 1. Flow diagram for identification of eligible patients.

Survival Analysis

In the recurrence group, time to treatment failure was significantly longer than that in the metastatic group (median 138 vs. 77 days, respectively, p = 0.017; fig. 2). The same was true of overall survival, with the recurrence group having a longer survival time than the metastasis group (median 270 vs. 185 days, p = 0.0003; fig. 3). The univariate analysis revealed recurrent or metastatic status to be an important factor influencing survival [hazard ratio (HR) 0.53, p = 0.0002]. Other factors, including KPS (HR 1.73), liver metastasis (HR 0.49), peritoneal metastasis (HR 0.65), white blood cell count (HR 0.63), hemoglobin (HR 1.48), albumin (HR 1.63), alkaline phosphatase (HR 0.49), aspartate aminotransferase (HR 0.69), alanine aminotransferase (HR 0.59), lactate dehy-

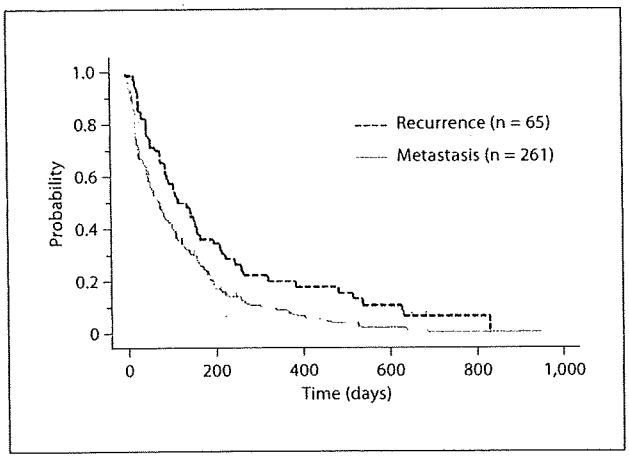

Fig. 2. Times to treatment failure in patients with recurrence and metastasis.

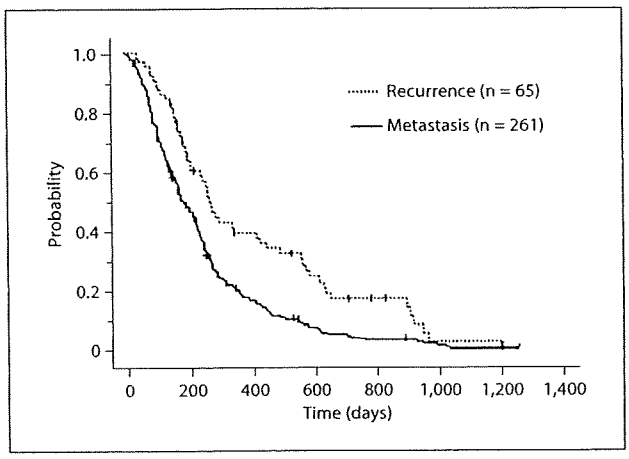

Fig. 3. Overall survival in patients with recurrence and metastasis.

Table 2. Baseline laboratory data of recurrence and metastasis groups

	Recurrence (n = 65)		Metastasis (n = 261)		p value
	median	range	median	range	
WBC, n/μl	5,500	2,700–11,200	6,300	1,800–35,500	0.0002
Hb, g/dl	12	8.4–15.1	12	6.7–16.1	0.66
Albumin, g/dl	3.7	2.2–4.6	3.6	2.4–4.6	0.048
ALP, U/l	327	154–2,751	378	38–3,593	0.43
AST, IU/l	26	13–149	27	11–226	0.89
ALT, IU/l	23	9–141	30	5–305	0.52
LDH, U/l	176	103–626	191	67–2,311	0.72
CRP, mg/dl	0.1	0.1–10.6	0.8	0.1–20.6	<0.001
CA 19-9, U/ml	30	1–52,400	148	1–1,620,000	0.018

WBC = White blood cells; Hb = hemoglobin; ALP = alkaline phosphatase; AST = asparate aminotransferase; ALT = alanine aminotransferase; LDH = lactate dehydrogenase; CRP = C-reactive protein; CA 19-9 = carbohydrate antigen 19-9.

drogenase (HR 0.51), CRP (HR 0.40) and CA 19-9 (HR 0.76), also correlated with survival (table 3). The multivariate analysis after stepwise selection identified KPS (HR 1.43, 95% CI 1.02–2.01), liver metastasis (HR 0.49, 95% CI 0.36–0.67), peritoneal metastasis (HR 0.38, 95% CI 0.27–0.53), alkaline phosphatase (HR 0.59, 95% CI 0.43–0.81), lactose dehydrogenase (HR 0.55, 95% CI 0.42–0.73) and CRP (HR 0.56, 95% CI 0.42–0.75) to be independent prognostic factors, while recurrent or metastatic status was not significant (HR 0.76, 95% CI 0.53–1.09, p = 0.14; table 4).

We also performed a subanalysis of the recurrence pattern only in patients with recurrences (n = 65). The local recurrence only group (n = 12) had somewhat longer survival than the distant only or local and distant recurrence group (n = 53) but the differences did not reach statistical significance (median 564 vs. 269 days, p = 0.67; fig. 4).

Adverse Events

In the recurrence group, the incidence of leukopenia was high compared to that of the metastatic cancer group

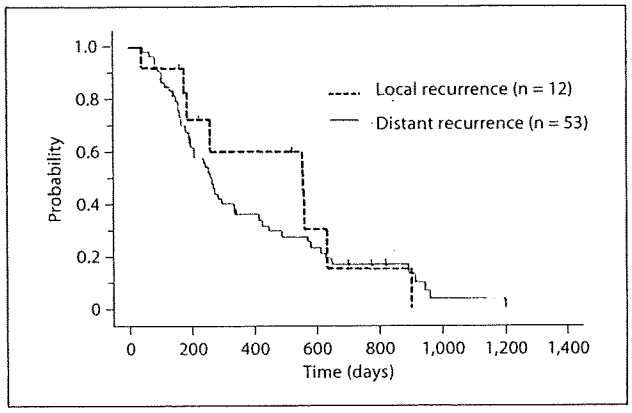

Fig. 4. Overall survival in the recurrence group (local recurrence only and distant metastasis with/without local recurrence).

Table 4. Results of multivariate analysis of survival

	HR	95% CI	p value
Recurrence vs. metastasis	0.76	0.53–1.09	0.14
KPS 70, 80 vs. 90, 100	1.43	1.02–2.01	0.038
Liver metastasis no vs. yes	0.49	0.36–0.67	<0.001
Peritoneal metastasis no vs. yes	0.38	0.27–0.53	<0.001
ALP ≤330 vs. >330	0.59	0.43–0.81	0.001
LDH ≤220 vs. >220	0.55	0.42–0.73	<0.001
CRP ≤1.0 vs. >1.0	0.56	0.42–0.75	0.001

KPS = Karnofsky performance status; ALP = alkaline phosphatase; LDH = lactate dehydrogenase; CRP = C-reactive protein.

Table 3. Results of univariate analysis of survival

	HR	95% CI	p value
Recurrence vs. metastasis	0.53	0.38–0.74	0.0002
Age ≤65 vs. >65	1.27	0.98–1.63	0.07
Male vs. female	1.01	0.79–1.30	0.95
KPS 70, 80 vs. 90, 100	1.73	1.26–2.38	0.008
Biliary drainage no vs. yes	0.98	0.72–1.34	0.91
Liver metastasis no vs. yes	0.49	0.37–0.65	<0.001
Peritoneal metastasis no vs. yes	0.65	0.48–0.89	0.006
Lung metastasis no vs. yes	1.28	0.91–1.81	0.16
WBC ≤6,000 vs. >6,000	0.63	0.49–0.80	0.002
Hb ≤12 vs. >12	1.48	1.15–1.90	0.002
Albumin ≤3.5 vs. >3.5	1.63	1.27–2.10	0.001
ALP ≤330 vs. >330	0.49	0.38–0.63	<0.001
AST ≤35 vs. >35	0.69	0.53–0.89	0.005
ALT ≤35 vs. >35	0.59	0.46–0.76	<0.001
LDH ≤220 vs. >220	0.51	0.39–0.66	<0.001
CRP ≤1.0 vs. >1.0	0.40	0.31–0.52	<0.001
CA 19-9 ≤1,000 vs. >1,000	0.76	0.59–0.98	0.04

KPS = Karnofsky performance status; WBC = white blood cells; Hb = hemoglobin; ALP = alkaline phosphatase; AST = asparate aminotransferase; ALT = alanine aminotransferase; LDH = lactate dehydrogenase; CRP = C-reactive protein; CA = carbohydrate antigen.

Table 5. Adverse events associated with gemcitabine treatment in each category

	Recurrence (n = 65)	Metastasis (n = 261)	p value
Leukopenia			
Grade 1–4	53 (81.5%)	170 (65.1%)	0.008
Grade 3/4	22 (33.8%)	32 (18.8%)	0.003
Neutropenia			
Grade 1–4	47 (72.3%)	169 (64.8%)	0.31
Grade 3/4	22 (33.8%)	94 (36.0%)	0.18
Anemia			
Grade 1–4	58 (89.2%)	225 (86.2%)	0.68
Grade 3/4	7 (10.8%)	22 (8.4%)	0.63
Thrombocytopenia			
Grade 1–4	35 (53.8%)	147 (56.3%)	0.78
Grade 3/4	3 (4.6%)	21 (8.0%)	0.58
Fatigue			
Grade 1–4	30 (46.2%)	135 (51.7%)	0.49
Grade 3/4	0 (0%)	1 (0.4%)	0.99
Anorexia			
Grade 1–4	27 (41.5%)	157 (60.2%)	0.008
Grade 3/4	1 (1.5%)	8 (3.1%)	0.99
Nausea			
Grade 1–4	16 (24.6%)	92 (35.2%)	0.11
Grade 3/4	0 (0%)	1 (0.4%)	0.99

(any grade: p = 0.008; grade 3–4: p = 0.003), although its incidence of neutropenia was similar (p = 0.31). Anorexia of any grade was frequently observed in the metastatic cancer patients (p = 0.008), although other non-hematological events were equally common (table 5). Grade 3 interstitial pneumonitis was observed in 3 metastatic cancer patients. There were 17 patients who died within 30 days after gemcitabine administration and all of them were considered to be cancer-related deaths. Overall, in both groups, gemcitabine monotherapy was well tolerated.

Discussion

This study revealed the relevance of recurrent and metastatic status to survival. Recurrent cancer patients had better survival than primary metastatic cancer patients (median 270 vs. 185 days, p = 0.0003). However, multivariate analysis demonstrated that recurrent or metastatic status was not an independent prognostic factor (HR 0.76, p = 0.14). On multivariate analysis, KPS, liver metastasis, peritoneal metastasis, alkaline phosphatase, lactose dehydrogenase and CRP were found to be important factors relating to survival, and all have previously been reported to be significant prognostic factors for pancreatic cancer [20, 21]. In our study, analysis of patient characteristics revealed that those with recurrent disease tended to have better prognostic factors, such as better KPS and lower CRP, than metastatic cancer patients. This may correlate with tumor burden, which is relatively low when recurrence is found, since patients who undergo resection usually receive close follow-up after surgery. In contrast, in most patients with primary metastasis, pancreatic cancer is diagnosed at an advanced stage after symptom development. Our findings suggest other factors, such as KPS, liver metastasis and CRP, to be more important influences on outcomes than recurrent or metastatic status.

Gemcitabine had a clear survival benefit in pancreatic cancer patients. To our knowledge, 15 phase III trials of systemic chemotherapy for advanced pancreatic cancer have been published [4–18]. All 15 studies included patients with unresectable locally advanced pancreatic cancer. In 8 studies, patients were stratified by locally advanced versus metastatic disease [4–10, 17, 18], and 2 other studies have suggested locally advanced versus metastatic cancer to be a significant prognostic factor in multivariate analysis [11, 12]. On the other hand, no trials have distinguished recurrent from metastatic disease as a prognostic factor. The rates of patients with history of pancreatectomy were described in 8 studies [4, 6–10, 17, 18]. The median percentage of such patients was 11% (range 6.1–20.8%). Of these, only the report by Van Cutsem et al. [4] referred to the relation between pancreatectomy history and prognosis. They examined the effect of tipifarnib given with gemcitabine in 688 patients with advanced pancreatic cancer, including 76 who underwent Whipple resection prior to study entry. The study demonstrated a trend toward better survival in those who had previously undergone surgical resection than in those who had not (median 229 vs. 185 days, HR 0.77, p = 0.09) [4].

In the present study, the response rate in the metastasis group was similar to that in the recurrence group. However, the disease control rate was higher in the recurrence group (82.5 vs. 68.9%, p = 0.04). The relatively low tumor burden in the recurrent cancer group may be one possible explanation for this high disease control rate. In the metastasis group, disease was found to be progressive, with multiple metastases, deterioration of general condition and inadequate organ function. In addition, the primary metastatic disease might tend to have aggressive biology with more rapid growth than the recurrent disease by nature. These factors may relate to difficulty delivering adequate chemotherapy, resulting in a higher progressive disease rate in the metastatic cancer group.

In contrast, 12 of 65 patients in the recurrence group had local recurrence alone. Of these 12 patients, 11 were radiographically evaluable. Ten showed stable disease and 1 achieved partial remission with gemcitabine treatment (data not shown). Therefore, patients with local recurrence alone may contribute to the good disease control rate in the recurrent cancer group. As to treatment delivery, the numbers of gemcitabine administrations and total dose of gemcitabine in the recurrence group were higher than those in the metastasis group (administration: median 15.9 vs. 11.8 times, p = 0.012; dosage: 15,318 vs. 11,297 mg, p = 0.021). The higher disease control rate in the recurrence group may be attributable, at least in part, to this prolonged delivery of gemcitabine since dose intensity did not differ between the 2 groups (576.9 vs. 655.7 mg/m^2/week, p = 0.09). As to adverse events, gemcitabine was well tolerated in both groups, although the rate of grade 3–4 leukopenia was slightly higher in the recurrence group.

In conclusion, we examined differences in clinical outcomes between patients with recurrent versus metastatic unresectable pancreatic cancer. Recurrent cancer patients had a somewhat better outcome, though this was not an independent prognostic factor. Responses to gemcitabine monotherapy were similar, as were drug delivery and toxicity. Although the present study has limitations due to it being a retrospective analysis using an insufficiently large sample size, it appears to be acceptable to treat recurrent and metastatic pancreatic cancer under the same category when conducting clinical trials.

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Four Cases of Pancreatic Acinar Cell Carcinoma Treated with Gemcitabine or S-1 as a Single Agent

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Pancreatic acinar cell carcinoma (ACC) is a comparatively rare tumor and account for ~1% of all cases of pancreatic cancer. Clinical presentation is usually related to either local spread or metastasis. The clinical features, especially those related to the prognosis and treatment outcomes, have not yet been fully clarified. There are no established treatments for unresectable pancreatic ACC. We administered gemcitabine monotherapy to four patients with ACC; however, the results were not satisfactory. Disease control without obvious tumor shrinkage was observed in one patient. Another patient showed severe renal damage caused by gemcitabine. On the other hand, fluoropyrimidine-based chemotherapy may have some activity against this tumor, because one of the three patients who received S-1 as second-line chemotherapy showed a partial response. Prospective clinical trials are necessary to confirm the effectiveness of fluoropyrimidine for the treatment of pancreatic ACC.

Key words: GI-pancreas – GI-pancreas-med – GI-pancreas-radoncol – chemo-GI tract

INTRODUCTION

Pancreatic acinar cell carcinoma (ACC) is a rare tumor. According to a survey conducted by the National Pancreatic Cancer Registry in Japan (1982–2002), pancreatic ACC accounted for 93 (0.4%) of all the 25 582 cases of resectable pancreatic cancer (1). In the USA, Cubilla and Fitzgeralf (2) reported that ACC accounted for ~1.2% of all cases of resectable pancreatic cancer. ACCs are defined as carcinomas in which the neoplastic cells show acinar differentiation and pancreatic enzyme production. On microscopic examination, these tumors are composed of cells arranged in nests and acinar structures, often showing lobulation, with thin strands of fibrovascular stroma. Periodic acid-Schiff staining with diastase digestion reveals fine zymogen granules in the cytoplasm of the tumor cells (3,5,6). All patients with ACC show positive staining with one or both of the stains available for pancreatic enzymes, namely trypsin and chymotrypsin. In case reports, ACCs are described as poorly defined, dense masses, well-defined masses with central necrosis, cystic masses surrounded by a thick hypervascular wall, well-defined hypodense masses with a thin, enhancing capsule, or well-defined, hypervascular solid masses on computed tomography (3). The radiological differential diagnosis of ACCs includes ductal adenocarcinoma, neuroendocrine tumor, solid and pseudopapillary tumor, pancreatic blastoma, mutinous cystic neoplasm and pseudocyst. It is important to differentiate among these neoplasms, because their treatments and prognoses differ significantly (3,4). However, with regard to the treatment of ACCs, no successful chemotherapeutic regimens have, unfortunately, been established yet. Therefore, there is clearly a need to establish effective treatment strategies for patients with recurrent or advanced unresectable disease. In this article, we report four patients with ACC of the pancreas who were treated with gemcitabine, which is regarded as the standard drug for the treatment of pancreatic ductal adenocarcinoma, and discuss potential treatment strategies for this disease.

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CASE REPORTS

The clinical characteristics of the four patients are summarized in Table 1.

CASE 1

A 48-year-old man with a large palpable abdominal mass in the epigastric region was referred to us. Dynamic computed tomography showed a huge, lobulated mass encircling the splenic artery, the splenic vein and the superior mesenteric

Table 1. Profiles of our four patients

Patient no.	Age/sex	UICC-TMN	Stage	Performance status	Surgery	First-line chemotherapy	Second-line chemotherapy	PFS for first-line chemotherapy with GEM	Survival time (days)
1	48/M	T3N0M0	II	0	Pancreatectomy and partial hepatectomy	GEM, 6 cycles SD	S-1, 1 cycle PD	6 months	>794
2	76/M	T4N0M0	III	0	—	GEM, 1 cycle NE	—	NE	412
3	67/M	T3N1M1	IV	0	—	GEM, 2 cycles PD	S-1, 8 cycles PR	2 months	407
4	61/F	T4N0M0	III	0	—	GEM, 6 cycles SD	S-1, 1 cycle PD	6 months	309

PFS, progression-free survival; GEM, gemcitabine; S-1, 1 M tegafur–0.4 M gimestat–1 M otastat potassium; SD, stable disease; PD, progression disease; NE, not evaluable; PR, partial response.

vein confluence in the pancreatic head and body (Fig. 1). Pancreaticoduodenectomy with excision of the tumor was performed in August 2005. Three metastatic nodules were found during the surgery and removed from the liver surface. The histological diagnosis was pancreatic ACC. After the operation, the patient received no chemotherapy, and 3 months later, multiple liver metastases were detected. Dynamic computed tomography showed metastases in the S6 segment of the liver. Chemotherapy was initiated with gemcitabine, which was administered by intravenous injection over 30 min at the dose of 1000 mg/m^2 on days 1, 8 and 15 of each 28-day cycle, for 6 months. After a long interval with stable disease (SD), progression of the liver metastases was confirmed and partial hepatectomy was performed at the patient's request. Although all the metastatic liver tumors were resected, a new metastatic lesion was found in the left upper quadrant of the abdomen at 3 months after the operation. Therefore, surgical resection for the recurrent tumor and the tumor extension to the small intestine and transverse colon was performed. Nonetheless, multiple liver metastases were confirmed again 7 months after the final resection, and oral chemotherapy was initiated with S-1 at the dose of 40 mg/m^2 twice a day from day 1 to day 28 of each 42-day cycle. However, the chemotherapy of S-1 was judged to be ineffective, because progression of the liver metastases was noted during the second course. The treatment plan was then switched to best supportive care.

Case 2

A 67-year-old man visited us with the chief complaint of epigastric discomfort of 6-month duration. He had received medical treatment for functional dyspepsia, because there were no abnormal findings on abdominal ultrasonography or

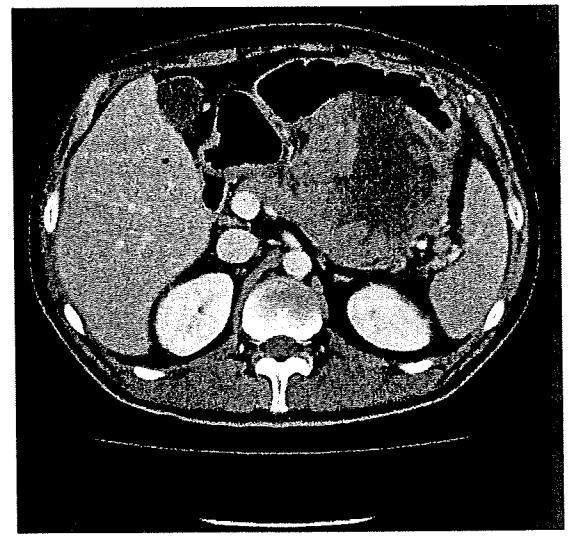

Figure 1. Abdominal computed tomography of Case 1 before treatment. The tumor appeared to be composed of cystic and solid components.

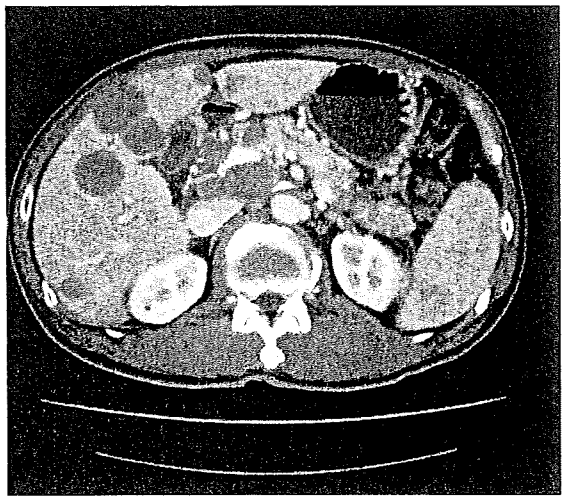

Figure 2. Abdominal computed tomography of Case 2 before treatment. Multiple liver tumors were noted at diagnosis.

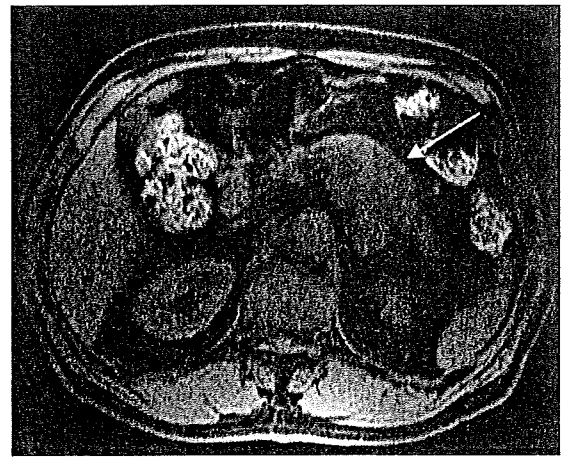

Figure 3. Abdominal magnetic resonance T1-weighted short T1 inversion recovery (STIR) imaging of Case 3 before treatment. This case was not a suitable candidate for intravenous iodine contrast use because of allergy. Tumor is detected in the pancreatic body (arrow) with weak contrast.

upper gastrointestinal (GI) endoscopy. However, since the patient showed no improvement, re-evaluation by abdominal ultrasonography was performed. The ultrasonography showed abdominal lymphadenopathy and multiple liver tumors and the patient was referred to our hospital for further examination. A final diagnosis of pancreatic tail cancer with liver, bone and lymph node metastases was made (Fig. 2). Histological diagnosis by needle aspiration biopsy confirmed the diagnosis of pancreatic ACC. Chemotherapy with gemcitabine was initiated in September 2007, the drug administered by intravenous injection over 30 min at the dose of 1000 mg/m^2 on days 1, 8 and 15 of each 28-day cycle. After the first chemotherapy cycle, progression of the ACC was noted, along with Grade 3 creatinine elevation. Thus, renal damage occurred suddenly and remained irreversible until the patient was discharged in November 2007. As gemcitabine was ineffective and had adverse effects, the patient was started on best supportive care.

Case 3

A 67-year-old man with chronic hepatitis C was referred to our hospital with elevation of the serum α-fetoprotein level. Dynamic magnetic resonance imaging revealed a focal uniformly dense hypovascular mass in the pancreatic body. Surgical treatment was considered to be contraindicated because the tumor was found to invade the superior mesenteric artery confluence (Fig. 3). The histological diagnosis made by needle aspiration biopsy was pancreatic ACC, and some tumor cells showed focally positive staining for α-fetoprotein. Therefore, we surmised that the elevation of serum α-fetoprotein level might be caused by secretion from the ACC. Therefore, chemotherapy was initiated with gemcitabine administered by intravenous injection over 30 min at the dose of 1000 mg/m^2 on days 1, 8 and 15 of each 28-day cycle. After two courses of chemotherapy with gemcitabine, dynamic computed tomography revealed tumor progression. After discontinuation of the chemotherapy with gemcitabine, oral chemotherapy was started with S-1 administered at the dose of 40 mg/m^2 twice a day from day 1 to day 28 of each 42-day cycle. The tumor size decreased by 34.1% [classified as a partial response (PR)] by the end of four courses of treatment. The serum α-fetoprotein level decreased from 5386 to 1367 ng/ml. After eight courses of chemotherapy with S-1, however, tumor progression was noted again. Therefore, chemotherapy was discontinued and the patient was started on best supportive care.

Case 4

A 61-year-old woman presented to us with high-colored urine. Physical examination revealed no abnormal findings. Upper GI endoscopy revealed deformation of the duodenal bulb, leading to the suspicion of. Dynamic computed tomography showed an irregularly lobulated mass in the pancreatic head encircling the splenic artery, the splenic vein and the superior mesenteric vein confluence (Fig. 4). The possibility of surgical treatment was ruled out because of tumor invasion into the superior mesenteric artery. The histological diagnosis made by needle aspiration biopsy was ACC of the pancreas. Chemotherapy was initiated with gemcitabine administered over 30 min at the dose of 1000 mg/m^2 on days 1, 8 and 15 of each 28-day cycle. The best overall response was SD through six courses. After six courses of chemotherapy, computed tomography revealed multiple liver metastases. Oral chemotherapy was started with S-1 administered at the dose of 40 mg/m^2 twice a day from day 1 to day 28 of each 42-day cycle. However, the multiple liver metastases continued to progress even after the start of S-1 treatment, and the patient was switched to best supportive care.

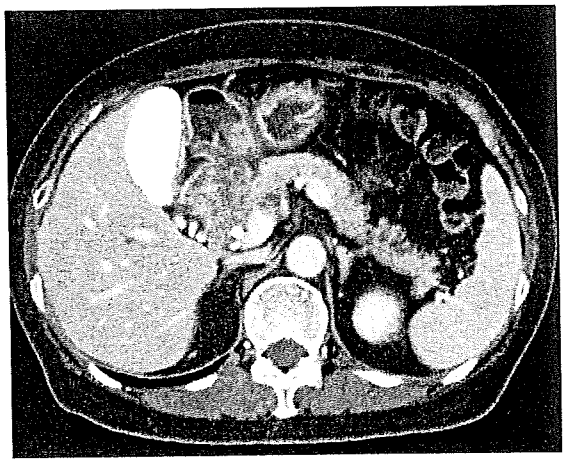

Figure 4. Abdominal computed tomography of Case 4 before treatment. The mass in the pancreatic head is contiguous with the duodenum and appears to invade the adjacent pancreatic tissue.

DISCUSSION

Surgical management remains the only curative therapy for patients with of ACC of pancreas with localized and resectable disease. Those who were treated by surgical resection were reported to show a median survival of longer than 30 months (7,8). This compares favorably with the data for patients with ductal adenocarcinoma of the pancreas; published reports on survival after resection have indicated a median survival of 12–18 months (10). More than 70% of the patients who undergo surgical resection are eventually confirmed to show recurrent disease (9). These results suggest that micrometastases are present even in cases where the disease is apparently localized to the pancreas (9).

In addition, most patients with pancreatic ACC present with unresectable locally advanced disease or obvious metastases. For patients at this stage of the disease, no adequate treatment strategies have been established yet (10,11), because no randomized controlled trials have been performed to confirm the efficacy of treatments. There are only a few reports on the use of single-drug chemotherapy, combination chemotherapy (9), chemoradiotherapy (10) or hepatic arterial injection chemotherapy (12–14) (Table 2). To the best of our knowledge, gemcitabine has never been demonstrated to show efficacy against ACC, although the drug has been widely used as the standard agent for the treatment of pancreatic adenocarcinoma. We administered chemotherapy with gemcitabine for patients with pancreatic ACC, and two of these patients showed SD without obvious tumor shrinkage. In addition, severe renal damage caused by gemcitabine was observed in one of our patients. Therefore, in our impression, chemotherapy with gemcitabine is not very promising for pancreatic ACC, although the true efficacy can only be determined by randomized controlled trials.

Some case studies have reported tumor shrinkage with fluoropyrimidine-based treatments. Holen et al. reviewed 22 chemotherapy regimens administered to 18 different patients and reported that there were no complete responses, only two PRs and seven SDs. The PRs were seen for combinations of irinotecan, 5-fluorouracil and leucovorin and also cytarabine, cisplatin and caffeine (9). It is noteworthy that ACCs of the pancreas tended to respond to 5-fluorouracil-based chemotherapies. The three patients showing PR reported in different case series had received combination therapies. Two of the patients had received 5-fluorouracil-based chemotherapy, and one patient had received combination therapies based on capecitabine (Table 2). In addition, we also observed good response to chemotherapy with S-1, a type of fluoropyrimidine. Further research is needed to clarify whether administration of fluoropyrimidine might improve the unsatisfactory results of chemotherapy for this disease.

For patients with unresectable, yet locally confined disease, radiotherapy may be one of the treatment options, as focal disease control may be expected. Holen et al. (9) also reported that all of the eight patients who were treated by radiation showed either PR or SD. Lee and Kim (10) reported two PR cases among locally advanced cases of ACC of the pancreas treated by concurrent capecitabine and radiation therapy. To obtain control of not only localized disease, but also of distant metastatic disease, radiotherapy combined with chemotherapy may be a potentially useful treatment strategy, but there are only a few reports of its use until date.

In conclusion, randomized controlled trials are needed to establish effective treatment strategies for unresectable

Table 2. Previous reports of chemotherapy or chemoradiotherapy for acinar cell carcinoma

Authors	Treatments	No. of patients	Antitumor response	Time to progression
Holen et al. (9)	5-Fluorouracil + radiation	2	PR, SD	NE
Sakon et al. (12)	Capecitabine + radiation	2	PR, PR	>15 months
Lee and Kim (10)	5-Fluorouracil[a] + mitomycin C[a] + cisplatin[a]	1	PR	18 months
Holen et al. (9)	Irinotecan + 5-fluorouracil + leucovorin	1	PR	7 months
Holen et al. (9)	Cytarabine + cisplatin + caffeine	1	PR	3 months

[a]Hepatic artery injection.

pancreatic ACC. However, it is not easy to recruit patients for trials, as pancreatic ACC is a very rarely occurring tumor. Multicenter and multinational cooperative trials are necessary to establish treatments to improve the dismal prognosis of patients with this disease.

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Conflict of interest statement

None declared.

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Regular Dose of Gemcitabine Induces an Increase in CD14$^+$ Monocytes and CD11c$^+$ Dendritic Cells in Patients with Advanced Pancreatic Cancer

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Objective: Chemotherapy and immunotherapy often seem to contradict each other. However, recent reports suggested that the anticancer effects in some chemotherapeutic agents were concerned with immune response. This study was designed to evaluate the immunological reaction by gemcitabine for future clinical trial of combination therapy with gemcitabine and cancer vaccines.

Methods: We evaluated several immunological parameters in patients with advanced pancreatic cancer who received a conventional dose of gemcitabine for 2 months. Twenty-eight patients with metastasis or locally advanced tumor, including 18 gemcitabine-naïve and 10 with a history of preceding gemcitabine treatment, were enrolled in this study. The patients received gemcitabine 1000 mg/m^2 for 3 weeks, followed by 1 week of rest. We monitored the kinetics of lymphocytes, natural killer cells, monocytes, dendritic cells (DC), human leukocyte antigen (HLA)-multimer conjugated with CMV or WT1 peptide, and intracellular cytokine production of interferon-γ and interleukin-4 by flow cytometry. The T cell receptor (TCR) repertoire was also analyzed.

Results: The absolute number and percentage of CD14$^+$ monocytes and CD11c$^+$ (myeloid) DC increased with gemcitabine treatment ($P = 0.033$ and $P = 0.021$). The percentage of CD123$^+$ (plasmacytoid) DC also increased ($P = 0.034$), whereas no significant change was observed in other immune parameters, including multimer, intracellular cytokine production and TCR repertoire.

Conclusions: Our finding that gemcitabine treatment induced the proliferation of CD14$^+$ monocytes and CD11c$^+$ DC could support combination therapy with gemcitabine and specific immunotherapy such as peptide vaccination against pancreatic cancers.

Key words: gemcitabine – dendritic cell – monocyte – pancreatic cancer – cancer vaccines

INTRODUCTION

Pancreatic cancer remains one of the most difficult cancers to treat. It is the fifth leading cause of cancer death in Japan (1) and the fourth in both sexes in the USA (2). Although gemcitabine has been the standard treatment option for unresectable disease for the past 10 years (3), the achieved benefit is suboptimal with objective response rates of <15% and a median survival of <6 months. Many clinical trials of cytotoxic or biologic agents combined with gemcitabine have not shown any survival advantage over gemcitabine alone (4–7). In 2005, the combination of erlotinib plus gemcitabine showed a statistically significant improvement in overall survival; however, the increase in median survival was marginal (6.24 vs. 5.91 months) (8).

Gemcitabine is a nucleotide analog which inhibits DNA synthesis. It is a relatively well-tolerated drug, except that Grade 3–4 neutropenia occurs in 26% of the patients (3). Plate et al. (9) reported the effects of gemcitabine treatment

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on immune cells, such as immune cell numbers and their function, as determined by intracellular cytokine production and ELISPOT assays. Although the absolute number of all lymphocyte subpopulations declined during the study period, there was no significant change in the proportion of most lymphocyte subtypes, except for $CD3^+CD45RO^+$ memory lymphocytes. In their experiment, immune-monitoring was limited to 21 days during the initial course of treatment, with no follow-up evaluation. In this study, we monitored immune parameters for 2 months in gemcitabine-naïve patients and in those who had already started treatment. Our data could provide a basis for planning a clinical trial of combination therapy with gemcitabine and various immunotherapies, including cancer vaccines.

PATIENTS AND METHODS

Patients, Treatment Schedule and Sampling

After written informed consent was obtained, peripheral blood (PB) samples were obtained from patients with pancreatic cancer who were planned to receive (gemcitabine-naïve group) or who had already started gemcitabine treatment (treated group) from August 2006 to September 2007 at the National Cancer Center Hospital (Tokyo, Japan). All of them had a histological or cytological diagnosis of pancreatic adenocarcinoma. None of them had received other chemotherapeutic agents or radiation therapy except for resection of the tumor and/or intraoperative radiation therapy. The patients were treated with gemcitabine 1000 mg/m^2 for 3 weeks followed by 1 week of rest. This cycle was repeated every 4 weeks. Before each treatment, blood counts were checked, and if neutropenia ($<1000/mm^3$) developed, the treatment was suspended. None of the patients used oral steroid, but some used intravenous dexamethasone treatment (8 mg) to prevent nausea before gemcitabine injection. Ten milliliters of heparinized blood and 7 ml EDTA blood were taken on days 0 and 14 of the first course, and on the first days of the second and third courses (Fig. 1). Heparinized blood was immediately used in the FACS analysis, and EDTA blood was lysed by Buffer EL (BD Biosciences PharMingen, San Diego, CA, USA), mixed with Buffer RLT (BD Biosciences PharMingen) and cryopreserved at $-80°C$ as an RNA lysate until the T cell receptor (TCR) repertoire analysis.

Flow Cytometry for Cell Phenotyping

The fluorescent-conjugated monoclonal antibodies (MAbs) used in this study are listed in Table 1. According to the manufacturer's protocol, PB samples were incubated with MAbs for 30 min at room temperature in the dark. Red blood cells were lysed by PhamLyse (BD). After being washed (CellWash from BD), the cells were fixed (CellFix from BD) and acquired on a flow cytometer (FACSCalibur, Becton Dickinson, CA, USA). Analyses were performed using CellQuest software. We calculated the absolute number of each cell type using the following formulas:

Lymphocyte phenotypes and natural killer (NK) cells:

$$\begin{aligned}\text{Count of each phenotype (cells/}\mu\text{l)} &= \text{WBC count}^{CD} \times \%\ \text{lymphocyte fraction}^{CD} \\ &\times \%\ \text{phenotype of lymphocyte gating}^{FM}\end{aligned}$$

$CD14^+$ monocytes:

$$\begin{aligned}\text{Count (cell/}\mu\text{l)} &= \text{WBC count}^{CD} \times \%\ \text{monocyte fraction}^{CD} \\ &\times \%\ CD14^+\ \text{cells in monocyte gating}^{FM}\end{aligned}$$

Dendritic cells (DC):

$$\begin{aligned}\text{Count (cell/}\mu\text{l)} &= \text{WBC count}^{CD} \times \%\ \text{of Lin1}^-\text{HLA-DR}^+ \\ &CD123^+\text{or Lin1}^-\text{HLA-DR}^+CD11c^{+FM}\end{aligned}$$

(where CD indicates the clinical data and FM indicates the flow cytometry data).

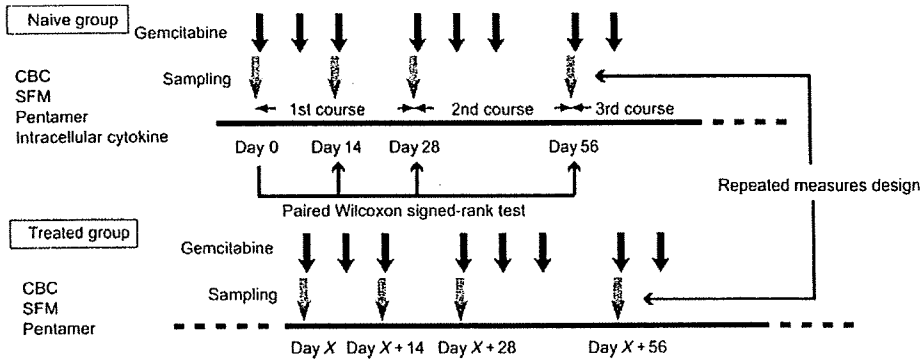

Figure 1. Schedules of gemcitabine treatment (brack arrows) and blood sampling (gray arrows), with a schematic of the analysis. Two cohorts (the naive group and the treated group) were examined in this study to confirm the long-term immunological effect of gemcitabine. CBC, complete blood count; SFM, surface maker staining (cell phenotype).

Table 1. The panel of fluorescent-conjugated monoclonal antibodies

No.	FITC	PE	PerCP	Analysis	Short names
1	IgG1[a]	IgG1[a]	IgG1[a]	$IgG1^+IgG1^+IgG1^-$	Negative control
2	CD4-FITC/CD8-PE[b]		CD3[a]	$CD4^-CD8^-CD3^+$	$CD4^+$ T cell
				$CD4^-CD8^+CD3^+$	$CD8^+$ T cell
3	CD16[a]	CD56[a]	CD3[a]	$CD16^+CD56^+CD3^-$	NK cell
4	Vα24[a]	CD161[a]	CD3[a]	$Vα24^-CD161^+CD3^+$	NKT cell
5	CD14[a]	CD20[a]		$CD14^+CD20^-$	$CD14^+$ monocyte
				$CD14^-CD20^+$	B cell
6	CD45RA[b]	CD45RO[b]	CD4[a]	$CD45RA^+CD45RO^-CD4^+$	Naive $CD4^+$ lymphocyte
				$CD45RA^-CD45RO^+CD4^+$	Memory $CD8^+$ lymphocyte
7	CD3[a]	CD69[a]	CD4[a]	$CD3^-CD69^+CD4^-$	$CD69^+CD4^+$ T cell
8	CD3[a]	CD69[a]	CD8[a]	$CD3^-CD69^+CD8^-$	$CD69^+CD8^+$ T cell
9	CD3[a]	CD25[a]	CD4[a]	$CD3^-CD25^+CD4^-$	$CD4^+CD25^+$ T cell
10	GITR[c]	CD25[a]	CD4[a]	$GITR^+CD25^+CD4^+$	
11	Lin-1[a]	CD123[a]	Anti-HLA-DR[a]	$Lin\text{-}1^-CD123^+HLA\text{-}DR^+$	$CD123^+$ dendritic cell
12	Lin-1[a]	CD11c[a]	Anti-HLA-DR[a]	$Lin\text{-}1^-CD11c^+HLA\text{-}DR^+$	$CD11c^-$ dendritic cell

Vα24, TCR Vα24; GITR, TNF receptor family-related; Lin-1, Lineage Cocktail 1.
[a]BD Biosciences, San Diego, CA, USA.
[b]Beckman–Coulter, Fullerton, CA, USA.
[c]R&D Systems, Minneapolis, MN, USA.

HLA Analysis

Before antigen-specific T cells were analyzed with regard to human leukocyte antigen (HLA)-pentamer and intracellular cytokine production, HLA typing was performed. Samples were stained with HLA-A02-FITC and HLA-A24-PE (BD) and analyzed by flow cytometry. Only HLA-A02 and -A24 patients were enrolled in the antigen-specific T cell analysis.

Pentamer Staining

Complexes of HLA-0201 CMV peptide (NLVPMVATV), WT1 peptide (RMFPNAPYL) and HLA-2402 WT1 peptide (CMTWNQMNL) were purchased from PROIMMUNE (Oxford, UK). Patients with HLA-A02 were examined for CMV and WT1, and patients with HLA-A24 were examined for WT1-specific TCR. CD3-FITC, CCR7-PE, CD3-PerCP and APC-conjugated pentamer were added to whole blood and incubated for 15 min at room temperature in the dark. The red blood cells were then lysed twice after antibody staining. After being washed with washing buffer, the cells were fixed and acquired on a flow cytometer. If there were enough pentamer-positive cells, we also performed CD45RA-FITC (Beckman–Coulter), CD57-FITC (BD), CD45RO-PE (Beckman–Coulter), CD27-PE (BD), CD8-PerCP and Pentamer-APC staining.

Peptides and CMV Lysate

The following >80% pure peptides, which were assessed as WT1 antigen epitope, were obtained using high-performance liquid chromatography (Qiagen, Hilden, Germany). The sequence of HLA-A0201-binding WT1 peptide was RMFPNAPYL [amino acids (AA) 126–134] and that of HLA-A2402-binding WT1 peptide was CMTWNQMNL (AA 235–243). CMV AD169 viral lysate was purchased from Advanced Biotechnologies (Alberta, Canada).

Intracellular Cytokine Staining

Intracellular cytokine staining was performed only in the naive group. Peripheral whole blood (1 ml) was stimulated for 6 h at 37°C with 1.0 μg/ml CMV lysate in the presence of 10 μg/ml co-stimulatory MAbs, CD28 and CD49d (BD). Patients with HLA-A02 or -A24 also received specific WT1-peptide stimulation at a final concentration of 1 ng/ml. 10 μg/ml breferdin A (Sigma, St Louis, MO, USA) was added during the last 4 h of incubation. Positive and negative controls were obtained by stimulating cells with 5 μl/ml staphylococcal enterotoxin B (Sigma) as a positive control or PBS (Ca^{2+}–Mg^{2+}) as a negative control. Samples were lysed by Lysing solution (BD), permeabilized by Permealyzing solution (BD) and stained with CD69-FITC, IL-4-PE, IFN-γ -PE, CD8-PerCP, CD4-PerCP and CD3-APC

(BD), before analysis with a FACSCalibur. Intracellular interferon (IFN)-γ- or interleukin (IL)-4-positive cells in $CD3^+CD4^+$ or $CD3^+CD8^+$ cells in lymphocyte gate were defined as cytokine-secreting T cells. CD69 was used as a marker for T cell activation.

T Cell Repertoire Analysis

Five samples were analyzed. Three milliliters of whole blood with EDTA were used for RNA extraction. RNA was extracted by a QIAamp RNA Blood Mini Kit (Qiagen), and an RT–PCR and PCR amplification were performed with a TaKaRa One Step RNA PCR Kit (AMV) (TaKaRa BIO Inc., Shiga, Japan) according to the supplier's protocol. The PCR conditions were: one cycle of 50°C for 30 min and 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s, with a final 1 min extension at 72°C and holding at 4°C (GeneAmp PCR System 9700, Applied Biosystems, CA, USA). We used 26 kinds of TCR-Vβ chain as forward primers with a Cβchain-conjugated 6FAM as a reverse primer (10,11). Vβ primers were synthesized by Invitrogen (CA, USA) and Cβ primer was synthesized by Applied Biosystems. One microliter of PCR product was mixed with 13.6 μl formamide and 0.4 μl size standard (Genescan-500 ROX, ABI), denatured at 94°C for 5 min and electrophoresed with a 3100-Avant Genetic Analyzer (ABI). The distribution was analyzed with GeneScan 7.7 (ABI).

Statistical Analysis

Progression-free survival was measured from the first day of gemcitabine treatment until the diagnosis of progressive disease, and data were analyzed by the Kaplan–Meier method. The log-rank test was used to analyze the differences between groups. Figure 1 shows a schematic of the analysis. In the naive group, we compared the immunological status pre-treatment to that after treatment by a paired Wilcoxon signed rank test. To compare the immunological status of the naive and treated groups, a repeated measures design was used. Data were analyzed with JMP 6.0 for Windows.

RESULTS

Patients

Twenty-eight patients were enrolled into this study (Table 2). Eighteen patients were receiving their first treatment with gemcitabine (naive group) and 10 had already received 3–20 previous courses of gemcitabine (treated group). In the naive group, three patients discontinued treatment due to early progression, pneumonia and entered the best supportive care, respectively. Five patients in the naive group and three in the treated group changed their schedule because they felt bad, showed fever associated with a common cold, had acute pancreatitis, showed stent obstruction and private matters. The patient data obtained during fever, pancreatitis and stent obstructions were removed from the analysis due to the suspension of gemcitabine treatment.

White Blood Cell Count, Neutrophils, Lymphocytes and Monocytes

We evaluated the white blood cell count (WBC), including a differential analysis of neutrophils, lymphocytes and monocytes (Fig. 2A–C and Supplementary Fig. S1A–C). The solid and dotted lines indicate the median number of cells in the naive and treated groups individually. The monocyte count in the naive group was lower than that in the treated group in a repeated measures analysis ($P = 0.001$). However, during the first 2 months, the number of monocytes increased significantly compared with the pre-treatment value at the beginning of the third course in the naive group ($P = 0.025$). Two months of gemcitabine treatment led to an increase in monocytes in the naive group to the same level as that in the treated group (Fig. 2C). The decrease in the lymphocyte count during the treatment course was transient, and only 5.6% of the patients in the naive group showed Grade 3–4 lymphopenia. Our data suggested that lymphocytes were not significantly influenced by gemcitabine treatment (Fig. 2B). There were no differences in the numbers of WBC, neutrophils, lymphocytes and monocytes between days 7 and 14, which suggested that these cells decreased by day 7 when the cell kinetics became stable.

Surface Markers

The counts of $CD69^+CD4^+$ T cells ($P = 0.002$), $CD69^+CD8^+$ T cells ($P = 0.012$) and $CD14^+$ monocytes ($P = 0.021$) (Fig. 2L, M and I), and two types of DC, $CD123^+$ and $CD11c^+$ ($P < 0.001$ and $P < 0.001$, Fig. 2P and Q) significantly increased in the treated group by a repeated measures analysis. The percentage of NK cells significantly decreased ($P = 0.008$) in the treated group (Supplementary Fig. S1F), but the absolute number was not significantly different ($P = 0.072$). Nevertheless, the tendency for the decrease in the treated group was clear (Fig. 2F). None of the other lymphocyte subtypes changed with regard to either number or percentage in the two groups. In comparison with pre-treatment and post-treatment in the naive group, the percentages of $CD4^+$ T cells ($P = 0.008$) and naive $CD4^+$ lymphocytes ($P < 0.001$) increased on day 14 (Supplementary Fig. S1D and J), whereas no significant change was observed in the other lymphocytes. In DC, the percentage of $CD123^+$ DC increased from the beginning of the second course ($P = 0.003$) and $CD11c^+$ DC increased from day 14 in the first course ($P < 0.001$) (Supplementary Fig. S1P and Q). The absolute numbers of most immune cells decreased. NK cells ($P = 0.018$)