should be assessed when comparing simultaneous resection and staged resections in SCLM.

The limitations of our study are its retrospective design and the relatively small number of patients studied.

Conclusion

The morbidity rate and the frequency of anastomotic leakage were high with simultaneous resection for SCLM, especially in patients with greater intraoperative blood loss or operation time greater than 8 h. For patients with SCLM, staged resections should be considered when simultaneous resection would involve excessive surgical stress.

References

1. Fong Y, Fortner J, Sun RL, Brennan MF, Blumgart LH. Clinical score for predicting recurrence after hepatic resection for metastatic colorectal cancer: analysis of 1001 consecutive cases. Ann Surg. 1999;230(3):309–318; discussion 18–21.
2. Nordlinger B, Guiguet M, Vaillant JC, Balladur P, Boudjema K, Bachellier P, Jaeck D. Surgical resection of colorectal carcinoma metastases to the liver. A prognostic scoring system to improve case selection, based on 1568 patients. Association Francaise de Chirurgie. Cancer. 1996;77(7):1254–1262.
3. Minagawa M, Makuuchi M, Torzilli G, Takayama T, Kawasaki S, Kosuge T, Yamamoto J, Imamura H. Extension of the frontiers of surgical indications in the treatment of liver metastases from colorectal cancer: long-term results. Ann Surg. 2000;231(4):487–499.
4. Scheele J, Stangl R, Altendorf-Hofmann A, Gall FP. Indicators of prognosis after hepatic resection for colorectal secondaries. Surgery. 1991;110(1):13–29.
5. Capussotti L, Vigano L, Ferrero A, Lo Tesoriere R, Ribero D, Polastri R. Timing of resection of liver metastases synchronous to colorectal tumor: proposal of prognosis-based decisional model. Ann Surg Oncol. 2007;14(3):1143–1150.
6. Chua HK, Sondenaa K, Tsiotos GG, Larson DR, Wolff BG, Nagorney DM. Concurrent vs. staged colectomy and hepatectomy for primary colorectal cancer with synchronous hepatic metastases. Dis Colon Rectum. 2004;47(8):1310–1316.
7. Lyass S, Zamir G, Matot I, Goitein D, Eid A, Jurim O. Combined colon and hepatic resection for synchronous colorectal liver metastases. J Surg Oncol. 2001;78(1):17–21.
8. Martin R, Paty P, Fong Y, Grace A, Cohen A, DeMatteo R, Jarnagin W, Blumgart L. Simultaneous liver and colorectal resections are safe for synchronous colorectal liver metastasis. J Am Coll Surg. 2003;197(2):233–41; discussion 41–42.
9. Fujita S, Akasu T, Moriya Y. Resection of synchronous liver metastases from colorectal cancer. Jpn J Clin Oncol. 2000;30(1):7–11.
10. Lambert LA, Colacchio TA, Barth RJ, Jr. Interval hepatic resection of colorectal metastases improves patient selection. Arch Surg. 2000;135(4):473–9; discussion 9–80.
11. Dindo D, Demartines N, Clavien PA. Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. Ann Surg. 2004;240 (2):205–213.
12. Kaplan E. Nonparametric estimation from incomplete observations. J Am Stat Assoc. 1958;53:457–481.
13. Tanaka K, Adam R, Shimada H, Azoulay D, Levi F, Bismuth H. Role of neoadjuvant chemotherapy in the treatment of multiple colorectal metastases to the liver. Br J Surg. 2003;90 (8):963–969.
14. Reddy SK, Pawlik TM, Zorzi D, Gleisner AL, Ribero D, Assumpcao L, Barbas AS, Abdalla EK, Choti MA, Vauthey JN, Ludwig KA, Mantyh CR, Morse MA, Clary BM. Simultaneous resections of colorectal cancer and synchronous liver metastases: a multi-institutional analysis. Ann Surg Oncol. 2007;14(12):3481–3491.
15. Thelen A, Jonas S, Benckert C, Spinelli A, Lopez-Hanninen E, Rudolph B, Neumann U, Neuhaus P. Simultaneous versus staged liver resection of synchronous liver metastases from colorectal cancer. Int J Colorectal Dis. 2007;22(10):1269–1276.
16. Allen PJ, Kemeny N, Jarnagin W, DeMatteo R, Blumgart L, Fong Y. Importance of response to neoadjuvant chemotherapy in patients undergoing resection of synchronous colorectal liver metastases. J Gastrointest Surg. 2003;7(1):109–115; discussion 16–7.
17. Mentha G, Majno PE, Andres A, Rubbia-Brandt L, Morel P, Roth AD. Neoadjuvant chemotherapy and resection of advanced synchronous liver metastases before treatment of the colorectal primary. Br J Surg. 2006;93(7):872–878.
18. Verhoef C, van der Pool AE, Nuyttens JJ, Planting AS, Eggermont AM, de Wilt JH. The "liver-first approach" for patients with locally advanced rectal cancer and synchronous liver metastases. Dis Colon Rectum. 2009;52(1):23–30.
19. Karanjia ND, Corder AP, Holdsworth PJ, Heald RJ. Risk of peritonitis and fatal septicaemia and the need to defunction the low anastomosis. Br J Surg. 1991;78(2):196–198.
20. Eberl T, Jagoditsch M, Klingler A, Tschmelitsch J. Risk factors for anastomotic leakage after resection for rectal cancer. Am J Surg. 2008;196(4):592–598.
21. Jung SH, Yu CS, Choi PW, Kim DD, Park IJ, Kim HC, Kim JC. Risk factors and oncologic impact of anastomotic leakage after rectal cancer surgery. Dis Colon Rectum. 2008;51 (6):902–908.
22. Kruschewski M, Rieger H, Pohlen U, Hotz HG, Buhr HJ. Risk factors for clinical anastomotic leakage and postoperative mortality in elective surgery for rectal cancer. Int J Colorectal Dis. 2007;22(8):919–927.
23. Lee WS, Yun SH, Roh YN, Yun HR, Lee WY, Cho YB, Chun HK. Risk factors and clinical outcome for anastomotic leakage after total mesorectal excision for rectal cancer. World J Surg. 2008;32(6):1124–1129.
24. Rullier E, Laurent C, Garrelon JL, Michel P, Saric J, Parneix M. Risk factors for anastomotic leakage after resection of rectal cancer. Br J Surg. 1998;85(3):355–358.
25. Meleagros L, Varty PP, Delrio P, Boulos PB. Influence of temporary faecal diversion on long-term survival after curative surgery for colorectal cancer. Br J Surg. 1995;82(1):21–25.
26. Montedori A, Cirocchi R, Farinella E, Sciannameo F, Abraha I. Covering ileo- or colostomy in anterior resection for rectal carcinoma. Cochrane Database Syst Rev. 2010(5):CD006878.

Pancreatology 12 (2012) 428–433

Contents lists available at SciVerse ScienceDirect

Pancreatology

journal homepage: www.elsevier.com/locate/pan

Original article

Treatment outcome for systemic chemotherapy for recurrent pancreatic cancer after postoperative adjuvant chemotherapy

Tomoko Katsui Taniyama [a], Chigusa Morizane [a,*], Kohei Nakachi [b], Satoshi Nara [c], Hideki Ueno [a], Shunsuke Kondo [a], Tomoo Kosuge [c], Kazuaki Shimada [c], Minoru Esaki [c], Masafumi Ikeda [b], Shuichi Mitsunaga [b], Taira Kinoshita [d], Masaru Konishi [d], Shinichiro Takahashi [d], Takuji Okusaka [a]

[a] Division of Hepatobiliary and Pancreatic Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
[b] Division of Hepatobiliary and Pancreatic Oncology, National Cancer Center Hospital East, Chiba, Japan
[c] Hepatobiliary and Pancreatic Surgery Division, National Cancer Center Hospital, Tokyo, Japan
[d] Hepatobiliary and Pancreatic Surgery Division, National Cancer Center Hospital East, Chiba, Japan

ARTICLE INFO

Article history:
Received 22 May 2012
Received in revised form
16 July 2012
Accepted 17 July 2012

Keywords:
Adjuvant chemotherapy
Chemotherapy
Gemcitabine
Recurrent pancreatic cancer
S-1

ABSTRACT

Objectives: A global consensus on how to treat recurrent pancreatic cancer after adjuvant chemotherapy with gemcitabine (ADJ-GEM) does not exist.
Methods: We retrospectively reviewed the clinical data of 41 patients with recurrences who were subsequently treated with chemotherapy.
Results: The patients were divided into two groups according to the time until recurrence after the completion of ADJ-GEM (ADJ-Rec): patients with an ADJ-Rec < 6 months ($n = 25$) and those with an ADJ-Rec ≥ 6 months ($n = 16$). The disease control rate, the progression-free survival after treatment for recurrence and the overall survival after recurrence for these two groups were 68 and 94% ($P = 0.066$), 5.5 and 8.2 months ($P = 0.186$), and 13.7 and 19.8 months ($P = 0.009$), respectively. Furthermore, we divided the patients with an ADJ-Rec < 6 months into two groups: patients treated with gemcitabine ($n = 6$) and those treated with alternative regimens including fluoropyrimidine-containing regimens ($n = 19$) for recurrent disease. Patients treated with the alternative regimens had a better outcome than those treated with gemcitabine.
Conclusions: Fluoropyrimidine-containing regimens may be a reasonable strategy for recurrent disease after ADJ-GEM and an ADJ-Rec < 6 months.

1. Introduction

Pancreatic cancer patients have an extremely poor prognosis. Although surgical resection is the only curative treatment, only 15%–20% of patients are candidates for resection. Even if a curative resection is performed, the 5-year-survival rate is only 10%–25%, and the median survival period is 11–20 months [1,2].

Various adjuvant chemotherapy or chemoradiotherapy regimens after surgical resection have been evaluated [2–6]. Recently, The Charite' Onkologie (CONKO)-001 trial was designed to determine the benefits of gemcitabine for patients with resected pancreatic cancer. Adjuvant chemotherapy with gemcitabine (ADJ-GEM) significantly improved the disease-free survival period, compared with surgery alone, in patients with resected pancreatic cancer. Although no significant difference in overall survival was seen at the time of publication, analysis after a longer follow-up period demonstrated a survival advantage for gemcitabine over observation-only (median progression-free survival, 22.8 months for ADJ-GEM vs. 20.2 months for observation-only; $P = 0.005$). At approximately the same time as the CONKO-001 trial, the Japanese Study Group of Adjuvant Therapy for Pancreatic Cancer (JSAP) conducted a randomized clinical trial evaluating adjuvant gemcitabine. Although no significant difference in overall survival was seen, the patients in the gemcitabine arm demonstrated a significantly longer disease-free survival period than the patients in the observation-only arm. These results were similar to those of the CONKO-001 trial and supported the concept that adjuvant chemotherapy using gemcitabine was effective in an Asian

* Corresponding author. Tel.: +81 3 3542 2511; fax: +81 3 3542 3815.
E-mail address: cmorizan@ncc.go.jp (C. Morizane).

1424-3903/$ – see front matter
http://dx.doi.org/10.1016/j.pan.2012.07.016

population [2,5]. Therefore, adjuvant therapy using gemcitabine for resected pancreatic cancer is now firmly established as a therapy that offers a modest but real improvement in overall survival [5,7].

In approximately 50% of patients, recurrent disease was reportedly seen within a year, even after receiving ADJ-GEM [5], and no global consensus exists regarding treatment strategies for recurrent disease after ADJ-GEM. If the length of time from the completion of adjuvant therapy until the detection of recurrence is less than 6 months, the NCCN guidelines recommend alternative chemotherapy using a fluoropyrimidine-based chemotherapy regimen. When this period is 6 months or greater, they recommend an alternative regimen or the same regimen as the previous therapy [8]. However, these recommendations have not been substantiated by actual clinical data.

In Japan, the oral fluoropyrimidine derivative S-1 is often used as an alternative regimen for gemcitabine-refractory cases. S-1 showed a non-inferiority to gemcitabine in terms of overall survival in a phase III trial and is considered an alternative to gemcitabine for chemonaïve patients with advanced pancreatic cancer [9]. Additionally, in gemcitabine-refractory metastatic cases, a recent phase II study of S-1 yielded results that demonstrated preferable activity, including a response rate of 9.5%–15% and a median overall survival time of 4.5–6.3 months [10,11]. Therefore, S-1 is widely used for the treatment of advanced pancreatic cancer in first-line and second-line settings in Japan.

We studied the current status of treatments for recurrent pancreatic cancer after curative resection followed by ADJ-GEM. The objective of this study was to examine the adequacy of the

Table 1
Patient characteristics at resection ($n = 41$).

		n (%)			
	Variables	All patients $n = 41$	ADJ-Rec < 6 months $n = 25$	ADJ-Rec ≥ 6 months $n = 16$	*P* value
Age (years)	Median (range)	65 (38–78)	64 (38–78)	65 (50–77)	0.96
Gender	Male	27 (66)	16 (64)	11 (69)	1.00
	Female	14 (34)	9 (36)	5 (31)	
PS[a] at recurrence	0	30 (73)	20 (80)	10 (63)	0.34
	1	5 (12)	3 (12)	2 (12)	
	Unknown	6 (15)	2 (8)	4 (25)	
Primary site	Head	26 (63)	17 (68)	9 (56)	0.51
	Body or -tail	15 (37)	8 (32)	7 (44)	
Type of Resection	PD[b]	26 (64)	17 (68)	9 (56)	0.66
	DP[c]	12 (29)	6 (24)	6 (38)	
	TP[d]	3 (7)	2 (8)	1 (6)	
Resection status	R0	36 (88)	22 (88)	14 (88)	1.00
	R1	5 (12)	3 (12)	2 (12)	
Histology	Adenocarcinoma	39 (95)	23 (92)	16 (100)	0.51
	Adenosquamous carcinoma	2 (5)	2 (8)	0 (0)	
Stage[e] at resection	IIA	5 (12)	0 (0)	5 (31)	0.006
	IIB	36 (88)	25 (100)	11 (69)	
CEA[f] (ng/mL)	Median (range)	2.7 (0.7–51.8)	2.7 (0.7–21.0)	2.4 (1.2–51.8)	0.98
CA19-9[g] (U/mL)	Median (range)	202 (0.5–6450)	212 (0.5–6450)	138 (17–3203)	0.56
Histological grade	Well	5 (12)	3 (12)	2 (12.5)	0.83
	Moderately	28 (71)	17 (68)	12 (75)	
	Poorly	7 (17)	5 (20)	2 (12.5)	
Lymph node ratio[h]	0	5 (12)	0 (0)	5 (31)	0.008
	0.1–0.199	23 (56)	14 (56)	9 (57)	
	0.2–0.299	8 (20)	7 (28)	1 (6)	
	0.3–	4 (10)	4 (16)	0 (0)	
	Unknown	1 (2)	0 (0)	1 (6)	
Recurrent pattern[i]	Locoregional	21 (51)	10 (40)	11 (69)	0.15
	Liver	18 (44)	14 (56)	4 (25)	
	Peritoneum	4 (10)	4 (16)	0 (0)	
	Lungs	11 (27)	7 (28)	4 (25)	
	Bones	1 (2)	1 (4)	0 (0)	
Cycles of ADJ-GEM	Median (range)	6 (3–9)	6 (3–6)	6 (3–9)	0.88
ADJ-Rec[j] (months)	Median (range)	3.7(0.1–36.1)	1.3 (0.1–4.9)	11.5 (6.3–36.1)	
Chemotherapy[k]	GEM	21 (51)	6 (24)	15 (94)	0.00
	Alternatives[l]	20 (49)	19 (76)	1 (6)	
	(S1)	17 (41)	17 (68)	1 (6)	
	(GEM + S1)	1 (2)	0 (0)	0 (0)	
	(S1 + Radiation)	1 (2)	1 (4)	0 (0)	
	(S1 + oxaliplatin)	1 (2)	1 (4)	0 (0)	

[a] PS, performance status.
[b] PD, pancreaticoduodenectomy.
[c] DP, distal pancreatectomy.
[d] TP, total pancreatectomy.
[e] Stage, UICC 7th.
[f] CEA, carcinoembryonic antigen at resection.
[g] CA-19-9, carbohydrate antigen 19-9 at resection.
[h] Lymph node ratio, number of metastatic lymph nodes divided by number of examined nodes.
[i] Recurrent pattern, numbers of locoregional, extra-pancreatic, and combined recurrences were 11, 20, and 10 patients.
[j] ADJ-Rec, period between the last date of ADJ-GEM and recurrence.
[k] Chemotherapy, chemotherapy for recurrent disease after adjuvant chemotherapy.
[l] Alternatives, all alternative regimens consisted of fluoropyrimidine-containing regimens.

NCCN guidelines for recurrent pancreatic cancer after adjuvant chemotherapy, which recommend that the treatment options should be determined by the period between the last date of ADJ-GEM and recurrence (ADJ-Rec), with a threshold of 6 months.

2. Patients and methods

2.1. Patients

A retrospective review was conducted for 113 pancreatic cancer patients who underwent curative resection followed by ADJ-GEM at the National Cancer Center Hospital (NCCH) and NCCH East in Japan between April 2002 and October 2010. Forty-two patients with no recurrence after ADJ-GEM, 10 patients with withdrawal from ADJ-GEM within 2 cycles, 6 patients with recurrence during ADJ-GEM, and 14 patients who changed hospitals after recurrence were excluded. We finally retrieved the clinical data of 41 patients with recurrences who were subsequently treated with chemotherapy at our hospitals.

2.2. Treatment

After resection, we started ADJ-GEM within 10 weeks. An initial gemcitabine dose of 1000 mg/m^2 was administrated intravenously for 30 min on days 1, 8 and 15 every 4 weeks for 3 to 6 cycles, in principle. A computed tomography examination was performed every 3–6 months. Once evidence of recurrence was revealed, treatment for recurrent disease was initiated.

2.3. Data collection and evaluation of tumor response

The following data were collected from the medical records: patient characteristics at resection, the resection status, the ADJ-Rec, the treatment regimen, and the outcome of treatment after the recurrence. We also compared the treatment outcomes according to the length of the ADJ-Rec and the treatment regimens. Tumor responses were evaluated according to the RECIST criteria, Ver.1.1. We evaluated the best overall response and the disease control rate (DCR). The DCR was defined as the rate of complete response + partial response + stable disease. When the disease status was stably maintained for more than 8 weeks, the patient was considered to have stable disease.

2.4. Statistical analysis

The Fisher exact test was used to assess the hypothesis of independence between categorical variables. For quantitative data such as age and the carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) levels, we used the Mann–Whiney test. ADJ-Rec was defined as the period between the last date of the administration of ADJ-GEM and the date on which local or distant recurrence was noted. The date of recurrence was defined as the date of documentation of recurrent disease using diagnostic imaging techniques. Progression-free survival (PFS) was defined as the period between the start of treatment for recurrent disease and the date of progression, the last follow-up visit, or death from any cause. Overall survival after recurrence (r-OS) was defined as the period between the start of treatment for recurrent disease and death from any cause or the last follow-up. Patients who were lost to follow-up were treated as censored cases. Survival curves were estimated using the Kaplan–Meier method, and the significances were evaluated using a log-rank test. All the analyses were performed using Stata/SE, Version 11.1 (StataCorp, USA).

3. Results

3.1. Patient characteristics

The characteristics at resection of the 41 eligible patients are listed in Table 1. R0 resection (complete resection with no microscopic residual tumor) was performed in 36 patients (88%). Concerning the pathological stage, 5 (12%) of the patients had stage IIA disease and 36 (88%) had stage IIB. The sites of recurrence were locoregional (21 patients), the liver (18 patients), and the lung (11 patients). Patients with an ADJ-Rec $\geq$ 6 months (16 patients) had a significantly better status than patients with an ADJ-Rec < 6 months (25 patients) with regard to disease stage ($P = 0.006$) and the lymph node ratio (the number of metastatic lymph nodes divided by the number of examined nodes) ($P = 0.0075$). As for the treatments for recurrent disease, 21 patients were treated with gemcitabine monotherapy and 20 patients were treated with alternative regimens. All the alternative regimens were fluoropyrimidine-containing regimens (17 patients received S-1 and 1 patient each received GEM + S-1, S-1 + radiation, and S-1 + oxaliplatin). The treatment strategy after recurrence depended on each oncologist's plan, without a unified policy. Among the 25 patients with an ADJ-Rec < 6 months, 6 were treated with gemcitabine monotherapy and 19 were treated with alternative regimens. Among the 16 patients with an ADJ-Rec $\geq$ 6 months, 15 were treated with gemcitabine monotherapy and 1 was treated with an alternative regimen.

3.2. Treatment efficacy and survival analysis of treatments for recurrence

Overall, 2 of the 41 patients responded to the treatments for recurrent disease (4.9%; 2 partial responses; 95% confidence interval (95% CI), 0.60%–16.53%). The DCR was 78% (32 of the 41 patients; 95% CI, 62.39%–89.44%). The median PFS and median r-OS were 5.5 months (95% CI, 3.7–8.1 months) and 18.3 months (95% CI, 13–19.8 months), respectively (Fig. 1).

We divided the patients into two groups according to the length of the ADJ-Rec: patients with an ADJ-Rec < 6 months ($n = 25$), and patients with an ADJ-Rec $\geq$ 6 months ($n = 16$). The DCRs were 68% and 94% ($P = 0.066$), and the median PFS periods were 5.5 and 8.2 months ($P = 0.186$; Fig. 2A), respectively. The median r-OS of the patients with an ADJ-Rec < 6 months was significantly shorter than

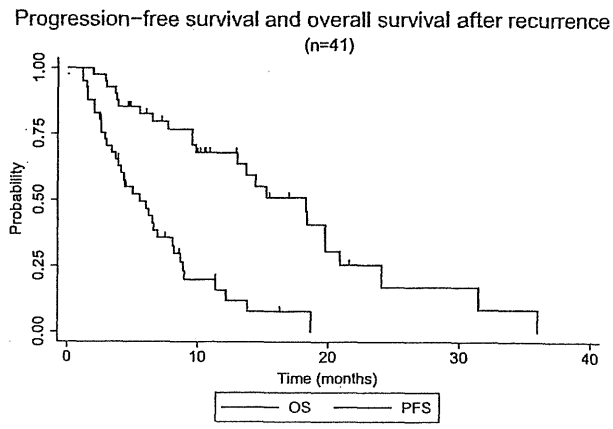

Fig. 1. Progression-free survival (PFS) and overall survival after recurrence (r-OS) in all patients ($n = 41$). The median PFS and r-OS were 5.5 and 18.3 months, respectively.

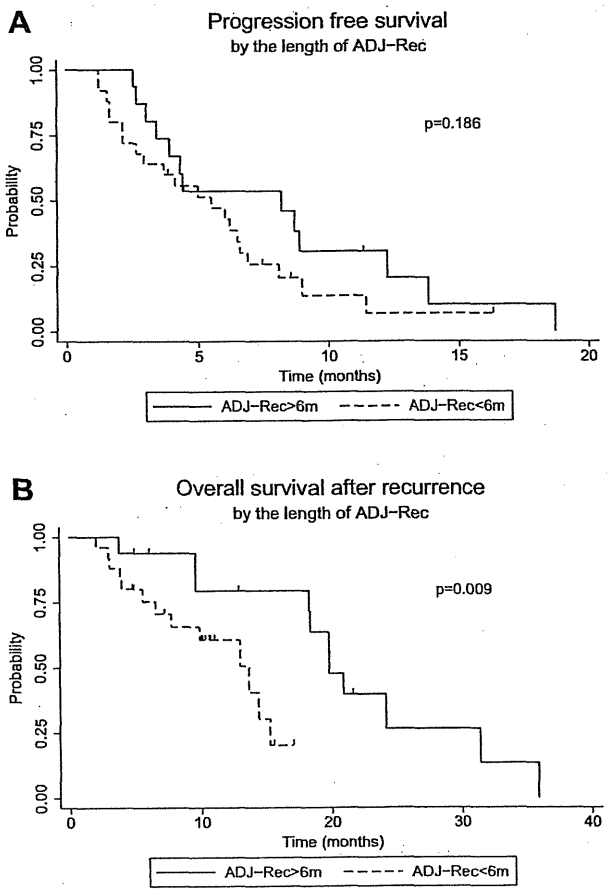

Fig. 2. Progression-free survival (PFS) and overall survival after recurrence (r-OS) according to the length of the ADJ-Rec: patients with an ADJ-Rec < 6 months ($n = 25$), and patients with an ADJ-Rec ≥ 6 months ($n = 16$). (A) The median PFS for each group was 5.5 and 8.2 months ($P = 0.186$), respectively. (B) The median r-OS was 13.7 and 19.8 months ($P = 0.009$), respectively.

that of the patients with an ADJ-Rec ≥ 6 months (13.7 and 19.8 months, $P = 0.009$; Fig. 2B).

Additionally, we divided the patients with an ADJ-Rec < 6 months into two groups according to the treatment regimens for recurrent disease: patients treated with gemcitabine ($n = 6$) and patients treated with alternative regimens ($n = 19$). The outcomes are shown in Table 2 and Fig. 3. For the patients treated with gemcitabine and those treated with alternative regimens, the DCR, median PFS and median r-OS were 67% and 68% ($P = 0.651$), 2.9 and 6.5 months ($P = 0.065$; Fig. 3A), and 7.7 and 13.0 months ($P = 0.242$; Fig. 3B), respectively.

4. Discussion

In this study, at first we examined the current status of the treatment strategy for pancreatic cancer patients with recurrence after adjuvant chemotherapy. Most patients with ADJ-Rec ≥ 6 months were placed on gemcitabine. Even for patients with an ADJ-Rec < 6 months, gemcitabine was resumed in 24% of these patients. Generally, patients who relapse within a short period after receiving adjuvant chemotherapy should be considered as being resistant to those drugs. The NCCN guidelines also recommend that the options for recurrent disease after adjuvant therapy should be assessed according to the ADJ-Rec. However, these guidelines are only the recommendation of the panel, and these strategies have not yet been substantiated by actual clinical data. In the case of ovarian cancer, a consensus based on actual clinical data exists with regard to the treatment strategy for relapsed disease. Patients who have relapsed within an interval of less than 6 months since the previous paclitaxel-plus-platinum chemotherapy should be considered as platinum resistant [12,13]. However, the chemosensitivity and the key drugs are quite different between pancreatic cancer and ovarian cancer. Therefore, actual clinical data for pancreatic cancer is needed.

The outcome of patients with a short ADJ-Rec was worse than that of the patients with a long ADJ-Rec. This finding suggests that patients with a long ADJ-Rec may owe their period of prolonged sensitivity to the adjuvant gemcitabine treatment, slow tumor growth, and a smaller quantity of residual tumor. Concerning advanced pancreatic cancer, similar findings have been reported in a previous study, which indicated that the progression-free survival period after first-line chemotherapy was an independent prognostic factor [14]. Additionally, patients with pathological stage IIA or a lymph node ratio of 0 had a long ADJ-Rec in the present study, possibly influencing the outcome. However, our results should be interpreted with caution because biases introduced by the different selection of treatment regimens between the two groups may exist.

Among the patients with an ADJ-Rec ≥ 6 months, we were unable to compare the treatment outcome according to regimens, since most of them (15 out of 16) received gemcitabine monotherapy and seldom received alternative options such a fluoropyrimidine-based regimens. In the present study, the patients treated with gemcitabine had a better DCR, PFS and r-OS than the metastatic or recurrent pancreatic cancer patients treated with gemcitabine in past studies [15,16]. Even after considering the possibility that an ADJ-Rec ≥ 6 months may be a good prognostic factor, these preferable outcomes suggest the appropriateness of a re-challenge with gemcitabine.

Among the patients with an ADJ-Rec < 6 months, patients receiving alternative regimens tended to have a better DCR, PFS,

Table 2
Outcomes of patients according to ADJ-Rec and treatment regimens.

	<6 months				≥6 months			
ADJ-Rec	All	GEM	Alternative	*P* value	All	GEM	Alternative	*P* value
n	25	6	19		16	15	1	
DCR (%)	68	67	68	1.00	94	93	(100)	1.00
95% CI	62.4–89.4	22.3–95.7	43.5–87.4		69.8–99.8	68.1–99.8	2.5–100	
Median PFS (m)	5.5	2.9	6.5	0.06	8.2	8.2	(12.2)	0.69
95% CI	2.6–6.6	1.5–	2.1–8.1		3.4–12.2	3.0–13.8		
Median r-OS(m)	13.7	7.7	13.0	0.24	19.8	20.9	(19.8)	0.67
95% CI	6.5–15.3	2.9–	6.5–		9.6–31.4	9.6–31.4		

ADJ-Rec, period between the last date of ADJ-GEM and recurrence; DCR, disease control rate; PFS, progression-free survival time; r-OS, survival time from recurrence; Alternative*, including S-1, GEM + S-1, S-1 + radiation, and S-1 + oxaliplatin.

and r-OS than those receiving gemcitabine monotherapy. Although the optimal ADJ-Rec threshold was not clarified, the present results support the recommendations of the NCCN guidelines, which recommend alternative regimens for patients with an ADJ-Rec < 6 months after previous treatment with gemcitabine. These findings suggest that a certain proportion of patients with a short ADJ-Rec may already have a gemcitabine-refractory status at the time of ADJ-GEM.

This study had some limitations. This study was a retrospective analysis with an insufficient sample size, and the treatment strategy after recurrence depended on each oncologist's plan, with no unified policy. Another limitation concerns the alternative treatment options after recurrence. The NCCN guidelines recommend alternative regimens as second-line therapies for metastatic disease. The recommended regimens consist of fluoropyrimidine-based therapies, such as 5-FU/leucovorin (LV)/oxaliplatin (Oxal) [17] or capecitabine/Oxal [18]. The CONKO-003 study revealed the survival advantage of 5-FU + LV + Oxal for gemcitabine-refractory pancreatic cancer. In Japan, these drugs have not yet been approved under the Japanese medical insurance system for the treatment of pancreatic cancer. S-1 monotherapy was mainly used as the alternative option in our study. Although S-1 demonstrated a non-inferiority to gemcitabine as a first-line treatment [8,9] and had a marginal activity as a second-line regimen for gemcitabine-refractory pancreatic cancer [10,11], it has not been accepted as a global standard therapy for gemcitabine-refractory pancreatic cancer.

In conclusion, patients with an ADJ-Rec ≥ 6 months had a relatively favorable outcome when treated with a gemcitabine rechallenge. Among the patients with an ADJ-Rec < 6 months, those patients receiving alternative regimens tended to have a better DCR, PFS, and r-OS, compared with those receiving gemcitabine. As a result, our results did not deny the appropriateness of strategies outline in the NCCN guidelines. A well-designed prospective study with a sufficient sample size is needed to identify the optimal regimen for the treatment of recurrent pancreatic cancer after postoperative adjuvant chemotherapy.

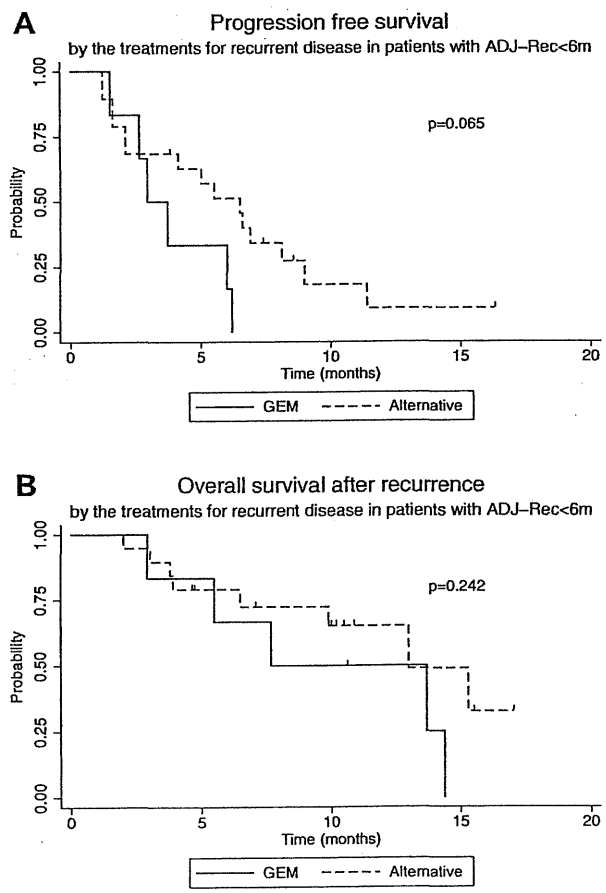

Fig. 3. Progression-free survival (PFS) and overall survival after recurrence (r-OS) according to treatments for recurrent disease in patients with an ADJ-Rec < 6 months: patients treated with gemcitabine ($n = 6$), and patients treated with alternative regimens ($n = 19$). (A) The median PFS for each group was 2.9 and 6.5 months ($P = 0.065$), respectively. (B) The median r-OS was 7.7 and 13.0 months ($P = 0.242$), respectively.

Grant support

None declared.

Conflict of interest

Takuji Okusaka had research findings and honoraria to disclose from Taiho pharmaceutical co. and Eli Lilly Japan.

Hideki Ueno had honoraria to disclose from Taiho pharmaceutical co. and Eli Lilly Japan, and had a consultation or advisory relationship to disclose from Taiho phatmaceutical co.

Tomoo Kosuge had honoraria to disclose from Taiho pharmaceutical co. and Eli Lilly Japan.

References

[1] Yeo Charles J, Cameron JL, Lillemoe KD. Pancreaticoduodenectomy for cancer of the head of the pancreas. Ann Surg 1995;221:721–33.
[2] Oettle H, Post S, Neuhaus P, Gellert K, Langrehr J, Ridwelski K, et al. Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. JAMA 2007;297:267–77.
[3] Kalser MH, Ellenberg SS. Pancreatic cancer. Adjuvant combined radiation and chemotherapy following curative resection. Arch Surg 1985;120:899–903.
[4] Neoptolemos JP, Stocken DD, Friess H, Bassi C, Dunn JA, Hickey H, et al. A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. N Engl J Med 2004;350:1200–10.
[5] Ueno H, Kosuge T, Matsuyama Y, Yamamoto J, Nakao A, Egawa S, et al. A randomised phase III trial comparing gemcitabine with surgery-only in patients with resected pancreatic cancer: Japanese study group of adjuvant therapy for pancreatic cancer. Br J Cancer 2009;101:908–15.
[6] Regine WF, Winter KA, Abrams RA, Safran H, Hoffman JP, Konski A, et al. Fluorouracil vs gemcitabine chemotherapy before and after fluorouracil-based chemoradiation following resection of pancreatic adenocarcinoma: a randomized controlled trial. JAMA 2008;299:1019–26.
[7] Riess H, Neuhaus P, Post S, Gellert K, Ridwelski K, Schramm H, et al. Conko-001: final results of the randomized, prospective, multicenter phase III trial of adjuvant chemotherapy with gemcitabine versus observation in patients with resected pancreatic cancer (PC). Ann Oncol 2008;19:45–6.
[8] NCCN clinical practice guidelines in oncology (NCCN guidelines®). Available at: http://www.nccn.org/professionals/physician_gls/f_guidelines.asp; December 7, 2011 [Accessed February 2012].
[9] Ioka T, Ikeda M, Ohkawa S, Yanagimoto H, Fukutomi A, Sugimori K, et al. Randomized phase III study of gemcitabine plus S-1 (GS) versus S-1 versus gemcitabine (GEM) in unresectable advanced pancreatic cancer (PC) in Japan and Taiwan: GEST study. J Clin Oncol 2011;29(suppl.). abstr 4007.
[10] Morizane C, Okusaka T, Furuse J, Ishii H, Ueno H, Ikeda M, et al. A phase II study of S-1 in gemcitabine-refractory metastatic pancreatic cancer. Cancer Chemother Pharmacol 2009;63:313–9.
[11] Sudo K, Yamaguchi T, Nakamura K, Denda T, Hara T, Ishihara T, et al. Phase II study of S-1 in patients with gemcitabine-resistant advanced pancreatic cancer. Cancer Chemother Pharmacol 2011;67:249–54.
[12] Harries M, Gore M. Part II: chemotherapy for epithelial ovarian cancer-treatment of recurrent disease. Lancet Oncol 2002;3:537–45.
[13] Parmar MK, Ledermann JA, Colombo N, du Bois A, Delaloye JF, Kristensen GB, et al. Paclitaxel plus platinum-based chemotherapy versus conventional platinum-based chemotherapy in women with relapsed ovarian cancer: the ICON4/AGO-OVAR-2.2 trial. Lancet 2003;361:2099–106.
[14] Reni M, Berardi R, Mambrini A, Pasetto L, Cereda S, Ferrari VD, et al. A multicentre retrospective review of second-line therapy in advanced pancreatic adenocarcinoma. Cancer Chemother Pharmacol 2008;62:673–8.
[15] Van Cutsem E, van de Velde H, Karasek P, Oettle H, Vervenne WL, Szawlowski A, et al. Phase III trial of gemcitabine plus tipifarnib compared

with gemcitabine plus placebo in advanced pancreatic cancer. J Clin Oncol 2004;22:1430–8.
[16] Hashimoto K, Ueno H, Ikeda M, Kojima Y, Hagihara A, Kondo S, et al. Do recurrent and metastatic pancreatic cancer patients have the same outcomes with gemcitabine treatment? Oncology 2009;77: 217–23.
[17] Pelzer U, Stieler J, Schwaner I, Heil G, Seraphin J, Gorner M, et al. Results of the conko 003 trial. A randomized second line trial in patients with gemcitabine refractory advanced pancreatic cancer. Onkologie 2008;31:98.
[18] Xiong HQ, Varadhachary GR, Blais JC, Hess KR, Abbruzzese JL, Wolff RA. Phase 2 trial of oxaliplatin plus capecitabine (XELOX) as second-line therapy for patients with advanced pancreatic cancer. Cancer 2008;113:2046–52.

Cancer Science
The official journal of the Japanese Cancer Association JCA

Impact of tumor-associated macrophages on invasive ductal carcinoma of the pancreas head

Kiyoshi Yoshikawa,[1,2] Shuichi Mitsunaga,[1,3] Taira Kinoshita,[2] Masaru Konishi,[2] Shinichiro Takahashi,[2] Naoto Gotohda,[2] Yuichiro Kato,[2] Masaki Aizawa[2] and Atsushi Ochiai[1,4]

[1]Pathology Division, Research Center for Innovative Oncology; [2]Department of Hepato-Biliary and Pancreatic Surgery; [3]Division of Hepatobiliary Pancreatic Oncology, National Cancer Center Hospital East, Kashiwa, Japan

(Received February 25, 2012/Revised July 4, 2012/Accepted July 18, 2012/Accepted manuscript online August 30, 2012/Article first published online October 4, 2012)

Tumor-associated macrophages (TAMs) are candidate histological factors in invasive ductal carcinoma (IDC) of the pancreas. Tumor-associated macrophages can be affected by cancer-related inflammation and pancreatitis and interact with important invasive behavior in a recurrent manner in pancreatic IDC. These features may help elucidate the aggressiveness of pancreatic IDC. The aim of this study was to characterize TAMs in pancreatic IDC in comparison with chronic pancreatitis (CP) and to reveal TAM-related factors and the clinical impact of TAMs. CD68 (a pan-macrophage marker) and CD204 (an M2 macrophage marker) immunohistochemistry was carried out in pancreas head specimens from 107 IDC cases and 11 CP cases. Immunopositive cell areas were calculated at the periphery and center of the tumor. The distributions of macrophages in IDC and CP and the relationship between TAMs and histological tumor factors, survival, and recurrence were evaluated. Macrophages were more frequently observed in the lesion periphery than the center in IDC and CP. The density of macrophages was elevated in IDC compared to CP. Dense M2 macrophages at the tumor periphery were frequently seen in large tumors and showed an independent impact on overall survival and disease-free time. Early recurrence in the liver or the local manipulated area was associated with high accumulation of peripheral M2 macrophages. More M2 macrophages were seen in IDC than in CP in both the periphery and the center. High numbers of peripheral M2 macrophages were associated with large tumor size, early recurrence in the liver, local recurrence, and shortened survival time in patients with pancreatic IDC. (*Cancer Sci* 2012; 103: 2012–2020)

The prognosis of patients undergoing resection for pancreatic invasive ductal carcinoma (IDC) remains poor.[1–5] Histological studies have been carried out to elucidate the aggressiveness of pancreatic IDC and have revealed prognostic factors including tumor size, lymph node involvement, nerve plexus invasion, positive resected margin, and low tumor grade.[1–8] Tumor-associated macrophages (TAMs) have recently been reported as a candidate factor in poor prognosis.[9]

Macrophages are the most abundant cancer stromal cells involved in the host immune system,[10] and TAMs have been found to play important roles in tumorigenesis, angiogenesis, matrix remodeling, and metastasis.[11–13] Tumor-associated macrophages have a prognostic impact in prostate, breast, and lung cancers, as well as pancreatic IDC.[9,14–16] The heterogeneity of macrophages has been discussed with regard to their different responses to various microenvironmental stimuli. Macrophages are classically activated towards the M1 phenotype by lipopolysaccharide and interferon-γ. M1 macrophages are characterized by high expression of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, IL-12, and tumor necrosis factor. Alternatively, macrophages are activated towards the M2 phenotype by IL-4, IL-13, and IL-10. M2 macrophages are characterized by high expression of IL-4 and IL-10 and low expression of IL-12.[12] Recent studies have revealed high CD204 expression in M2 macrophages and have shown that TAMs are polarized to the M2 phenotype.[12,17,18]

The distribution of TAMs was recently evaluated as a prognostic index in various cancers. A high number of TAMs in the peripheral area of the tumor is correlated with poor prognosis in gastric cancer,[19] hepatocellular carcinoma,[20] and non-small-cell lung cancer,[21] although an increased number of TAMs in the invasive front of colon cancer is associated with favorable prognosis.[22] Increased numbers of TAMs in many cancers are linked to reduced patient survival. In pancreatic IDC, high accumulation of TAMs in the periphery of the tumor is correlated with extrapancreatic invasion, lymph vessel invasion, lymph node involvement, and shortened survival time.[9] Tumor-associated macrophages may be a key to elucidating the aggressiveness of pancreatic IDC. Detailed clinicopathological studies should be carried out to estimate the role of TAMs. First, the distribution of macrophages should be compared between mass-forming chronic pancreatitis (CP) and pancreatic IDC. Macrophages accumulate at the inflammatory site and play crucial roles in the diverse phase.[23,24] Pancreatitis is prevalent in pancreatic IDC and CP due to obstruction of the main pancreatic duct.[25] Tumor-associated macrophages in pancreatic IDC can be affected by both pancreatitis and inflammatory mediators from tumor cells; macrophages in CP are affected by pancreatitis only. The comparison of macrophages between pancreatic IDC and CP may provide evidence that tumor cells mainly lead to TAM accumulation in pancreatic IDC. Second, TAM-related tumor factors should be examined in detail. Tumor-associated macrophages are attracted to and retained in avascular and necrotic areas where they are exposed to tumor hypoxia.[26,27] Our previous clinicopathological study showed that tumor necrosis is frequent in large tumors.[7] Tumor size may be associated with TAM accumulation. Identification of the precise TAM-related tumor factors is useful for estimating microenvironmental interactions between TAMs and pancreatic IDC. Third, the impact of TAMs on tumor relapse should be evaluated. The prognostic value of TAMs may indicate that TAMs are predictive markers of recurrence. The impact of TAMs on recurrence will reinforce the clinical significance of TAMs. Finally, multivariate analysis should be carried out to confirm the impact of TAMs on prognosis. The prognostic value of TAMs has only been tested with univariate analysis. Establishment of the prognostic importance needs to show independence among various tumor factors with multivariate analysis.

The aim of this study was to characterize TAMs in pancreatic IDC in comparison with CP and to reveal TAM-related

[4]To whom correspondence should be addressed.
E-mail: aochiai@east.ncc.go.jp

doi: 10.1111/j.1349-7006.2012.02411.x

Table 1. Characteristics of patients who underwent pancreaticoduodenectomy with curative intent for a pancreatic head tumor

Parameter	Invasive ductal carcinoma	Chronic pancreatitis
Number	107	11
Age (years), median (range)	64.0 (37–82)	52.0 (38–72)
Gender (male/female)	64/44	10/1
CEA (ng/mL), median (range)	3.5 (0.8–60.3)	3 (0.9–15.7)
CA19-9 (U/mL) (median, range)	109.0 (1.0–21400.0)	14.0 (5.0–245.8)
Combined resection (portal vein/inferior vena cava/colon/liver)	51/2/2/2	0
Intraoperative radiotherapy	30	0
Adjuvant chemotherapy	10 (GEM:8, S-1:2)	0
Stage (UICC 6th) (IA/IB/IIA/IIB/III/IV)	0/0/19/79/1/8	

S-1, an oral anti-cancer drug that combines tegafur, a prodrug of fluorouracil, with 5-chloro-2,4-dihydropyrimidine and potassium oxonate in a molar ratio of 1.0:0.4:1.0 (Taiho Pharmaceutical, Tokyo, Japan). CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; GEM, gemcitabine.

factors and the clinical impact of TAMs on tumor relapse and prognosis.

Materials and Methods

Patients. Between September 1992 and December 2007, 116 patients with a pathological diagnosis of pancreatic IDC who underwent a pancreaticoduodenectomy with curative intent at our institution were investigated, because pancreatitis due to obstruction of the main pancreatic duct is evident in the pancreatic head lesions of IDC and CP cases. Three in-hospital deaths, two patients with incomplete follow-up data, two patients who died of non-cancerous causes within 5 years of the pancreaticoduodenectomy (one due to liver cirrhosis and one due to brain infarction), and two patients whose surgical specimens were of poor quality were excluded from the study. The remaining 107 patients were investigated. For the CP cases in this study, 11 patients who underwent pancreaticoduodenectomy during the same period and were pathologically diagnosed with CP were assessed. Chronic pancreatitis was diagnosed according to The Revised Japanese Clinical Diagnostic Criteria for Chronic Pancreatitis.[28] All CP cases showed fibrosis that was distributed primarily in the interlobular spaces, showing a nodular pattern of lobules called cirrhosis due to the disruption of dense interlobular fibrosis or the loss of exocrine parenchyma with irregular fibrosis. All patients signed an institutional review board-approved informed consent form. The median age of the IDC patients was 64.0 years (range, 37–82 years), and 44 were women (41.1%). The median age of the CP patients was 52.0 years (range, 38–72 years), and 1 (9.1%) was a woman (Table 1). None of the 107 IDC patients received neoadjuvant chemotherapy or radiotherapy; 30 received intraoperative radiotherapy,[29] and 10 received adjuvant chemotherapy. Extended lymphadenectomy including regional and peripancreatic lymph node dissection was carried out with pancreaticoduodenectomy, according to the Japanese Classification of Pancreatic Cancer.[30] Combined resection of the portal vein, inferior vena cava, colon, and para-aortic lymph node was carried out for macroscopically curative resection.

To assess initial recurrence of the tumor, follow-up contrast computed tomography was done every 3 months after surgery or earlier if clinically indicated by examination, symptoms, or a rise in tumor markers, such as serum carcinoembryonic antigen and serum carbohydrate antigen 19-9, which were checked every month. If necessary, further examination such as cytology was carried out to diagnose peritoneal dissemination.

Evaluation of clinicopathological features. Clinical characteristics and pathological examination results were retrieved from the clinical records. Lymphatic (ly), venous (v), and intrapancreatic nerve invasion (ne) were classified into four groups according to the definition of the Japan Pancreas Society and were based on the most extensively involved area observed

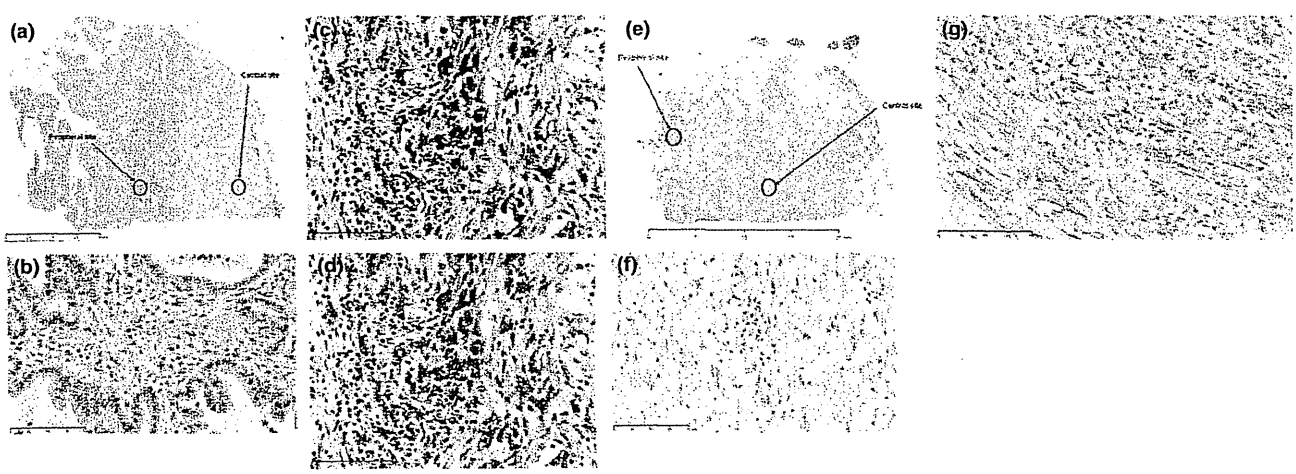

Fig. 1. Objective measurement of the area ratio of immunopositive cells. (a) Using the section showing the maximum diameter of the invasive ductal carcinoma tumor that was stained with anti-CD204, hot spots in the center and the periphery of the tumor were observed at a magnification of ×40. Center (b) and periphery (c) of invasive ductal carcinoma of the pancreas (magnification, ×400). We measured the area of immunopositive cell bodies at this magnification using the Automeasure function of Axio Vision 4.7.1. Axio Vision software visualized the CD204-positive area as red-colored areas (d) and objectively calculated the positive area ratio (summed area of immunopositive cells/measured area). CD204 expression in chronic pancreatitis (CP) tumors was measured using the section with the maximum diameter of the CP tumor (e–g). In the entire image of the CD204-stained CP section (E; magnification, ×40), hot spots of CD204 expression at the central and the peripheral sites of the CP (f, g; magnification ×400) were selected. The positive area ratio of CD204 in the selected image was objectively calculated with Axio Vision software.

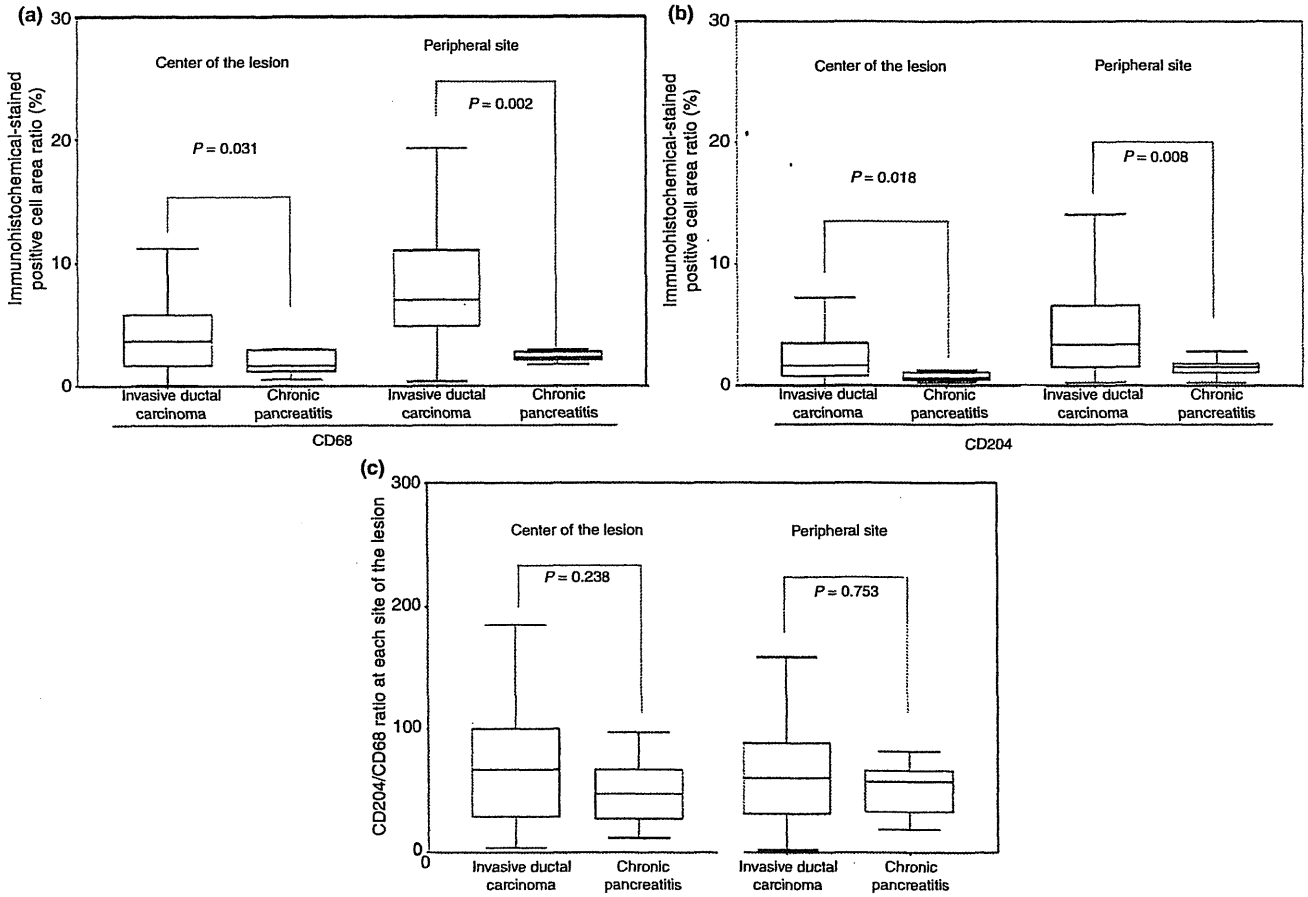

Fig. 2. Distribution of CD68- and CD204-positive cells at the center and periphery of the lesion in invasive ductal carcinoma and chronic pancreatitis. In each site of the lesion and for each immunohistochemical stain, significantly more immunopositive cells were observed in invasive ductal carcinoma than in chronic pancreatitis. (a,b) The CD204/CD68 ratio does not show a significant difference between these two types of lesions (c).

under low-power magnification (×100): 0, no invasion of cancer cells; (i) invasion of a few cancer cells (1–3 points); (ii) moderate invasion of cancer cells (4–8 points); and (iii) marked invasion of multiple cancer cells (>8 points).[30]

The following clinicopathological factors were investigated retrospectively to assess their impact on survival: age ($\leq$64 years *vs* >64 years); sex; serum carcinoembryonic antigen ($\leq$3.5 ng/mL *vs* >3.5 ng/mL), serum carbohydrate antigen 19-9 ($\leq$109 U/mL *vs* >109 U/mL); grade of tumor differentiation (well *vs* moderate or poor); tumor size ($\leq$3 cm *vs* >3 cm); serosal invasion (absent *vs* present); retropancreatic tissue invasion (absent *vs* present); portal vein invasion (absent *vs* present); lymphatic invasion (ly0, 1 *vs* ly2, 3); venous invasion (v0, 1 *vs* v2, 3); intrapancreatic nerve invasion (ne0, 1 *vs* ne2, 3); extrapancreatic nerve plexus invasion (absent *vs* present); and lymph node involvement (absent *vs* present).

Antibodies and immunohistochemistry. Paraffin-embedded blocks of tumor at the maximum diameter were cut into 3-μm serial sections. The sections were deparaffinized in xylene, dehydrated in a graded ethanol series, and immersed in 0.3% hydrogen peroxide in methanol for 15 min to inhibit endogenous peroxidase activity. For antigen retrieval, the slides were heated at 95°C for 15 min in a microwave oven (H2800 Microwave Processor; Energy Beam Sciences, East Granby, CT, USA) in 0.1 M citric acid buffer then allowed to cool for 1 h at room temperature. After washing the slides three times in PBS, non-specific binding was blocked by pre-incubating in 2% normal swine serum in PBS (blocking buffer) for 30 min at room temperature. Individual slides were then incubated overnight at 4°C in mouse anti-human CD68 antibody (1:400 in blocking buffer; Dako, Glostrup, Denmark) or mouse anti-human CD204 antibody (Scavenger Receptor class A-E5, 1:400 in blocking buffer; Transgenic, Kumamoto, Japan). The slides were again washed three times with PBS and incubated with EnVision (Dako) for 1 h at room temperature. After extensive washing with PBS, the color reaction was developed with 2% 3, 3'-diaminobenzidine in 50 mM Tris-buffer (pH 7.6) containing 0.3% hydrogen peroxide. The sections were then counterstained with Mayer's hematoxylin, dehydrated, and mounted.

Definition of center of lesion and peripheral site. To identify the center of the lesion, H&E stained sections were scanned at a magnification of ×40, and the margin of the tumor was marked on each slide. The intersection of the major and minor axes was defined as the center of the lesion, and four fields including the center at a magnification of ×100 were defined as the center of the lesion. Peripheral sites were defined as fields that included cancer cells and adjacent non-cancerous cells at a magnification of ×100. In the pancreatitis cases, the same procedure was used to identify the center and the margin of the dense fibrosing area.

Evaluation of immunohistochemistry (IHC). The IHC-positive cells were quantified by determining the percentage of IHC-positive cells in an area (IHC%) and the IHC-positive cell

doi: 10.1111/j.1349-7006.2012.02411.x

Table 2. Distribution of the percentage of the CD68-positive cell area at the center and periphery of lesions in pancreatic tumors according to clinicopathological features

Parameter	Category	*n*	Central CD68%, median (range)	*P*	Peripheral CD68%, median (range)	*P*
Age (years)	≤64	58	3.75 (0.22–18.60)	0.574	6.25 (0.47–18.70)	0.422
	>64	49	3.63 (0.22–16.40)		7.58 (0.37–25.10)	
Gender	Male	63	3.47 (0.22–18.60)	0.582	6.54 (0.47–25.10)	0.695
	Female	44	3.96 (0.22–17.80)		7.26 (0.37–18.40)	
CEA (ng/mL)	≤3.5	57	3.64 (0.22–11.20)	0.980	6.92 (0.86–25.10)	0.450
	>3.5	50	3.82 (0.22–18.60)		6.76 (0.37–23.20)	
CA19-9 (U/mL)	≤109	53	3.86 (0.22–18.60)	0.815	6.19 (0.47–23.20)	0.108
	>109	54	3.64 (0.42–17.80)		7.50 (0.37–25.20)	
Differentiation	Well	31	3.88 (0.22–0.42)	0.752	7.97 (0.86–23.20)	0.374
	Moderate/Poor	76	3.64 (0.42–18.60)		6.34 (0.37–25.10)	
Tumor size (cm)	≤3.0	66	3.72 (0.22–18.60)	0.414	6.20 (0.69–25.10)	0.526
	>3.0	41	3.25 (0.42–17.80)		7.42 (0.37–18.70)	
Serosal invasion	Absent	84	3.65 (0.22–18.60)	0.554	6.49 (0.37–25.10)	0.451
	Present	23	3.86 (0.22–17.80)		7.42 (0.93–18.40)	
Retroperitoneal invasion	Absent	9	3.86 (0.47–12.10)	0.556	8.39 (0.37–14.30)	0.827
	Present	98	3.65 (0.22–18.60)		6.54 (0.47–25.10)	
Lymphatic invasion	ly0/1	60	3.80 (0.22–18.60)	0.660	6.25 (0.47–23.20)	0.332
	ly2/3	47	3.47 (0.44–17.80)		7.59 (0.37–25.10)	
Vessel invasion	v0/1	10	3.49 (0.64–12.10)	0.822	6.75 (1.56–16.60)	0.756
	v2/3	97	3.65 (0.22–18.60)		6.92 (0.37–25.10)	
Intrapancreatic nerve invasion	ne0/1	27	3.78 (0.22–16.40)	0.917	7.09 (0.93–25.20)	0.346
	ne2/3	80	3.65 (0.22–18.60)		6.92 (0.37–25.10)	
Extrapancreatic nerve	Absent	48	3.72 (0.22–18.60)	0.975	7.06 (0.69–25.10)	0.643
Plexus invasion	Present	59	3.64 (0.22–17.80)		6.22 (0.37–23.20)	
Portal vein invasion	Absent	81	3.56 (0.22–18.60)	0.437	7.37 (0.47–25.10)	0.079
	Present	26	3.92 (0.42–11.00)		5.92 (0.37–15.90)	
Lymph node involvement	Absent	22	1.82 (0.22–11.20)	0.091	5.45 (0.69–19.30)	0.045*
	Present	85	3.78 (0.22–18.60)		7.42 (0.37–25.10)	

*$P < 0.05$. Differences between the two groups were evaluated using the Mann–Whitney *U*-test. CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; ly, lymphatic; ne, intrapancreatic nerve; v, venous.

count, which was generally used to evaluate immunohistochemical staining.

Tumor-associated macrophages identified as CD68- or CD204-positive cells were defined as cells with oval to round nuclei that showed strong membranous/cytoplasmic staining but no nuclear staining. After scanning the immunostained slide at a magnification of ×100, the three areas with the greatest number of macrophages in both the center of the lesion and the peripheral site were selected as hot spots. The Automeasure function in Axio Vision 4.7.1 software (Carl Zeiss, Oberkochen, Germany) was used to distinguish the immunopositive area and to objectively calculate the summed areas of the immunopositive cells in each hot spot at a magnification of ×400. The IHC % (summed area of CD68- or CD204-positive cells/measured area ×100) was then calculated for each site (Fig. 1).

The number of macrophages was counted in three hot spots at ×400 magnification using a micrometer. The mean number of infiltrating macrophages was then calculated.

Statistical analysis. Correlations between IHC% and macrophage count for CD68 and CD204 in the center of the lesion and the peripheral sites were evaluated using Spearman's rank correlation coefficients. Differences in macrophage infiltration between the two groups were evaluated using the Mann–Whitney *U*-test. Overall survival time was calculated from the date of pancreaticoduodenectomy to August 24, 2010. Parameters that were significantly associated with disease-free survival (DFS) or overall survival rates evaluated in univariate analyses using log–rank tests were further analyzed with multivariate analysis using the Cox proportional hazard regression model. Crude overall survival curves were plotted using the Kaplan–Meier method. All *P*-values were two-sided, and the significance level was set at $P < 0.05$. All statistical analyses were carried out using the Statistical Package for the Social Sciences 11.5 J for Windows software (SPSS Inc., Chicago, IL, USA).

Results

Comparison of the area ratio of IHC-positive cells and IHC-positive macrophage count. To validate auto-measurement of IHC-positive cell areas, the correlation between IHC-positive cell numbers and IHC% was examined. The median CD68 count was 21.0 (range, 1.7–64.0) at the center and 42.0 (range, 13.3–94.3) at the periphery of the lesion. The median CD204 count was 14.0 (range, 0.3–48.3) at the center and 24.7 (range, 4.0–75.3) at the periphery. The CD68% and CD204% strongly correlated with the number of CD68- and CD204-positive cells at the center and the periphery of the tumor in pancreatic IDCs ($P < 0.001$, R [correlation coefficient] >0.4). To ensure objectivity, auto-measurement of the IHC% was used to quantify immunoreactivity in this study (Fig. S1).

Distribution of CD68- and CD204-positive cells in pancreatic IDC and CP. A series of 107 IDC specimens of the pancreas and 11 specimens of CP were examined for CD68 and CD204 expression in the center and periphery of the lesion. In the IDC series, the median CD68% was 3.65% (range, 0.05–18.6%) at the center of the lesion and 9.92% (range, 0.37–25.1%) at the periphery, whereas the median CD68% of the CP series was 1.62% (range, 0.55–6.20%) at the center of the lesion and 2.29% (range, 1.13–19.5%) at the periphery ($P = 0.031$ at the center, $P = 0.002$ at the periphery). The median CD204% was 1.64% (range, 0.06–18.1%) at the center of the lesion and

Table 3. Distribution of central and peripheral CD204-positive cell area ratios in pancreatic tumors according to clinicopathological features

Parameter	Category	*n*	Central CD204%, median (range)	*P*	Peripheral CD204%, median (range)	*P*
Age (years)	≤64	58	1.54 (0.06–18.10)	0.970	3.43 (0.34–12.80)	0.846
	>64	49	1.65 (0.22–9.010)		3.27 (0.27–14.00)	
Gender	Male	63	1.51 (0.06–9.310)	0.364	3.27 (0.27–14.00)	0.552
	Female	44	1.77 (0.19–18.10)		3.59 (0.43–14.00)	
CEA (ng/mL)	≤3.5	57	1.45 (0.10–11.90)	0.064	3.31 (0.27–14.00)	0.469
	>3.5	50	2.02 (0.06–18.10)		3.80 (0.34–14.00)	
CA19-9 (U/mL)	≤109	53	1.56 (0.10–9.31)	0.983	3.37 (0.27–14.00)	0.400
	>109	54	1.66 (0.06–18.10)		3.41 (0.34–14.00)	
Differentiation	Well	31	1.38 (0.06–18.10)	0.477	3.43 (0.27–14.00)	0.995
	Moderate/Poor	76	1.69 (0.10–9.31)		3.33 (0.44–14.00)	
Tumor size (cm)	≤3.0	66	1.45 (0.13–9.31)	0.110	3.10 (0.27–14.00)	0.031*
	>3.0	41	2.10 (0.06–18.10)		3.38 (0.34–12.70)	
Serosal invasion	Absent	84	1.66 (0.06–11.90)	0.797	3.34 (0.27–14.00)	0.575
	Present	23	1.34 (0.10–18.10)		4.23 (0.55–12.20)	
Retroperitoneal invasion	Absent	9	1.68 (0.22–8.84)	0.439	6.10 (1.21–11.80)	0.346
	Present	98	1.60 (0.06–18.10)		3.37 (0.27–14.00)	
Lymphatic invasion	ly0/1	60	1.45 (0.22–11.90)	0.201	3.10 (0.27–14.10)	0.151
	ly2/3	47	1.96 (0.06–18.10)		4.26 (0.34–12.80)	
Vessel invasion	v0/1	10	1.17 (0.19–7.98)	0.309	3.33 (0.43–7.18)	0.460
	v2/3	97	1.66 (0.06–18.10)		3.43 (0.27–14.00)	
Intrapancreatic nerve invasion	ne0/1	27	1.64 (0.43–9.31)	0.659	3.38 (0.43–11.80)	0.954
	ne2/3	80	1.64 (0.06–18.10)		3.40 (0.27–14.00)	
Extrapancreatic nerve	Absent	48	1.73 (0.06–11.90)	0.641	3.34 (0.34–14.00)	0.925
plexus invasion	Present	59	1.56 (0.10–18.10)		3.43 (0.27–14.00)	
Portal vein invasion	Absent	81	1.44 (0.06–18.10)	0.012*	3.31 (0.27–14.0)	0.263
	Present	26	2.56 (0.44–9.01)		4.13 (0.55–14.0)	
Lymph node involvement	Absent	22	1.12 (0.13–4.20)	0.018*	0.94 (0.44–8.83)	0.003*
	Present	85	1.86 (0.06–18.10)		4.06 (0.27–14.0)	

*$P < 0.05$. Differences between the two groups were evaluated using the Mann–Whitney *U*-test. CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; ly, lymphatic; ne, intrapancreatic nerve; v, venous.

3.38% (range, 0.27–14.0%) at the periphery in the IDC series, whereas the median CD204% in the CP series was 0.60% (range, 0.26–3.78%) at the center of the lesion and 1.59% (range, 0.32–3.54%) at the periphery ($P = 0.018$ at the center, $P = 0.008$ at the periphery). In each series, CD68- and CD204-positive cells were more frequently observed in the periphery than at the center of the lesions (Fig. 2). The CD204/CD68 ratios at the center and periphery were compared between IDC and CP cases to evaluate the population of cells with the M2 phenotype. In IDC cases, the median CD204/CD68 ratio was 67.6% (range, 3.6–185.4%) at the center of the lesion and 59.9 (range, 2.1–158.5%) at the peripheral sites, whereas the median CD204/CD68 ratio was 47.3% (range, 12.2–96.9%) at the center and 57.6% (range, 18.1–81.7%) at the periphery in CP cases. These differences were not significant ($P = 0.238$ at the center, $P = 0.753$ at the periphery; Fig. 2).

Distribution of CD68% and CD204% according to clinicopathological features. The relationship between clinicopathological features and macrophage infiltration was evaluated using Mann–Whitney *U*-tests (Tables 2, 3). The IDCs with lymph node involvement showed elevated expression of peripheral CD68 ($P = 0.045$), central CD204 ($P = 0.018$), and peripheral CD204 ($P = 0.003$). Cases with tumors >3.0 cm were significantly correlated with high peripheral CD204 expression ($P = 0.031$), and those with portal vein invasion were significantly correlated with high central CD204 expression ($P = 0.012$).

Univariate and multivariate analyses of parameters significantly associated with overall survival and DFS. The median IHC% of infiltrating macrophages was used to divide the cases into two groups, high (above the median value) and low (equal to or below the median value). Univariate analyses using log–rank tests were carried out to compare survival according to IHC% (Table 4), and overall survival curves were obtained with the Kaplan–Meier method (Fig. 3). Univariate analysis (Table 4) produced the following candidates for predicting prognosis: tumor size > 3.0 cm ($P = 0.0001$); lymph node involvement ($P = 0.0106$); lymphatic invasion ($P = 0.0171$); extrapancreatic nerve plexus invasion ($P = 0.0025$); and high central and peripheral CD204 expression ($CD204^{high}$) ($P = 0.0248$ at the center, $P < 0.0001$ at the periphery). Multivariate analysis (Table 5) revealed the following independent prognostic factors: tumor size > 3.0 cm (hazard ratio [HR], 2.017; $P = 0.002$); extrapancreatic nerve plexus invasion (HR, 1.992; $P = 0.002$); and peripheral $CD204^{high}$ (HR, 2.781; $P < 0.001$).

Univariate analysis (Table 4) showed that tumor size > 3.0 cm ($P = 0.0058$), serosal invasion ($P = 0.0427$), extrapancreatic nerve plexus invasion ($P = 0.0057$), and peripheral $CD204^{high}$ ($P = 0.0010$) were correlated with shorter DFS. Multivariate analysis (Table 5) revealed that extrapancreatic nerve plexus invasion (HR, 1.882; $P = 0.008$) and peripheral $CD204^{high}$ (HR, 1.864; $P = 0.010$) were independent risk factors for DFS. Initial recurrent sites of IDC were considered to be liver metastasis ($n = 38$), local recurrence ($n = 38$), or peritoneal dissemination ($n = 20$). The DFS curves for these groups were plotted using the Kaplan–Meier method to determine any significant impact of high CD204 expression at the peripheral site. The peripheral $CD204^{high}$ group had a significantly shorter DFS period than the peripheral $CD204^{low}$ group when stratified by initial liver metastasis and local recurrence (Fig. 4).

doi: 10.1111/j.1349-7006.2012.02411.x

Table 4. Univariate analyses of overall survival (OS) and disease-free survival (DFS) in patients with invasive ductal carcinoma of the pancreas

Factor	Category	*n*	OS, median (range)	*P* (uni)	DFS, median (range)	*P* (uni)
Age (years)	≤64	58	15.0 (3–145)	0.1561	7.5 (2–145)	0.1678
	>64	49	16.0 (1–90)		8.0 (1–90)	
Gender	Female	44	14.0 (1–77)	0.6205	8.0 (1–77)	0.4528
	Male	63	16.0 (2–145)		8.0 (1–145)	
CEA (ng/mL)	≤3.5	57	16.0 (2–145)	0.1757	11.0 (1–145)	0.1374
	>3.5	50	11.5 (1–90)		5.5 (1–90)	
CA19-9 (U/mL)	≤109	53	19.0 (1–90)	0.9288	8.0 (1–90)	0.3710
	>109	54	13.0 (3–145)		7.5 (2–145)	
Differentiation	Well	31	20.0 (1–77)	0.2594	15.0 (1–77)	0.1694
	Moderate/Poor	76	12.5 (2–145)		6.5 (1–145)	
Tumor size (cm)	≤3.0	66	19.0 (2–145)	0.0001*	11.0 (2–145)	0.0058*
	>3.0	41	10.0 (1–52)		6.0 (2–34)	
Serosal invasion	Absent	84	16.0 (1–145)	0.1058	10.0 (1–145)	0.0427*
	Present	23	12.0 (2–39)		6.0 (2–34)	
Retroperitoneal invasion	Absent	9	8.0 (4–53)	0.6294	6.0 (2–53)	0.5389
	Present	98	15.5 (1–145)		8.0 (1–145)	
Portal vein invasion	Absent	81	16.0 (1–90)	0.0745	8.0 (1–90)	0.4140
	Present	26	12.0 (3–145)		6.5 (2–145)	
Lymphatic invasion	0/1	60	20.0 (1–145)	0.0171*	9.5 (1–145)	0.1598
	2/3	47	11.0 (2–63)		6.0 (1–64)	
Vessel invasion	0/1	10	26.0 (6–77)	0.1072	17.0 (3–77)	0.2669
	2/3	97	13.0 (1–145)		8.0 (1–145)	
Intrapancreatic nerve invasion	0/1	27	15.0 (4–145)	0.1198	10.0 (2–145)	0.1001
	2/3	80	15.5 (1–77)		8.0 (1–77)	
Lymph node involvement	Absent	22	26.0 (4–90)	0.0106*	12.0 (1–90)	0.0645
	Present	85	13.0 (1–145)		8.0 (1–145)	
Extrapancreatic nerve plexus invasion	Absent	48	19.0 (3–145)	0.0025*	11.5 (2–145)	0.0057*
	Present	59	12.0 (1–53)		7.0 (1–53)	
CD68% at center	≤3.65%	53	19.0 (2–90)	0.5247	8.0 (1–90)	0.6641
	>3.65%	54	13.0 (1–145)		7.5 (1–145)	
CD68% at periphery	≤6.92%	54	19.0 (3–145)	0.3471	8.0 (2–145)	0.4213
	>6.92%	53	12.0 (1–77)		8.0 (1–77)	
CD204% at center	≤1.64%	54	19.0 (1–90)	0.0248*	8.0 (1–90)	0.6195
	>1.64%	53	11.0 (3–145)		6.0 (1–145)	
CD204% at periphery	≤3.39%	54	21.0 (3–90)	<0.0001*	13.5 (1–90)	0.0010*
	>3.39%	53	10.0 (1–145)		6.0 (1–145)	

*$P < 0.05$. Univariate analysis (uni) was carried out using the log–rank test. CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; CD68%, summed area of CD68-positive cells/measured area ×100; CD204%, summed area of CD204-positive cells/measured area ×100.

Discussion

This was the first study to evaluate the distributions of M2 macrophages (CD204-positive cells) in pancreatic IDC and CP. M2 macrophages preferentially accumulated in peripheral rather than central sites in pancreatic IDC and CP. This finding may indicate that non-cancerous cells play an important role in the recruitment of macrophages and the polarization toward M2 macrophages in pancreatic IDC and CP. In CP, macrophages are recruited using chemoattractants produced by myofibroblasts.[31] Myofibroblasts are considered to be the activated state of pancreatic stellate cells (PSCs), and PSCs are activated by pancreatitis[31] and pancreatic cancer cells.[32] Macrophages in pancreatic IDC may have infiltrated because of chemoattractants produced by myofibroblasts derived from PSCs. The polarization toward M2 macrophages may be responsible for the cells producing IL-4 and IL-10 in both IDC and CP tumors. We considered mast cells and PSCs as candidates. Mast cells accumulate in peripheral areas of IDC[33] and intestinal areas of CP[34] and can produce IL-10.[35] Activated PSCs are abundant in IDC and CP tumors and lead to IL-4 production by T cells.[36] Mast cells and PSCs may play important roles in M2 accumulation in IDC and CP. In this study, most peripheral M2 macrophages in pancreatic IDC were dense along the stroma but not along tumor cells, a finding that may reinforce the above speculation.

Accumulated M2 macrophages in pancreatic IDC were more numerous than in CP. In pancreatic IDC, a large tumor was significantly correlated with dense peripheral M2 macrophages. These results indicate that the tumor volume affects accumulation of M2 macrophages. Recent studies have shown that monocyte recruitment is driven by several chemoattractants such as MIP-2, CCL3, and hypoxia-inducible factor-2α, which are secreted by malignant cells and stromal cells and induced by tumor hypoxia.[26,27,37] Tumor-associated macrophages are recruited to tumors by multiple growth factors and chemokines that are often produced by tumor cells themselves.[38,39] Tumor necrosis is increased in large tumors,[7] and TAMs are attracted to and retained in avascular and necrotic areas where they are exposed to tumor hypoxia.[26,27] Large tumors may increase expression of inflammatory mediators from tumor cells, stroma cells, and tumor hypoxia. Thus, increased tumor volume may promote accumulation of M2 macrophages.

The independent impact of M2 macrophages on survival and time to relapse was first revealed with multivariate analysis in pancreatic IDC. Dense accumulation of peripheral M2 macrophages was established as a good predictive mar-

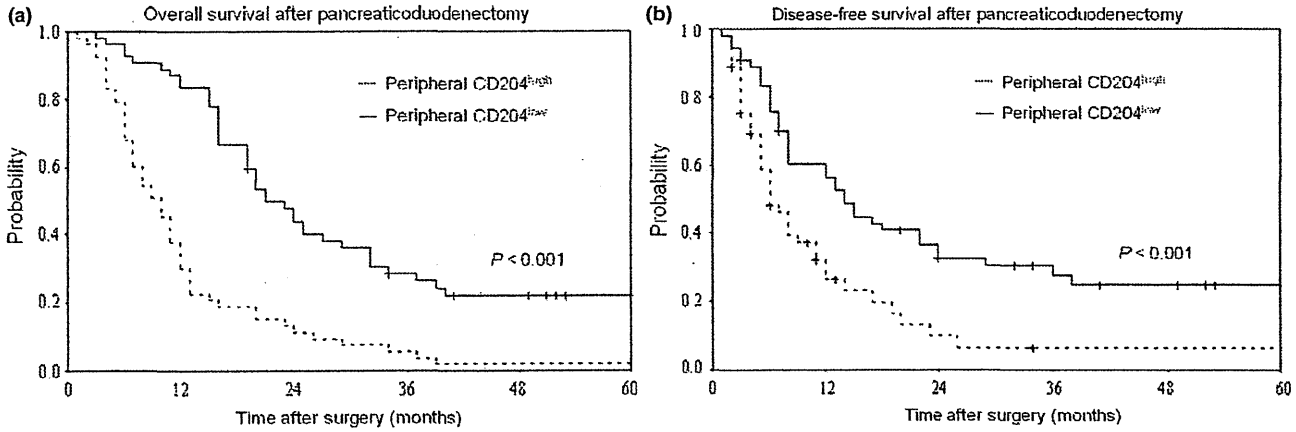

Fig. 3. *Overall survival (a) and disease-free survival (b) curves for invasive ductal carcinoma of the pancreas according to the area ratio of peripheral CD204-positive cells. Disease-free survival periods were significantly shorter in patients with peripheral CD204^{high} than in patients with CD204^{low}. Prognosis was significantly worse in patients with peripheral CD204^{high} than for those with CD204^{low}.*

ker of survival and recurrence. According to the type of initial recurrence, dense peripheral M2 macrophages were associated with early relapse in liver and the manipulated area of the pancreaticoduodenectomy. This suggests that M2 macrophages may accelerate liver metastasis and local recurrence. Tumor-associated macrophages are important producers of proteases, including MMPs, and of a wide variety of growth factors, such as fibroblast growth factor and epidermal growth factor (EGF) receptor family ligands that can stimulate the growth and motility of tumor cells.[38] Tumor-associated macrophages have been reported to be the most significant source of EGF in tumors,[40] and they are associated with EGF receptor expression and poor outcome in breast cancer.[41] Pollard *et al.* showed that tumor cells respond to macrophage-produced EGF ligands *in vivo* by chemotaxis and invasion, and that macrophages are often associated with vessels.[38,42] Thus, M2 macrophages may provide chemotactic signals that recruit tumor cells to blood vessels and enhance their egress into vasculature, leading to tumor hematogenous metastasis and further local invasion. These effects of M2 macrophages may shorten DFS and overall survival.

Lymph node involvement was significantly correlated with high CD204 expression in peripheral sites of the lesion. Tumor-associated macrophages within the invasive tumor front have a profound influence on the regulation of tumor angiogenesis and lymphangiogenesis by production of vascular endothelial growth factor-C and -D.[9,37,41,43] Elevated lymphangiogenesis by TAMs may promote lymph node metastasis.

The independent prognostic values of large tumor size and extrapancreatic nerve plexus invasion were reported in our previous study[7] and reconfirmed by this study. Time to recurrence was associated with the presence of extrapancreatic nerve plexus invasion. Large tumor size did not show an impact on DFS, because high accumulation of peripheral M2 macrophages correlated with large tumor size.

In conclusion, dense M2 macrophages in peripheral sites were significantly correlated with large tumor size, lymph node involvement, and poor prognosis due to accelerated liver metastasis and local recurrence. The number of accumulated M2 macrophages was associated with tumor volume, but the distribution of M2 macrophages in CP was similar to that in IDC.

Acknowledgments

Supported by Grants-In-Aid for Cancer Research and by a Third Term Comprehensive 10-year Strategy for Cancer Control grant from the Ministry of Health, Labor and Welfare of Japan.

Disclosure Statement

The authors have no conflict of interest.

Table 5. Multivariate analyses of independent significant factors associated with overall survival and disease-free survival in patients with invasive ductal carcinoma of the pancreas

	Overall survival			Disease-free survival		
	HR	95% CI	*P* (multi)	HR	95% CI	*P* (multi)
Tumor size (>3.0 cm)	2.017	1.301–3.127	0.002*	1.492	0.920–2.419	0.105
Serosal invasion present				1.667	0.960–2.896	0.070
Lymph node involvement present	1.112	0.612–2.020	0.727			
Extrapancreatic nerve plexus invasion present	1.992	1.283–3.095	0.002*	1.882	1.176–3.013	0.008*
Central CD204^{high}	1.035	0.673–1.592	0.874			
Peripheral CD204^{high}	2.781	1.740–4.445	<0.001*	1.864	1.164–2.986	0.010*

*$P < 0.05$. Multivariate analyses (multi) were carried out using the Cox regression hazard model. Central CD204^{high}, percentage of CD204-positive cells area over 1.64%; CI, confidence interval; HR, hazard ratio; Peripheral CD204^{high}, percentage of CD204-positive cells area over 3.39%.

doi: 10.1111/j.1349-7006.2012.02411.x

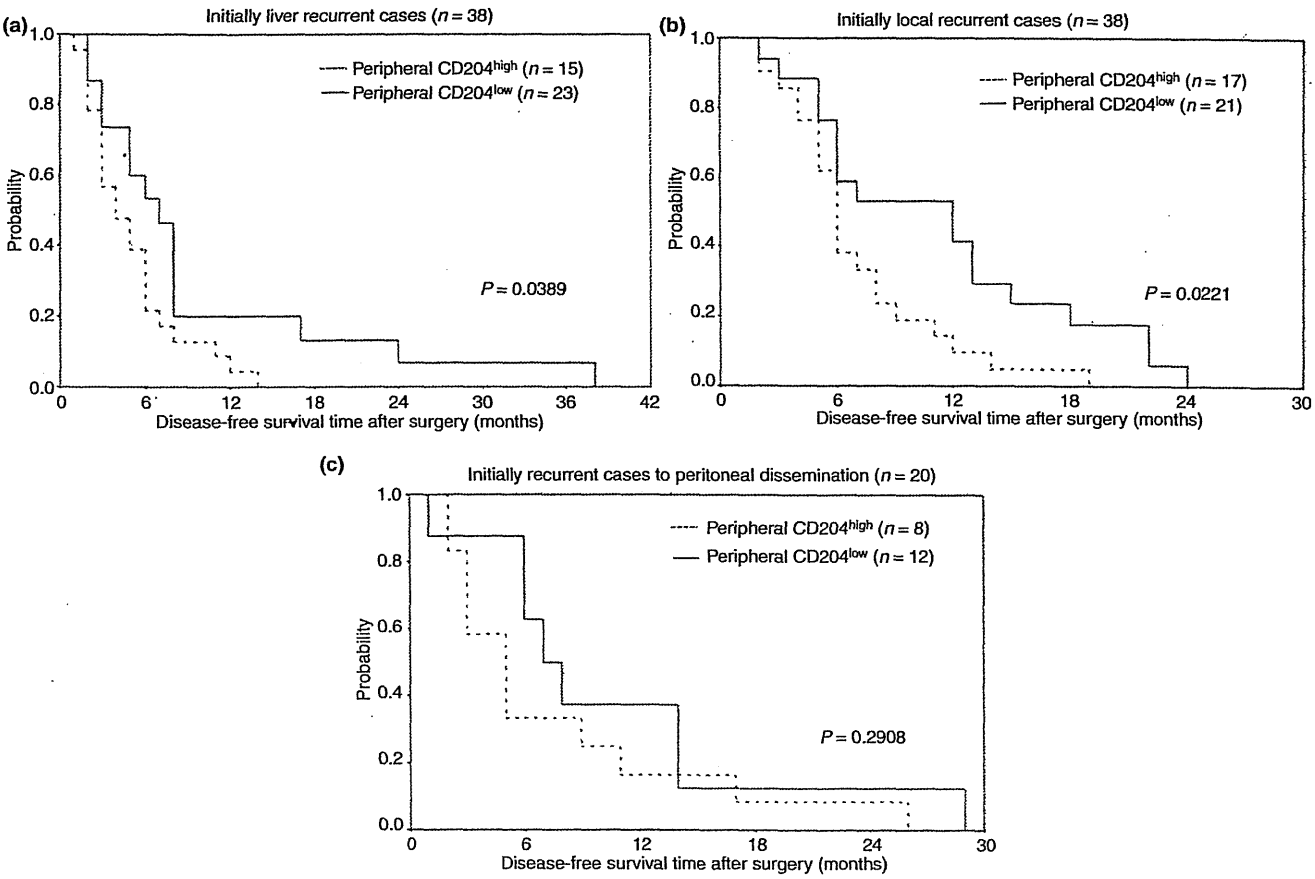

Fig. 4. Disease-free survival curves of invasive ductal carcinoma of the pancreas according to the area ratio of peripheral CD204-positive cells >3.39% and ≤3.39% in three groups that showed initial recurrence in the liver (a), local recurrence (b), and peritoneal dissemination (c). Peripheral CD204^{high} cases showed significantly shorter disease-free survival times in the groups with initial liver metastasis and initial local recurrence.

References

1 Cleary SP, Gryfe R, Guindi M *et al.* Prognostic factors in resected pancreatic adenocarcinoma: analysis of actual 5-year survivors. *J Am Coll Surg* 2004; **198**: 722–31.

2 Han SS, Jang JY, Kim SW, Kim WH, Lee KU, Park YH. Analysis of long-term survivors after surgical resection for pancreatic cancer. *Pancreas* 2006; **32**: 271–5.

3 Katz MH, Wang H, Fleming JB *et al.* Long-term survival after multidisciplinary management of resected pancreatic adenocarcinoma. *Ann Surg Oncol* 2009; **16**: 836–47.

4 Schnelldorfer T, Ware AL, Sarr MG *et al.* Long-term survival after pancreatoduodenectomy for pancreatic adenocarcinoma: is cure possible? *Ann Surg* 2008; **247**: 456–62.

5 Shimada K, Sakamoto Y, Nara S, Esaki M, Kosuge T, Hiraoka N. Analysis of 5-year survivors after a macroscopic curative pancreatectomy for invasive ductal adenocarcinoma. *World J Surg* 2010; **34**: 1908–15.

6 Raut CP, Tseng JF, Sun CC *et al.* Impact of resection status on pattern of failure and survival after pancreaticoduodenectomy for pancreatic adenocarcinoma. *Ann Surg* 2007; **246**: 52–60.

7 Mitsunaga S, Hasebe T, Iwasaki M, Kinoshita T, Ochiai A, Shimizu N. Important prognostic histological parameters for patients with invasive ductal carcinoma of the pancreas. *Cancer Sci* 2005; **96**: 858–65.

8 Conlon KC, Klimstra DS, Brennan MF. Long-term survival after curative resection for pancreatic ductal adenocarcinoma. Clinicopathologic analysis of 5-year survivors. *Ann Surg* 1996; **223**: 273–9.

9 Kurahara H, Shinchi H, Mataki Y *et al.* Significance of M2-polarized tumor-associated macrophage in pancreatic cancer. *J Surg Res* 2011; **167**: e211–9.

10 Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* 2001; **357**: 539–45.

11 Allavena P, Sica A, Solinas G, Porta C, Mantovani A. The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. *Crit Rev Oncol Hematol* 2008; **66**: 1–9.

12 Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* 2002; **23**: 549–55.

13 Sica A, Schioppa T, Mantovani A, Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. *Eur J Cancer* 2006; **42**: 717–27.

14 Lissbrant IF, Stattin P, Wikstrom P, Damber JE, Egevad L, Bergh A. Tumor associated macrophages in human prostate cancer: relation to clinicopathological variables and survival. *Int J Oncol* 2000; **17**: 445–51.

15 Tsutsui S, Yasuda K, Suzuki K, Tahara K, Higashi H, Era S. Macrophage infiltration and its prognostic implications in breast cancer: the relationship with VEGF expression and microvessel density. *Oncol Rep* 2005; **14**: 425–31.

16 Ohtaki Y, Ishii G, Nagai K *et al.* Stromal macrophage expressing CD204 is associated with tumor aggressiveness in lung adenocarcinoma. *J Thorac Oncol* 2010; **5**: 1507–15.

17 Komohara Y, Ohnishi K, Kuratsu J, Takeya M. Possible involvement of the M2 anti-inflammatory macrophage phenotype in growth of human gliomas. *J Pathol* 2008; **216**: 15–24.

18 Kawamura K, Komohara Y, Takaishi K, Katabuchi H, Takeya M. Detection of M2 macrophages and colony-stimulating factor 1 expression in serous and mucinous ovarian epithelial tumors. *Pathol Int* 2009; **59**: 300–5.

19 Ohno S, Inagawa H, Dhar DK *et al.* Role of tumor-associated macrophages (TAM) in advanced gastric carcinoma: the impact on FasL-mediated counterattack. *Anticancer Res* 2005; **25**: 463–70.
20 Ding T, Xu J, Wang F *et al.* High tumor-infiltrating macrophage density predicts poor prognosis in patients with primary hepatocellular carcinoma after resection. *Hum Pathol* 2009; **40**: 381–9.
21 Dai F, Liu L, Che G *et al.* The number and microlocalization of tumor-associated immune cells are associated with patient's survival time in non-small cell lung cancer. *BMC Cancer* 2010; **10**: 220.
22 Zhou Q, Peng RQ, Wu XJ *et al.* The density of macrophages in the invasive front is inversely correlated to liver metastasis in colon cancer. *J Transl Med* 2010; **8**: 13.
23 Mosser DM. The many faces of macrophage activation. *J Leukoc Biol* 2003; **73**: 209–12.
24 Lucas T, Waisman A, Ranjan R *et al.* Differential roles of macrophages in diverse phases of skin repair. *J Immunol* 2010; **184**: 3964–77.
25 Dominguez-Munoz JE. Pancreatic exocrine insufficiency: diagnosis and treatment. *J Gastroenterol Hepatol* 2011; **26** (Suppl 2): 12–6.
26 Murdoch C, Lewis CE. Macrophage migration and gene expression in response to tumor hypoxia. *Int J Cancer* 2005; **117**: 701–8.
27 Imtiyaz HZ, Williams EP, Hickey MM *et al.* Hypoxia-inducible factor 2alpha regulates macrophage function in mouse models of acute and tumor inflammation. *J Clin Invest* 2010; **120**: 2699–714.
28 Shimosegawa T, Kataoka K, Kamisawa T *et al.* The revised Japanese clinical diagnostic criteria for chronic pancreatitis. *J Gastroenterol* 2010; **45**: 584–91.
29 Furuse J, Kinoshita T, Kawashima M *et al.* Intraoperative and conformal external-beam radiation therapy with protracted 5-fluorouracil infusion in patients with locally advanced pancreatic carcinoma. *Cancer* 2003; **97**: 1346–52.
30 Japan Pancreas Society. *Classification of Pancreatic Carcinoma*, 2nd edn. Tokyo: Kanehara, 2003.
31 Andoh A, Takaya H, Saotome T *et al.* Cytokine regulation of chemokine (IL-8, MCP-1, and RANTES) gene expression in human pancreatic periacinar myofibroblasts. *Gastroenterology* 2000; **119**: 211–9.
32 Apte MV, Park S, Phillips PA *et al.* Desmoplastic reaction in pancreatic cancer: role of pancreatic stellate cells. *Pancreas* 2004; **29**: 179–87.
33 Cai SW, Yang SZ, Gao J *et al.* Prognostic significance of mast cell count following curative resection for pancreatic ductal adenocarcinoma. *Surgery* 2011; **149**: 576–84.
34 Hoogerwerf WA, Gondesen K, Xiao SY, Winston JH, Willis WD, Pasricha PJ. The role of mast cells in the pathogenesis of pain in chronic pancreatitis. *BMC Gastroenterol* 2005; **5**: 8.
35 Grimbaldeston MA, Nakae S, Kalesnikoff J, Tsai M, Galli SJ. Mast cell-derived interleukin 10 limits skin pathology in contact dermatitis and chronic irradiation with ultraviolet B. *Nat Immunol* 2007; **8**: 1095–104.
36 Tang D, Yuan Z, Xue X *et al.* High expression of Galectin-1 in pancreatic stellate cells plays a role in the development and maintenance of an immunosuppressive microenvironment in pancreatic cancer. *Int J Cancer* 2012; **130**: 2337–48.
37 Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. *Nat Rev Cancer* 2008; **8**: 618–31.
38 Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer* 2004; **4**: 71–8.
39 Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. *J Clin Invest* 2007; **117**: 1155–66.
40 O'Sullivan C, Lewis CE, Harris AL, McGee JO. Secretion of epidermal growth factor by macrophages associated with breast carcinoma. *Lancet* 1993; **342**: 148–9.
41 Leek RD, Hunt NC, Landers RJ, Lewis CE, Royds JA, Harris AL. Macrophage infiltration is associated with VEGF and EGFR expression in breast cancer. *J Pathol* 2000; **190**: 430–6.
42 Wyckoff J, Wang W, Lin EY *et al.* A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res* 2004; **64**: 7022–9.
43 Schoppmann SF, Birner P, Stockl J *et al.* Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. *Am J Pathol* 2002; **161**: 947–56.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Correlation between immunopositive cell count and cell area ratio.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

doi: 10.1111/j.1349-7006.2012.02411.x

Furukawa *et al. Molecular Cancer* 2012, **11**:88
http://www.molecular-cancer.com/content/11/1/88

MOLECULAR CANCER

RESEARCH Open Access

Targeting of MAPK-associated molecules identifies SON as a prime target to attenuate the proliferation and tumorigenicity of pancreatic cancer cells

Toru Furukawa[1,2*], Etsuko Tanji[1], Yuko Kuboki[1,2], Takashi Hatori[2], Masakazu Yamamoto[2], Kyoko Shimizu[2], Noriyuki Shibata[3] and Keiko Shiratori[2]

Abstract

Background: Pancreatic cancer is characterized by constitutive activation of mitogen-activated protein kinase (MAPK). Activation of MAPK is associated with the upregulation of genes implicated in the proliferation and survival of pancreatic cancer cells. We hypothesized that knockdown of these MAPK-associated molecules could produce notable anticancer phenotypes.

Methods: A RNA interference-mediated knockdown screening of 78 MAPK-associated molecules previously identified was performed to find molecules specifically associated with proliferation of pancreatic cancer cells *in vitro*. Expression of an identified molecule in pancreatic cancer tissues was examined by immunohistochemistry. *In vivo* tumorigenicity of cancer cells with stable knockdown of the molecule was assayed by using xenograft models. Flow cytometry and live cell imaging were employed to assess an association of the molecule with cell cycle.

Results: The knockdown screening revealed that knockdown of *SON*, the gene encoding SON, which is a large serine/arginine-rich protein involved in RNA processing, substantially suppressed pancreatic cancer cell proliferation and survival *in vitro* and tumorigenicity *in vivo*. SON expression was higher in ductal adenocarcinomas than in cells of normal ducts and precursor lesions in pancreatic cancer tissues. Knockdown of *SON* induced G2/M arrest and apoptosis in cultured cancer cells. The suppressive effect of *SON* knockdown on proliferation was less pronounced in cultured normal duct epithelial cells. SON formed nuclear speckles in the interphase of the cell cycle and dispersed in the cytoplasm during mitosis. Live cell imaging showed that SON diffusely dispersed in the early mitotic phase, accumulated in some foci in the cytoplasm in the late mitotic phase, and gradually reassembled into speckles after mitosis.

Conclusion: These results indicate that SON plays a critical role in the proliferation, survival, and tumorigenicity of pancreatic cancer cells, suggesting that SON is a novel therapeutic molecular target for pancreatic cancer.

Keywords: SON, MAPK, RNA interference, Speckle, Cell cycle

* Correspondence: furukawa.toru@twmu.ac.jp
[1]Institute for Integrated Medical Sciences, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan
[2]Institute of Gastroenterology, Tokyo Women's Medical University, Tokyo 162-8666, Japan
Full list of author information is available at the end of the article

Background

Pancreatic cancer is a leading cause of cancer-related deaths [1,2]. Despite advancements in diagnostic and therapeutic modalities, the 5-year survival rate of patients with pancreatic cancer is less than 10% [3]. This poor prognosis elicits an urgent need for the development of effective diagnostic and therapeutic measures to improve patient survival. Molecular medicine may be able to fulfill this need, as exemplified by imatinib in the treatment of chronic myeloid leukemia [4]. Pancreatic cancer is characterized by constitutive activation of mitogen-activated protein kinase (MAPK), due to gain-of-function mutations in *KRAS* or *BRAF* and loss-of-function of dual specificity phosphatase 6 (DUSP6) [5-7]. Active MAPK translocates to the nucleus, activates transcription factors, and induces the expression of a variety of genes [8]. In a previous study, we screened the genome for downstream targets of MAPK and identified 78 molecules specifically associated with MAPK activity in pancreatic cancer cells [9]. These MAPK-associated molecules include molecules implicated in DNA replication, RNA editing, spindle formation, mitosis, signal transduction, and membrane trafficking. These biological processes play critical roles in the survival, maintenance, and proliferation of pancreatic cancer cells. We hypothesized that molecular targeting of these MAPK-associated molecules could result in notable anticancer phenotypes, as we previously observed by targeting *AURKA* [9,10]. In this study, we performed a systematic knockdown screening of MAPK-associated molecules in pancreatic cancer cells.

Results

Knockdown screening of MAPK-modulated genes in pancreatic cancer cells

We performed knockdown screening using a pancreatic cancer cell line, MIA PaCa-2, and custom-designed short interfering RNAs (siRNAs) targeting all the 78 MAPK-modulated genes that were previously identified and isolated in the cell line (Additional file 1: Table S1) [9]. The cells were transiently transfected with each of the 78 siRNAs, and *in vitro* proliferation was subsequently examined for 5 consecutive days. This screening showed that proliferation of cancer cells was suppressed to variable degrees depending on the individual gene targeted (Figure 1). Knockdown of *AURKB*, *CENPA*, *EBNA1BP2*, *GOLT1A*, *KIF11*, *NEDD4L*, *SON*, *TPX2*, or *WDR5* suppressed proliferation by more than 50% compared with control. Among these targets, we focused on *SON* for further study because it showed the most substantial suppressive effect. This gene encodes a nuclear speckle protein, SON, which is involved in RNA processing.

Knockdown of *SON* attenuates proliferation in vitro, considerably in pancreatic cancer cells but less remarkably in normal phenotype cells

The *in vitro* suppressive effect of siRNA targeting *SON* on proliferation was reanalyzed in detail by using MIA PaCa2; PCI-35, a pancreatic cancer cell line with an aggressive phenotype; and HPDE, an immortalized normal human pancreatic duct epithelial cell line [7,11-13]. The suppressive effects of *SON* knockdown on cell proliferation appeared to be fatal in MIA PaCa-2, static in PCI-35, and insignificant in HPDE (Figure 2A). The effects of siRNA on SON expression were assayed by an immunoblotting method, which showed 77%, 10%, and 48% reduction of SON expression in MIA PaCa-2, PCI-35, and HPDE, respectively (Figure 2B). These results indicated that *SON* knockdown attenuated the *in vitro* proliferation of pancreatic cancer cells. The attenuation of proliferation depended on the efficiency of SON knockdown in pancreatic cancer cells, but was less remarkably affected in normal phenotype cells.

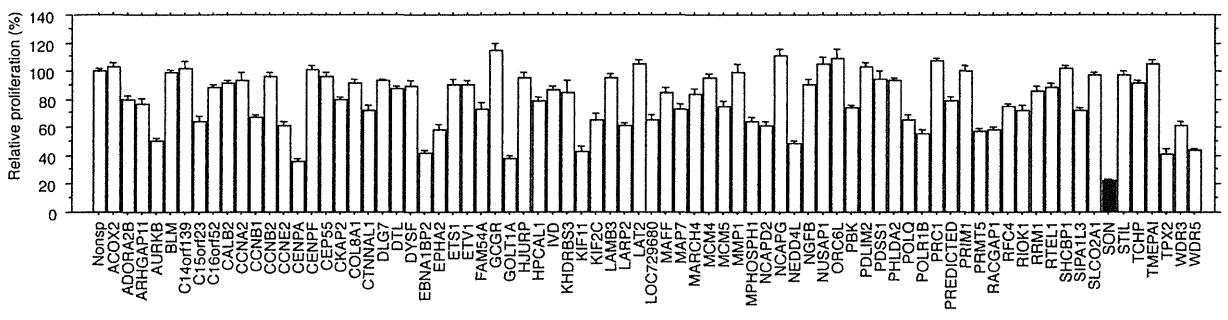

Figure 1 Knockdown screening of MAPK-associated genes in pancreatic cancer cells. Proliferation of MIA PaCa-2, a pancreatic cancer cell line, transfected with siRNA targeting various genes associated with MAPK (indicated on the horizontal axis), was determined by MTT assay on day 5 post-transfection. Plotted values are expressed relative to cells infected with control siRNA to a nonspecific sequence (Nonsp). Plots represent an average of 2 independent experiments; each experiment includes data from 8 independent transfection wells. Knockdown of *SON* (closed column) showed the most remarkable anti-proliferative phenotype.

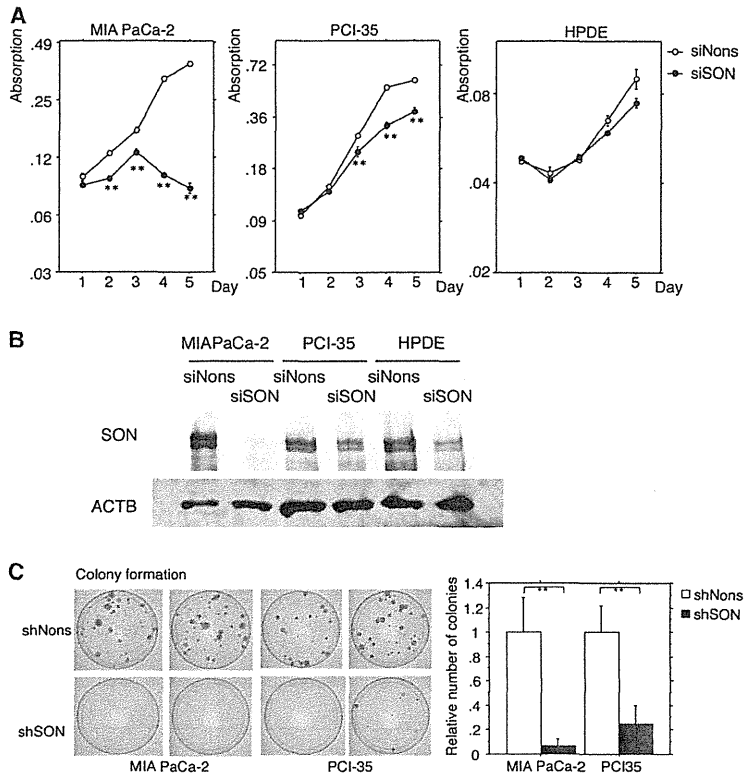

Figure 2 A. Proliferation of pancreatic cancer cells (MIA PaCa-2 and PCI-35) and normally phenotypic duct epithelial cells (HPDE) transfected with siRNA against *SON* (siSON) or a nonspecific sequence (siNons) and measured by MTT assay. The plots represent an average of 2 independent experiments; experiment includes data from 8 independent transfection wells. **B.** Expression of *SON* in cells transfected with siSON or siNons is shown in immunoblots probed with anti-SON antibody (SON) or anti-beta actin antibody (ACTB). **C.** Colony formation assay of pancreatic cancer cells transfected with vectors expressing shRNA targeting *SON* (shSON) or a non-specific sequence (shNons).

Stable knockdown of *SON* reduces the survival of pancreatic cancer cells in vitro

We next constructed a vector expressing short hairpin RNA (shRNA) identical to the *SON* siRNA when processed. We examined the effect of stable knockdown of *SON* on the survival of pancreatic cancer cells *in vitro* using a colony formation assay. We found that stable knockdown of *SON* strongly attenuated the survival of cancer cells, even in PCI-35 cells, in which transient transfection of siRNA targeting *SON* modestly suppressed proliferation (Figure 2C).

SON is overexpressed in pancreatic ductal adenocarcinomas

To establish the native expression of SON in pancreatic cancer, we examined 34 tissues with pancreatic ductal adenocarcinoma that were surgically resected. Immunohistochemistry showed that SON was strongly expressed in the nuclei of cancer cells in most ductal adenocarcinomas significantly more obviously than in the nuclei of non-neoplastic ducts or pancreatic intraepithelial neoplasia (PanIN), a precursor lesion of ductal adenocarcinoma ($p < 0.001$ by ANOVA) (Figure 3 and Table 1). This result indicates that SON is specifically overexpressed in pancreatic cancer.

Knockdown of *SON* retards the tumorigenicity of pancreatic cancer cells in vivo

We then performed a tumorigenicity assay using stably transfected pancreatic cancer cell clones carrying the shRNA vector targeting *SON*. Several stably transfected clones of MIA PaCa-2 and PCI-35 cells were obtained, and expression of *SON* was determined by real-time quantitative PCR. *SON* expression was lowest, reduced by 50%, in an MIA PaCa-2 clone (Figure 2D). We could not obtain any stably transfected PCI-35 clones in which *SON* expression was obviously reduced. This was probably because PCI-35, unlike MIA PaCa-2, could not survive modest knockdown of *SON*, which strongly suppresses the survival of cancer cells *in vitro*. The stably transfected clone of MIA PaCa-2 was inoculated into the subcutis of nude mice, and tumorigenicity was monitored. After 4 weeks, tumorigenicity was significantly retarded (Figure 4A).

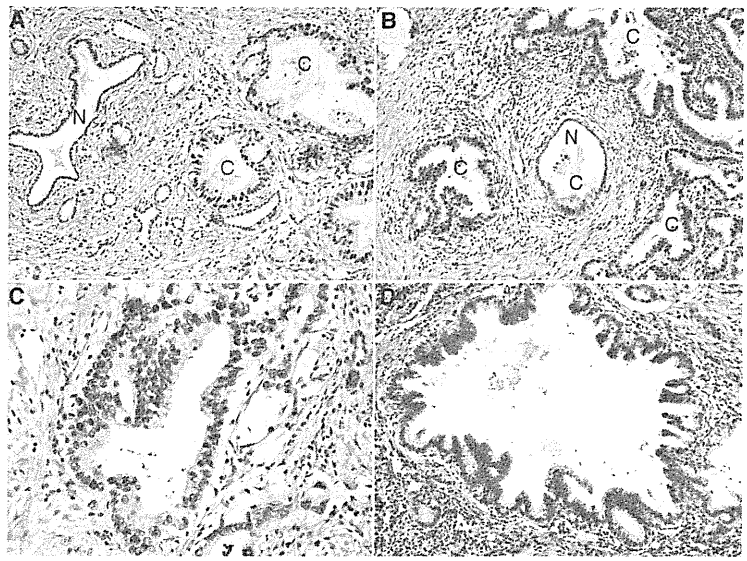

Figure 3 Immunohistochemical examination of SON expression in pancreatic cancer tissues. Diaminobenzidine and hematoxylin was used as a chromogen and a counter stain, respectively. **A** and **B**. Ductal adenocarcinomas (ducts labeled in C) strongly express SON in nuclei, more obviously than normal ductal cells (ducts labeled in N). The normal duct in panel B was partially (lower half) involved with carcinoma cells (original magnification, 100×). **C**. A high-powered view of ductal adenocarcinoma shows strong expression of SON in nuclei (original magnification, 200×). **D**. Pancreatic intraepithelial neoplasia, a precursor lesion of ductal adenocarcinoma, shows less obvious expression of SON (original magnification, 100×).

Knockdown of *SON* induces cell cycle arrest and apoptosis

To determine the mechanism by which *SON* knockdown suppresses the proliferation and survival of pancreatic cancer cells, the DNA content of siRNA-transfected MIA PaCa-2 and PCI-35 cells was measured by flow cytometry, and the cell cycle was assessed. Knockdown of *SON* increased the fraction of cells in G2/M and sub-G1, indicating that the cells were in G2/M arrest and apoptosis (Figure 4B).

SON shuttles between the nucleus and cytoplasm depending on the cell cycle

To investigate the dynamics of intracellular SON expression and its role in mitosis, a vector expressing SON, tagged with enhanced green fluorescence protein (EGFP) at the amino terminus (EGFP-SON), was constructed and transfected into 293 cells. The dynamics of intracellular SON expression were then analyzed. Expression of EGFP-SON was confirmed by immunoblotting by using specific antibodies against SON or EGFP (Figure 5A). Confocal laser scanning images showed that EGFP-SON was expressed as speckles in the nuclei of cells in the interphase and was dispersed in the cytoplasm of cells in the mitotic phase (Figure 5B). Time-lapse live imaging of cells expressing EGFP-SON showed that SON dispersed diffusely in the cytoplasm in metaphase and anaphase, accumulated in some foci in the cytoplasm during telophase and cytokinesis, and gradually reassembled in nuclear speckles after cytokinesis as foci in the cytoplasm faded (Figure 5C). From metaphase, the reassembly into nuclear speckles took approximately 2 hours. These results indicate that SON shuttles between the nucleus and the cytoplasm depending on the phase of the cell cycle, transitioning from nuclear speckles and through diffuse dispersion and subsequent temporal accumulation in the cytoplasm, to slow reassembly into nuclear speckles during mitosis and the early G1 phase.

Table 1 Expression of SON in ductal lesions evaluated by immunohistochemistry

Ductal lesion	Total number of lesions	Intensity score			*P* (ANOVA)
		1, weak	2, moderate	3, strong	
Ductal adenocarcinoma	34	0	3	31	< 0.001
PanIN	23	15	8	0	
Normal	29	24	5	0	

Discussion

In this study, among many genes associated with MAPK, we found that knockdown of *SON* remarkably suppressed the proliferation, survival, and tumor formation of pancreatic cancer cells. The suppressive effect was less pronounced in normally phenotypic ductal cells. In primary pancreatic cancer tissues, *SON* was overexpressed in