Table II. EGFR immunostaining in the surgically resected cancers and the far advanced cancers obtained at autopsy.

	No. of cases (%)							
		Membranous EGFR reactivity				Cytoplasmic EGFR reactivity		
	Total	0	1+	2+	3+	0	1+	2+
Surgically resected cancers	44	24 (55)	7 (16)	12 (27)	1 (2)	22 (50)	12 (27)	10 (23)
Far advanced cancers	40	6 (15)	15 (38)	7 (18)	12 (30)	4 (10)	23 (58)	13 (33)
Primary cancers[a]	20	3 (15)	9 (45)	3 (15)	5 (25)	2 (10)	13 (65)	5 (25)
Hepatic metastases[a]	20	3 (15)	6 (30)	4 (20)	7 (35)	2 (10)	10 (50)	8 (40)

[a]No significant difference between membranous and cytoplasmic EGFR reactivity.

Table III. Expression of EGFR stratified according to histological grading between the surgically resected cancers and the far advanced cancers obtained at autopsy.

		No. of tumors (%)			
Histological grade	Total	Membranous EGFR overexpression	P-value[a]	Cytoplasmic EGFR overexpression	P-value[a]
Surgically resected cancers	44	13 (30)[b]		10 (23)[c]	
Grade 1	12	1 (8)	0.07	1 (8)	0.2
Grade 2/3	32	12 (38)		9 (28)	
Far advanced cancers	40	19 (48)[b]		13 (33)[c]	
Grade 1	7	3 (43)	0.8	1 (14)	0.3
Grade 2/3	33	16 (48)		12 (36)	

[a]P-value indicates comparisons between Grade 1 and Grade 2/3 tumors. No significant difference was noted between EGFR expression and histological grade (Grade 1 vs. 2/3). [b]P=0.09, statistically significant difference between surgically resected and advanced cancers. [c]P=0.3, statistically significant difference between surgically resected and advanced cancers.

cells, a score of 0 was assigned, regardless of the intensity of the staining. If faint or barely perceptible membranous staining was detected in >10% of the tumor cells, a score of 1+ was assigned. Scores of 2+ and 3+ were assigned when weak to moderate staining and strong staining, respectively, were observed on the entire membrane in >10% of the tumor cells (Fig. 1). Cases showing a score of 2+ or 3+ were defined as showing overexpression.

Cytoplasmic staining was divided into 3 grades (0, 1+ and 2+), as grading of the intensity of the immunoreaction was difficult for the cytoplasm. The level of cytoplasmic staining was categorized as follows: when cytoplasmic staining was observed in <10% of the tumor cells, a score of 0 was assigned. If faint or barely perceptible cytoplasmic staining was detected in >10% of tumor cells, a score of 1+ was assigned. A score of 2+ was assigned when moderate or strong staining, respectively, was observed in >10% of the tumor cells. Cytoplasmic granular staining was also scored as 2+. Cases showing a score of 2+ were judged as showing overexpression (Fig. 2).

Statistical analysis. We used the χ^2 test or Fisher's exact test to determine the correlation between EGFR expression and histological grade. Differences were considered to indicate statistical significance at a P-value <0.05. All statistical analyses were performed using Statview 5.0 software (SAS Institute Inc., Cary, NC, USA).

Results

The expression profiles of membranous and cytoplasmic EGFR in both surgically resected cancers and far advanced cancers obtained at autopsy are shown in Table II. In the 44 surgically resected cancers, 13 (30%) exhibited membranous overexpression of EGFR, comprising 1 case (2%) of score 3+ and 12 cases (27%) of score 2+ and 10 (23%) exhibited cytoplasmic overexpression of EGFR.

In the primary tumors in the 20 far advanced cancers, the percentage of samples with positivity for membranous EGFR overexpression was 40%, (8 of 20), comprising 3 cases (15%) of score 2+ and 5 cases (25%) of score 3+, and the percentage of samples showing positivity for cytoplasmic EGFR overexpresion was 25% (5 of 20). In the hepatic metastases in the 20 far advanced cancers, the positivity of membrane EGFR overexpression was 55%, (11 of 20), comprising 4 cases (20%) of score 2+ and 7 cases (35%) of score 3+, and the positivity of cytoplasmic EGFR overexpresion was 40% (8 of 20).

In a total of 40 tumors at a far advanced stage, the percentage of samples showing positivity for membranous EGFR overexpression was 48% (19 of 40) comprising 7 cases (18%) of score 2+ and 12 cases (30%) of score 3+, and the percentage of samples showing positivity for cytoplasmic EGFR overexpresion was 33% (13 of 40). Therefore, the far advanced tumors tended to show membranous and cytoplasmic EGFR overexpression more frequently than the surgically resected tumors, although the difference was not significant.

When these cases were stratified according to histological grade, higher grade (Grades 2 and 3) cancer tissues tended to show membranous EGFR overexpression more frequently (12 of 32, 38%) than the lower grade (Grade 1) cancer tissues (1 of 12, 8%) in the surgically resected pancreatic cancers, although the difference was statistically marginal (P=0.07). The percentage of cytoplasmic EGFR overexpression did not differ statistically between the low grade (Grade 1) tumors (1 of 12, 8%) and higher grade (Grades 2 and 3) tumors (9 of 32, 28%) in the surgically resected cases.

The tissues of the far advanced cancers showed similar rates of membranous and cytoplasmic overexpressions, regardless of histological grade. In the 40 far advanced tumors, membranous EGFR overexpression was detected in 3 (43%) of 7 Grade 1 cases and in 16 (48%) of Grade 2 or 3 cases. In these far advanced tumors, cytoplasmic overexpression of EGFR was detected in 14% (1 of 7) of Grade 1 tumors and 36% (12 of 33) of Grade 2 or 3 tumors (Table III).

Discussion

In the present study, we demonstrated that EGFR overexpression in the cell membrane and cytoplasm was common in both surgically resected and far advanced pancreatic carcinomas. The occurrences of membranous and cytoplasmic EGFR overexpression tended to be higher in the tumors at far advanced stages than in the tumors that were at surgically resectable stages as determined using a global standard kit for EGFR assay.

Cytoplasmic EGFR expression in the far advanced cancers may be explained by the hypothesis of epithelial-to-mesenchymal transition which is thought be an important mechanism for promoting cancer invasion and metastasis (23). Persistently activated EGFR can decrease intercellular adhesion between tumor cells and enhance cancer cell migration. Willmarth *et al* showed that EGF-activated EGFR in MCF10A cells enhanced signal transduction predominantly from the endosomes rather than from the membrane (24). Barr *et al* (25) suggested that continuously EGF-treated EGFR induced endocytosis of E-cadherin, a cell-to-cell adhesion protein, which enhanced invasiveness in several human cancer cell lines. Ueda *et al* previously reported that EGFR overexpression in the cytoplasm of pancreatic cancers was associated with poorer clinical outcome of patients (10,26). The present study corroborated that not only membranous overexpression but also cytoplasmic overexpression of EGFR is important for the acquisition of highly aggressive and metastatic properties of pancreatic carcinomas.

In the present study, the rate of EGFR overexpression in surgically resected cancers tended to be higher in higher grade (Grades 2 and 3) tumors than in low grade (Grade 1) tumors. It is not surprising that poorly differentiated pancreatic cancers exhibited a higher incidence of EGFR overexpression as the patients with pancreatic carcinoma with altered EGFR activity tend to show a more aggressive clinical course and a poorer clinical outcome (27). Aggressive tumors appear to require the activation of an EGFR-mediated autocrine signaling in order to maintain proliferation. Therefore, we suppose the possibility that cytoplasmic EGFR protein, which is newly synthesized within the endoplasm reticulum, would be processed at the cellular surface. Some investigators reported that binding of EGF to EGFR activates its receptor tyrosine kinases and accelerates its internalization through clathrin-coated pits followed by the efficient lysosomal targeting of internalized receptors, which results in receptor downregulation and degradation. Thus, the ligand-induced internalization of EGFR, so-called endocytosis trafficking, is characterized as activated EGFR (28-30). If the EGFR ligands dissociated EGFR localized in endosomes, EGFR would be deactivated and recycled to the plasma membrane.

We should consider the possibility that EGFR localization and its activity in advanced or metastatic pancreatic cancers may be modulated by chemotherapy or radiation therapy which those patients had received. It is known that ionizing radiation, hypoxia and oxidative stress can also phosphorylate EGFR with ligand independence, which is sequentially internalized and shuttled into the nucleus (31). Li *et al* (32) reported that the non-small cell lung cancer H226 cells which acquire resistance to cetuximab, an anti-EGFR antibody, showed decreased membranous EGFR accompanied by EGFR expressed with nuclear localization. These findings imply that EGFR localization of cancer cells may be an important determinant of responsiveness to specific therapies.

In conclusion, we demonstrated using immunohistochemistry that membranous and cytoplasmic EGFR overexpression was frequently noted in surgically resected and far advanced pancreatic cancers. These findings suggest that membranous and cytoplasmic overexpression of EGFR may be indicative of the potential aggressiveness of pancreatic cancers.

Acknowledgements

This study was supported in part by a grant-in-aid for defense medicine from the Ministry of Defense and a grant-in-aid from the Foundation for Promotion of Defense Medicine. The authors thank Dr Tatsuro Takahashi and Mr. Ryuji Saito, Department of Central Laboratory, Japan Labor Health and Welfare Organization Kushiro Rosai Hospital, Kushiro, Hokkaido, Japan, for technical assistance with the immunohistochemistry.

References

1. Matsuno S, Egawa S, Fukuyama S, *et al*: Pancreatic Cancer Registry in Japan: 20 years of experience. Pancreas 28: 219-230, 2004.
2. Bramhall SR, Allum WH, Jones AG, Allwood A, Cummins C and Neoptolemos JP: Treatment and survival in 13,560 patients with pancreatic cancer, and incidence of the disease, in the West Midlands: an epidemiological study. Br J Surg 82: 111-115, 1995.
3. Parkin DM, Bray FI and Devesa SS: Cancer burden in the year 2000. The global picture. Eur J Cancer 37 (Suppl 8): S4-S66, 2001.

4. Burris HA III, Moore MJ, Andersen J, *et al*: Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol 15: 2403-2413, 1997.
5. Oettle H, Post S, Neuhaus P, *et al*: Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. JAMA 297: 267-277, 2007.
6. Ueno H, Kosuge T, Matsuyama Y, *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 101: 908-915, 2009.
7. Kindler HL, Friberg G, Singh DA, *et al*: Phase II trial of bevacizumab plus gemcitabine in patients with advanced pancreatic cancer. J Clin Oncol 23: 8033-8040, 2005.
8. Moore MJ, Goldstein D, Hamm J, *et al*: Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol 25: 1960-1966, 2007.
9. Salomon DS, Brandt R, Ciardiello F and Normanno N: Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol 19: 183-232, 1995.
10. Ueda S, Ogata S, Tsuda H, *et al*: The correlation between cytoplasmic overexpression of epidermal growth factor receptor and tumor aggressiveness: poor prognosis in patients with pancreatic ductal adenocarcinoma. Pancreas 29: e1-e8, 2004.
11. Mendelsohn J and Baselga J: Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. J Clin Oncol 21: 2787-2799, 2003.
12. Cappuzzo F, Hirsch FR, Rossi E, *et al*: Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small cell lung cancer. J Natl Cancer Inst 97: 643-655, 2005.
13. Hirsch FR, Varella-Garcia M, McCoy J, *et al*: Increased epidermal growth factor receptor gene copy number detected by fluorescence in situ hybridization associates with increased sensitivity to gefitinib in patients with bronchioloalveolar carcinoma subtypes: a Southwest Oncology Group Study. J Clin Oncol 23: 6838-6845, 2005.
14. Tsao MS, Sakurada A, Cutz JC, *et al*: Erlotinib in lung cancer – molecular and clinical predictors of outcome. N Engl J Med 353: 133-144, 2005.
15. Lynch TJ, Bell DW, Sordella R, *et al*: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small cell lung cancer to gefitinib. N Engl J Med 350: 2129-2139, 2004.
16. Han SW, Kim TY, Hwang PG, *et al*: Predictive and prognostic impact of epidermal growth factor receptor mutation in non-small cell lung cancer patients treated with gefitinib. J Clin Oncol 23: 2493-2501, 2005.
17. Mitsudomi T, Kosaka T, Endoh H, *et al*: Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small cell lung cancer with postoperative recurrence. J Clin Oncol 23: 2513-2520, 2005.
18. Paez JG, Janne PA, Lee JC, *et al*: EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 304: 1497-1500, 2004.
19. Takano T, Ohe Y, Sakamoto H, *et al*: Epidermal growth factor receptor gene mutations and increased copy numbers predict gefitinib sensitivity in patients with recurrent non-small cell lung cancer. J Clin Oncol 23: 6829-6837, 2005.
20. Takano T, Ohe Y, Tsuta K, *et al*: Epidermal growth factor receptor mutation detection using high-resolution melting analysis predicts outcomes in patients with advanced non-small cell lung cancer treated with gefitinib. Clin Cancer Res 13: 5385-5390, 2007.
21. Sobin LH and Wittekind Ch (eds): International Union Against Cancer (UICC). TNM Classification of Malignant Tumors. 6th edition. Wiley-Liss, New York, 2002.
22. Tsuda H, Akiyama F, Terasaki H, *et al*: Detection of HER-2/neu (c-erb B-2) DNA amplification in primary breast carcinoma. Interobserver reproducibility and correlation with immunohistochemical HER-2 overexpression. Cancer 92: 2965-2974, 2001.
23. Dembinski JL and Krauss S: Characterization and functional analysis of a slow cycling stem cell-like subpopulation in pancreas adenocarcinoma. Clin Exp Metastasis 26: 611-623, 2009.
24. Willmarth NE, Baillo A, Dziubinski ML, Wilson K, Riese DJ II and Ethier SP: Altered EGFR localization and degradation in human breast cancer cells with an amphiregulin/EGFR autocrine loop. Cell Signal 21: 212-219, 2009.
25. Barr S, Thomson S, Buck E, *et al*: Bypassing cellular EGF receptor dependence through epithelial-to-mesenchymal-like transitions. Clin Exp Metastasis 25: 685-693, 2008.
26. Ueda S, Hatsuse K, Tsuda H, *et al*: Potential crosstalk between insulin-like growth factor receptor type 1 and epidermal growth factor receptor in progression and metastasis of pancreatic cancer. Mod Pathol 19: 788-796, 2006.
27. Holbro T, Civenni G and Hynes NE: The ErbB receptors and their role in cancer progression. Exp Cell Res 284: 99-110, 2003.
28. Madshus IH and Stang E: Internalization and intracellular sorting of the EGF receptor: a model for understanding the mechanisms of receptor trafficking. J Cell Sci 122: 3433-3439, 2009.
29. Sorkin A and Goh LK: Endocytosis and intracellular trafficking of ErbBs. Exp Cell Res 315: 683-696, 2009.
30. Sorkin A and von Zastrow M: Endocytosis and signalling: intertwining molecular networks. Nat Rev Mol Cell Biol 10: 609-622, 2009.
31. Dittmann K, Mayer C, Kehlbach R and Rodemann HP: Radiation-induced caveolin-1 associated EGFR internalization is linked with nuclear EGFR transport and activation of DNA-PK. Mol Cancer 7: 69, 2008.
32. Li C, Iida M, Dunn EF, *et al*: Nuclear EGFR contributes to acquired resistance to cetuximab. Oncogene 28: 3801-3813, 2009.

World J Surg (2012) 36:2187–2191
DOI 10.1007/s00268-012-1622-0

World Journal of Surgery

The Implications of Positive Peritoneal Lavage Cytology in Potentially Resectable Pancreatic Cancer

Ryuji Yoshioka · Akio Saiura · Rintaro Koga · Junichi Arita · Nobuyuki Takemura · Yoshihiro Ono · Junji Yamamoto · Toshiharu Yamaguchi

Published online: 4 May 2012

Abstract
Background The clinical implications of peritoneal lavage cytology (CY) status in patients with potentially resectable pancreatic cancer have not been established.
Method We retrospectively reviewed clinical data from 254 consecutive patients who underwent macroscopically curative resection for pancreatic cancer from February 2003 to December 2010 in our institution. Correlations between CY status and survival and clinicopathological findings were investigated.
Results Of the 254 patients, 20 were CY+ (7.9 %). There were no significant differences between CY+ and CY− patients in background data (age, sex, the level of preoperative tumor marker, and adjuvant chemotherapy). Patients with positive serosal invasion were more likely to be CY+ than those with negative serosal invasion ($P < 0.001$) by univariate analysis. The median overall survival of CY+ patients and CY− patients was 23.8 months (95 % CI = 17.6–29.8) and 26.5 months (95 % CI = 20.7–32.3), respectively ($P = 0.302$). The median recurrence-free survival of CY+ and CY− patients was 8.1 months (95 % CI = 0.0–17.9) and 13.5 months (95 % CI = 11.5–15.5), respectively ($P = 0.089$).
Conclusion CY+ status without other distant metastasis does not necessarily preclude resection in patients with pancreatic cancer.

Presented at the European Society for Medical Oncology 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain, June 2011.

R. Yoshioka · A. Saiura (✉) · R. Koga · J. Arita · N. Takemura · Y. Ono · T. Yamaguchi
Department of Gastroenterological Surgery, Cancer Institute Hospital, Japanese Foundation for Cancer Research, 3-8-31 Ariake, Koto-ku, Tokyo 135-8550, Japan
e-mail: saiura-tky@umin.ac.jp

J. Yamamoto
Department of Hepato-Biliary-Pancreatic Surgery, National Defense Medical College Hospital, Tokorozawa, Japan

Introduction

Pancreatic adenocarcinoma is the fourth leading cause of cancer-related death in the United States and is associated with an extremely poor prognosis [1]. Surgical resection offers the only chance for cure; however, only 10–20 % of patients are considered candidates for surgical resection. Patients with locally advanced/unresectable disease (30–40 %) or metastatic disease (50–60 %) are excluded from consideration for resection [2].

Peritoneal lavage cytology (CY) is used widely in the diagnosis and staging of ovarian and gastric cancer [3–5]. In pancreatic cancer, malignant cancer cells have been identified in 7–30 % of peritoneal washings [6–16], and The American Joint Committee on Cancer (AJCC) staging of pancreatic cancer includes positive CY findings (CY+) as indicative of stage IV disease [5]. Indeed, many studies have associated CY+ status with advanced disease and poor survival and concluded that CY+ status in potentially resectable pancreatic cancer should be considered a contraindication for radical surgery [6, 8, 10, 11, 14].

On the other hand, several authors reported that CY status is not always associated with poor prognosis in patients with potentially resectable pancreatic cancer, claiming that CY+ in the absence of macroscopic peritoneal metastasis or liver metastasis is not a contraindication for radical surgery [12, 13, 15, 16]. The clinical implications of CY status in potentially resectable pancreatic cancer without other distant metastasis thus remain controversial, and the present study sought to clarify this issue.

Patients and methods

Clinical data collected prospectively from 254 consecutive patients who underwent macroscopically curative resection for invasive ductal carcinoma of the pancreas in the absence of macroscopic liver or peritoneal metastasis from February 2003 to December 2010 in our institution were reviewed retrospectively. Correlations between CY status and survival and clinicopathological findings were investigated. Curative resection was defined as the macroscopic removal of all gross tumors without liver metastases or macroscopic peritoneal spreading.

Postoperative follow-up

Routine follow-up consisted of laboratory studies, including tumor markers at 3-month intervals and computed tomography imaging at 3–6-month intervals. Until March 2005, our institute participated in a multicenter randomized phase III trial comparing gemcitabine with surgery alone in patients with macroscopically resected pancreatic cancer [17]. After the study, gemcitabine-based adjuvant chemotherapy was routinely performed. Postoperative peritoneal recurrence was defined as the recurrence detected macroscopically by imaging.

Peritoneal lavage cytology

Peritoneal washing and cytological analysis were routinely performed at the time of surgical exploration using a normal saline introduced into the abdominal cavity. After gentle agitation, as much fluid as possible was collected by syringe and centrifuged. Cytological smears were prepared from the centrifuged deposit and examined by an experienced pathologist after Papanicolaou staining.

Statistical analysis

For univariate analysis, binomial variables were compared using Pearson's χ^2 test and Fisher's exact test. Continuous variables were compared using the Mann–Whitney U test. Only those variables with P values of 0.10 or less by univariate analysis were entered into multivariate analyses in a backward stepwise manner until all variables remaining in the model were significant. Survival curves were calculated using the Kaplan-Meier method and compared using log rank tests. P values less than 0.05 were considered significant. Statistical analyses were performed using SPSS v19.0 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

There was no postoperative mortality. The patients were followed for a mean period of 24.7 months (range = 0.8–97.4 months). The conclusive stages, according to AJCC staging, of the 254 patients who underwent resection were IA in 10 patients, IB in 14 patients, IIA in 49 patients, IIB in 142 patients, III in 3 patients, and IV in 36 patients. Of the 254 patients, 20 were CY+ (7.9 %). Table 1 summarizes the patient demographics and clinical characteristics. There was no significant difference between the CY+ and CY− groups in age, sex, serum level of preoperative tumor markers, and the presence or absence of adjuvant chemotherapy. R1 resections were significantly more frequent in CY+ patients (10 of 20 patients; 50 %) than in CY− patients (58 of 234 patients; 24.8 %; Table 2).

The correlation between cytological status and clinicopathological parameters was analyzed (Table 3). Patients with positive serosal invasion were more likely to be CY+ than those with negative serosal invasion ($P < 0.001$). Multivariate analysis identified serosal invasion as the only independent factor associated with CY status ($P < 0.001$;

Table 1 Patient characteristics

Age (years) [mean (range)]	66.4 (42–89)
Gender	
Male	138
Female	116
Stage	
IA	10
IB	14
IIA	49
IIB	142
III	3
IV	36
Operation	
Pancreatoduodenectomy	172
Distal pancreatectomy	78[a]
Total pancreatectomy	3
Central pancreatectomy	1
CY	
Negative	234
Positive	20
R0/1	
R0	186
R1	68
Survival time (months) [median (95 % CI)]	24.7 (19.8–29.6)

CY+ positive peritoneal lavage cytology

[a] Including three patients of Appleby procedure

Table 2 Comparison of background data between CY+ and CY− patients

	CY−	CY+	*P*
Age (years) [mean (range)]	66.6 (42–87)	63.4 (42–89)	0.212
Gender			
Male	124	14	
Female	110	6	0.143
Preoperative tumor marker [mean (range)]			
CEA (ng/ml)	6.0 (0.0–145.4)	3.8 (0.9–17.0)	0.699
CA19-9 (U/ml)	1136.0 (0.1–50,000)	4786.1 (2.0–45,894.7)	0.151
CA125 (U/ml)	33.2 (4.5–1,108.0)	17.7 (6.5–37.8)	0.957
Adjuvant chemotherapy			
Yes	166	16	
No	68	4	0.388
R0/1			
R0	176	10	
R1	58	10	0.015*

CY+ positive peritoneal lavage cytology

* Statistically significant

Table 3 Univariate analysis of correlation between cytological status and pathological parameters

	CY−	CY+	*P* value
Differentiation			
Well/mod	187	17	
Other	47	3	0.773
Tumor size			
<2 cm	24	1	
>2 cm	240	19	0.703
Serosal invasion			
Negative	201	10	
Positive	33	10	<0.001*
Retroperitoneal invasion			
Negative	72	6	
Positive	162	14	0.943
Bile duct invasion			
Negative	140	14	
Positive	94	6	0.372
Duodenal invasion			
Negative	165	11	
Positive	69	9	0.118
Portal vein invasion			
Negative	182	12	
Positive	52	8	0.097
Arterial invasion			
Negative	230	19	
Positive	4	1	0.339
Perineural invasion			
Negative	159	15	
Positive	75	5	0.515
Lymph node metastasis			
Negative	72	3	
Positive	162	17	0.138

CY+ positive peritoneal lavage cytology

*Statistically significant

odds ratio [OR] = 6.091; 95 % confidence interval [CI] = 2.354–15.760).

The median overall survival of CY+ patients and CY− patients was 23.8 (95 % CI = 17.6–29.8) months and 26.5 (95 % CI = 20.7–32.3) months, respectively ($P = 0.302$), while the median recurrence-free survival (RFS) of CY+ and CY− patients was 8.1 (95 % CI = 0.0–17.9) months and 13.5 (95 % CI = 11.5–15.5) months, respectively ($P = 0.089$; Fig. 1).

Table 4 summarizes the distribution of initial recurrence site after resection. Peritoneal recurrence was more significantly frequent in CY+ patients (7 of 20 patients; 35 %) than in CY− patients (16 of 234 patients; 7 %).

Discussion

Several studies have evaluated the prognostic value of CY status in pancreatic cancer, with many associating CY+ status with advanced disease and poor prognosis. The National Comprehensive Cancer Network (NCCN) pancreatic adenocarcinoma guidelines state that "The panel considers positive cytology from washings obtained at laparoscopy or laparotomy to be equivalent to M1 disease" [18]. Many studies also concluded that CY+ status is a contraindication for resection [6–11, 14, 19]. Ferrone et al. [19] pointed out that CY+ patients who underwent resection without other distant metastasis had significantly worse survival than CY− patients and had a survival rate similar to that of patients with stage IV disease. On the other hand, Yachida et al. [15] suggested that this conclusion could be premature because only a small number of CY+ patients had undergone resection in the relevant studies. In addition, several studies found no significant correlation between CY status and survival in patients who had undergone potentially curative resection [12, 15, 16]. According to these three reports, the overall survival of CY− and CY+ patients who underwent pancreatic resection without liver metastasis and/or macroscopic peritoneal metastasis was not significantly different (Table 5), and the authors concluded that CY+ status without other distant metastasis is not a contraindication for radical surgery. The present study supports the latter results, and we therefore consider that the indication for resecting CY+ pancreatic

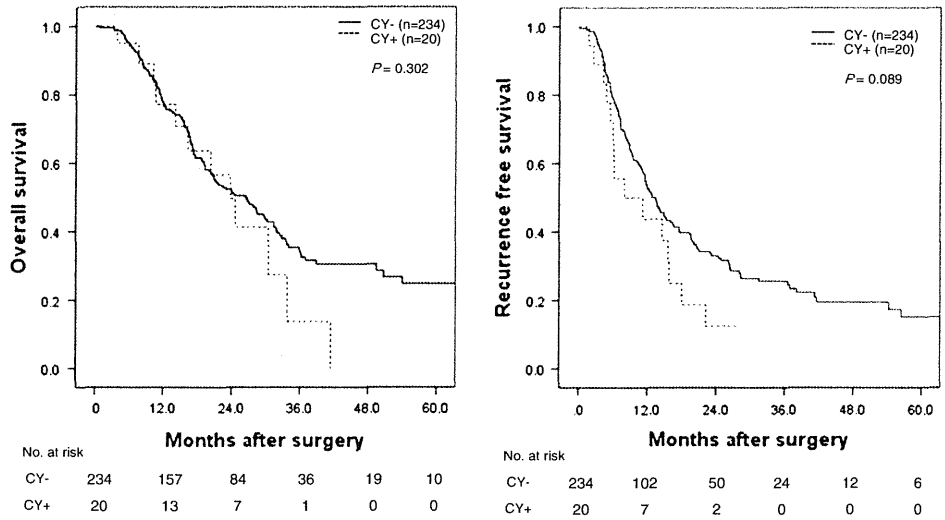

Fig. 1 Overall and recurrence-free survival of patients with negative (CY-) or positive (CY+) peritoneal lavage cytology following pancreatic resection. The median overall survival of CY+ patients and CY- patients was 23.8 (95 % CI, 17.6–29.8) months and 26.5 (95 % CI, 20.7-32.3) months, respectively ($P = 0.302$). The median recurrence-free survival of CY+ and CY- patients was 8.1 (95 % CI, 0.0–17.9) months and 13.5 (95 % CI, 11.5–15.5) months, respectively ($P = 0.089$)

Table 4 The first recurrence site after operation

	CY− *n* (%)	CY+ *n* (%)	*P*
Liver	67 (28)	6 (30)	1.000
Local	29 (12)	3 (15)	0.745
Lymph node	18 (8)	2 (10)	0.675
Lung	23 (10)	0 (0)	0.232
Peritoneum	16 (7)	7 (35)	0.001*
Other	15 (6)	1 (5)	

CY+ positive peritoneal lavage cytology

* Statistically significant

Table 5 Published studies analyzing the correlation between survival and CY status in patients who underwent resection

Author	Journal	Year		*n*	Median survival (months)	*P* value
Yachida et al. [15]	Br J Surg	2002	CY−	114	NA	0.347
			CY+	16	NA	
Meszoely et al. [12]	Am Surg	2004	CY−	122	19	0.055
			CY+	13	15	
Ferrone et al. [19]	J Gastrointest Surg	2006	CY−	207	16	<0.001
			CY+	10	8	
Yamada et al. [16]	Ann Surg	2007	CY−	136	13.5	0.269
			CY+	21	13.6	
Present study		2011	CY−	234	26.5	0.302
			CY+	20	23.8	

NA not available, *CY+* positive peritoneal lavage cytology

cancer without distant metastases remains controversial. To our knowledge, there is no report that compares the prognosis between resected CY+ patients and unresected CY+ patients without other distant metastasis. The same limitation is equally true of the present study.

Generally, patients with metastatic disease have a short median survival of 3–6 months [2], and FOLFIRINOX, the most promising treatment regimen at present, increased that time to only 11.1 months [20]. Patients with locally advanced nonmetastatic disease have a median survival of 6–10 months without resection, while those who undergo neoadjuvant therapy followed by R0 resection have an overall survival of 20 months [2, 21]. At present, surgical resection remains the only potentially curative treatment for pancreatic cancer, indicating that the median overall survival of 23.8 months in the CY+ patients who underwent resection in the present study was a promising result.

In the present study, CY+ patients showed a higher number of peritoneal recurrences with R1 resection, and the median RFS was shorter in CY+ patients than in CY− patients (8.1 vs. 13.5 months), although the difference was not statistically significant ($P = 0.089$). This insignificant difference might be due to the small number of cases from a single institution and/or the short follow-up period of 24.7 months in the present study.

In conclusion, positive peritoneal lavage cytology was not associated with overall survival after resection, despite of the increased frequency of peritoneal recurrence. CY+ status without other distant metastasis does not necessarily preclude resection in patients with pancreatic cancer.

Acknowledgments This study was supported by a grant-in-aid for cancer research from the Ministry of Health, Welfare and Labor of Japan and by the Japanese Foundation for Promotion of Cancer Research.

Conflict of interest The authors declare no personal conflict of interest and no financial support for the study.

References

1. Siegel R, Ward E, Brawley O, Jemal A (2011) Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. CA Cancer J Clin 61: 212–236
2. Gillen S, Schuster T, Meyer Zum Buschenfelde C et al (2010) Preoperative/neoadjuvant therapy in pancreatic cancer: a systematic review and meta-analysis of response and resection percentages. PLoS Med 7:e1000267
3. Aletti GD, Gallenberg MM, Cliby WA et al (2007) Current management strategies for ovarian cancer. Mayo Clin Proc 82:751–770
4. Japanese Gastric Cancer Association (2011) Japanese classification of gastric carcinoma: 3rd English edition. Gastric Cancer 14:101–112
5. American Joint Committee on Cancer (2010) Exocrine and endocrine pancreas. In: The AJCC Cancer Staging Manual, 7th ed. Springer, New York, pp 241–249
6. Clark CJ, Traverso LW (2010) Positive peritoneal lavage cytology is a predictor of worse survival in locally advanced pancreatic cancer. Am J Surg 199:657–662
7. Fernandez-del Castillo C, Rattner DW, Warshaw AL (1995) Further experience with laparoscopy and peritoneal cytology in the staging of pancreatic cancer. Br J Surg 82:1127–1129
8. Jimenez RE, Warshaw AL, Fernandez-Del Castillo C (2000) Laparoscopy and peritoneal cytology in the staging of pancreatic cancer. J Hepatobiliary Pancreat Surg 7:15–20
9. Leach SD, Rose JA, Lowy AM et al (1995) Significance of peritoneal cytology in patients with potentially resectable adenocarcinoma of the pancreatic head. Surgery 118:472–478
10. Makary MA, Warshaw AL, Centeno BA et al (1998) Implications of peritoneal cytology for pancreatic cancer management. Arch Surg 133:361–365
11. Merchant NB, Conlon KC, Saigo P et al (1999) Positive peritoneal cytology predicts unresectability of pancreatic adenocarcinoma. J Am Coll Surg 188:421–426
12. Meszoely IM, Lee JS, Watson JC et al (2004) Peritoneal cytology in patients with potentially resectable adenocarcinoma of the pancreas. Am Surg 70:208–213 discussion 213–204
13. Nakao A, Fujii T, Sugimoto H et al (2006) Oncological problems in pancreatic cancer surgery. World J Gastroenterol 12: 4466–4472
14. Warshaw AL (1991) Implications of peritoneal cytology for staging of early pancreatic cancer. Am J Surg 161:26–29 discussion 29–30
15. Yachida S, Fukushima N, Sakamoto M et al (2002) Implications of peritoneal washing cytology in patients with potentially resectable pancreatic cancer. Br J Surg 89:573–578
16. Yamada S, Takeda S, Fujii T et al (2007) Clinical implications of peritoneal cytology in potentially resectable pancreatic cancer: positive peritoneal cytology may not confer an adverse prognosis. Ann Surg 246:254–258
17. Ueno H, Kosuge T, Matsuyama Y et al (2009) 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 101:908–915
18. Tempero MA, Arnoletti JP, Behrman S et al (2010) Pancreatic adenocarcinoma. J Natl Compr Cancer Netw 8:972–1017
19. Ferrone CR, Haas B, Tang L et al (2006) The influence of positive peritoneal cytology on survival in patients with pancreatic adenocarcinoma. J Gastrointest Surg 10:1347–1353
20. Conroy T, Desseigne F, Ychou M et al (2011) FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med 364:1817–1825
21. Katz MH, Pisters PW, Evans DB et al (2008) Borderline resectable pancreatic cancer: the importance of this emerging stage of disease. J Am Coll Surg 206:833–846 discussion 846–838

Surgical Technique

Importance of Maintaining Left Gastric Arterial Flow at Appleby Operation Preserving Whole Stomach for Central Pancreatic Cancer

Akifumi Kimura[1], Junji Yamamoto[1], Suefumi Aosasa[3], Kazuo Hatsuse[1], Makoto Nishikawa[1], Kiyoshi Nishiyama[1], Hironori Tsujimoto[1], Tomoyuki Moriya[1], Kazuo Hase[1], Hiroshi Shinmoto[2] and Tatsumi Kaji[2]

[1]Department of Surgery and [2]Department of Radiology, National Defense Medical College, Saitama, Japan
[3]Department of Surgery, Social Health Insurance OMIYA General Hospital, Saitama, Japan

Corresponding author: Akifumi Kimura, Department of Surgery, National Defense Medical College, 359-8513, Namiki 3-2, Tokorozawa, Saitama, Japan; E-mail: akifumi@muse.ocn.ne.jp

Key Words: Appleby operation; Pancreatic cancer; Left gastric artery; Celiac axis.

Abbreviations: Three Dimensional (3D); Celiac Axis (CA); Common Hepatic Artery (CHA); Computed Tomography (CT); Distal Pancreatectomy with Celiac Axis Resection (DP-CAR); Gastroduodenal Artery (GDA); Hounsfield Unit (HU); Left Gastric Artery (LGA); Superior Mesenteric Artery (SMA); Splenic Artery (SPA); Quality of Life (QOL).

SUMMARY

The safety of whole stomach-preserving Appleby operation with resection of the left gastric artery (LGA) for pancreatic cancer cannot be assured. The anatomy of the celiac axis (CA) with special regard to the position of the origin of the LGA was examined. Using 3D images of the vascular architecture reconstructed from volume data of helical CT, the length of the CA and the position of the origin of the LGA from the CA were measured in 53 patients. Among 53 patients, 47 patients (89%) had classical anatomy of the CA branches. The mean length (±standard deviation) of the CA and the distance from the root of the LGA to the bifurcation of the CA were 25.2mm (±4.9) (range 14.6-36.5) and 10.3mm (±4.5) (range 2.4-21.9), respectively. In 23 (45%) cases, the LGA arose farther than 10mm away from the bifurcation of the CA. Among six patients with anatomical variation of the arteries, two (4%) had the LGA directly arising from the aorta. Conservation of the LGA at modified Appleby operation would give complete cancer removal by *en bloc* resection of the nerve plexus, without risk of ischemic complications of the stomach and liver.

INTRODUCTION

According to the TNM atlas of the UICC (1), the body of the pancreas is assigned to the parenchyma between the left border of the portal vein and the aorta. This part is astride the important part of the abdominal aorta from which the celiac axis (CA) and superior mesenteric artery (SMA) arise. As a result of such anatomical features and its invasive biological propensity (2), cancer of the body of the pancreas often involves major arterial structures, including the celiac axis (CA) and superior mesenteric artery (SMA), thus being found as unresectable T4 carcinoma (3).

In 1953, the Canadian surgeon Appleby firstly presented a collective report about the feasibility and safety of resection of the CA and CHA with total gastrectomy and distal pancreatectomy for gastric cancer with bulky lymph node metastases around these vessels (4). Nimura *et al.* first adopted Appleby's operation preserving the whole stomach in a patient with T4 pancreatic body carcinoma invading the CA (5) and several authors have reported this procedure (6-10).

Hirano *et al.* (7) reported an excellent outcome of locally advanced central pancreatic carcinoma in the largest series of patients who had undergone such operation, which they named DP-CAR (distal pancreatectomy with celiac axis resection). According to their results, this operation could be safely performed with no mortality. However, postoperative morbidity was high (48%), among which ischemic gastritis was found in 13% of patients. We herein evaluate the anatomy of celiac axis by CT reconstructed angiography and present three patients who underwent Appleby operation preserving the whole stomach with the left gastric artery for cancer of the body of the pancreas and discuss the feasibility and safety of this procedure.

SURGICAL TECHNIQUE

Evaluation of anatomy of celiac axis by CT reconstructed angiography

The anatomy of the CA was analyzed in 53 patients who underwent helical CT for preoperative evaluation of hepatobiliary disease (bile duct cancer (n=29), gallbladder cancer (n=14) and cancer of the papilla of Vater (n=10)) which did not invade near the CA, SMA and their branches. CT images were acquired using two 64-channel multidetector scanners (Aquillion 64™; Toshiba Medical System, Tokyo, Japan) at our hospital. A total of 1.8mL of non-ionic contrast material (iopamidol (300-370mg iodine per mL)) per kilogram body weight was injected into an antecubital vein at a rate of 4.0mL/sec using a power injector. Bolus tracking was used to assure appropriate imaging timing and early and late arterial-phase imaging was performed 5 and 22 seconds after the contrast density in the abdominal aorta reached a predefined threshold of 120 Hounsfield units (HU). From the obtained volume data, 3D images of the vascular architecture, which could be rotated in any direction, were reconstructed. Using these 3D images, the length of the CA and the position of the origin of the LGA from the CA were measured (**Figure 1**).

Hepato-Gastroenterology 2012; 59:2650-2652 doi 10.5754/hge12164

RESULTS

Among 53 patients, six had anatomical variation of the arteries; two had the LGA directly arising from the aorta, two had the splenic artery (SPA) arising before the LGA from the CA and the remaining two had the SPA and the CHA arising from the SMA, respectively. The remaining 47 patients who had classical anatomy of the CA branches were analyzed. The mean length (±standard deviation) of the CA and the distance from the root of the LGA to the bifurcated end of the CA were 25.2mm (±4.9) (range 14.6-36.5) and 10.3mm (±4.5) (range 2.4-21.9), respectively. In 23 (45%) cases, the LGA arose more than 10mm from the bifurcated end of the CA. The longer the CA, the farther the LGA from the bifurcation of the CA ($p<0.05$).

Three patterns of modified Appleby operation (Table 1)

From August 2010 to January 2011, three patients underwent Appleby operation preserving the whole stomach with LGA. All of these cases had advanced pancreatic body cancer without dissemination or distant metastasis and had been suspected tumor invading around the neural plexus of the CA, CHA, or LGA. The details of each patient are shown in **Table 1** and **Figure 2**. Patient 1 had the LGA separately originating from the aorta; thus, the CA was severed at the origin of the aorta. Patient 2 had ordinary vascular anatomy, and the CA was severed distal to the LGA branching. Patient 3 had reconstruction of the LGA with the right inferior phrenic artery after extirpation of the CA at its origin. Postoperative liver dysfunction was mild, with a maximum level of aspartate transaminase of 380IU/L (normal range 9-38 IU/L) in patient 2. The nutritional state of all three patients was good, with percent body weight loss of 0%, 1% and 6% at the last follow-up, respectively. Resection of the CHA and CA was necessary for complete removal of the tumor in all patients.

DISCUSSION

In 1953, Appleby first, and with foresight, presented a collective case series proving that resection of the distal pancreas and total stomach with extirpation of the entire celiac axis and its branches with all of its attendant tissues could be performed with impunity in block dissection for gastric carcinoma. In this operation, hepatopetal arterial flow could be preserved through a double arcade of arterial network surrounding the pancreatic head anteriorly and posteriorly (4). After Nimura *et al.* first succeeded in resecting a central pancreatic carcinoma invading the CA using a similar procedure in which the whole stomach was preserved (5), several authors reported the same operative procedure with various names (6-8,10,13). A total of 48 cases of such operations for central to left-sided pancreatic carcinoma have been reported to date.

Stomach-preserving Appleby operation has been conducted with zero mortality but with high morbidity (7,14). Ischemia of the stomach or the liver is an occasional cause of such complications. In their detailed report, Kondo *et al.* (14) emphasized the uncommonness of ischemic gastropathy after stomach-preserving Appleby's operation except when additional arteries supplying the stomach were divided. However, according to Eleisi *et al.* (15), the arterial blood supply of the stomach is about 60% from the LGA, about 20% from the splenic artery and only about 15% from the right gastric and right gastroepiploic arteries. About 80% of additional replenishing blood flow from the right-side gastric arteries might reduce hepatopetal blood supply through the pancreatic arcades from the SMA; thus, the safety of such procedure for the remnant stomach cannot be assured. Conservation of left gastric arterial flow would help boost arterial irrigation to the liver, thus alleviating both gastric and hepatic ischemia. Postoperative liver ischemia was mild in the present series, the maximum serum value of aspartate aminotransferase being ten times the maximum normal value.

Appleby operation preserving the whole stomach with LGA is indicated for tumors: 1) confined to the neck, body and tail of the pancreas; 2) not invading beyond the bifurcation of the proper hepatic artery and gastroduodenal artery; and 3) not invading the stomach and SMA. There

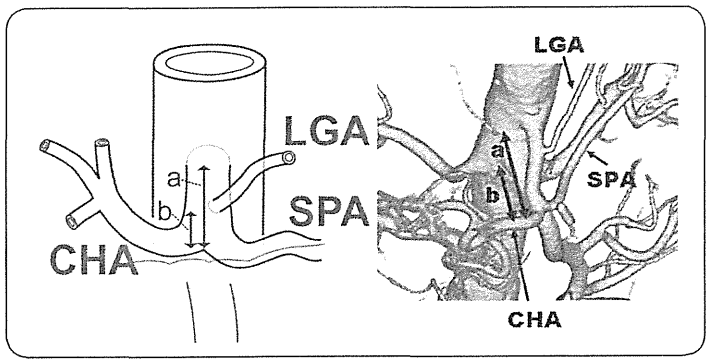

FIGURE 1. Measurement of celiac axis. Using 3D images of the vessel, the length of the celiac axis and the position of the origin of the left gastric artery of the celiac axis were measured. Length (a) is the distance between the root and the end of the celiac axis. Length (b) is the distance from the left gastric artery to the bifurcation of the celiac axis. CHA: Common Hepatic Artery; LGA: Left Gastric Artery; SPA: Splenic Artery.

TABLE 1. Demographic and clinicopathologic data of patients who underwent modified Appleby operation preserving whole stomach with left gastric artery

	Case 1	Case 2	Case 3
Age; Gender	75; F	66; M	60; F
Diagnostic sign	Elevation of CA19-9	Elevation of CA19-9	Appetite loss
Preop CA19-9 (IU/mL)	196.3	4265	1300
Blood loss/op. time	185g/306min	410g/427min	173g/477min
Peak postop. AST*	84	380	135
Complication	Grade B	Grade B	Grade B
Per os start	6 POD	7 POD	6 POD
Time to discharge	30 POD	31 POD	95 POD
Recurrence	None	None	Liver metastasis
Present status	Alive 15 months	Alive 13 months	Alive 9 months
% Weight loss¶	0	1	6
Max. tumor size	23mm	45mm	30mm
Invaded artery	Splenic artery	None	Splenic artery
Invaded vein	Splenic vein	Splenic vein (occlusion) Portal vein	none
(Peri) neural invasion	Celiac axis Splenic artery	Splenic artery	Common hepatic artery Splenic artery
Positive lymph nodes Number of positive nodes/all harvested nodes	Superior peripancreatic 1/27	Superior peripancreatic 9/45	Superior peripancreatic 8/30
Surgical margin	Negative	Negative	Negative

*Normal range of AST (aspartate aminotransferase) is 9 to 38IU/L; ¶Body weight of each patient was measured at the last follow-up.

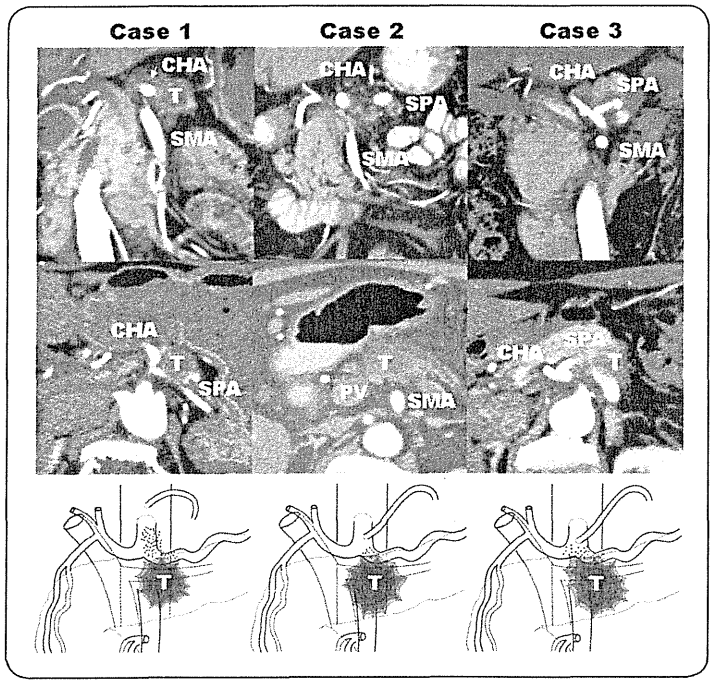

FIGURE 2. Preoperative image and schema of tumor location and its extent of invasion. Three cases of preoperative coronal (upper column) and axial (middle column) image and schema of tumor location and arteries of upper abdomen (lower column) are shown. The dots represent the invasion of the tumors. Each case was suspected tumor invaded around the CA. T: Tumor; CA: Celiac Axis; SPA: Splenic Artery; CHA: Common Hepatic Artery; SMA: Superior Mesenteric Artery.

are several ways to preserve the left gastric arterial flow in stomach-preserving Appleby operation. This operation is most frequently used for a central pancreatic carcinoma, invading or closely approaching the CHA or bifurcation of the CA. Analysis of 3D reconstructed arteriographic images showed an extra surgical margin of 1cm with the LGA-preserving procedure, compared to ordinary distal pancreatectomy. In case of a variation of the LGA directly arising from the aorta (4% of cases), the LGA can be preserved with extirpation of the CA at its origin from the aorta.

As the anatomies of three cases of Appleby operations preserving the whole stomach with the LGA were compatible with those of CT reconstructed angiography respectively, we could estimate the transecting region of artery and the need for reconstruction of the LGA until the operation. These cases have been reported herein, pertaining to the feasibility, safety and effectiveness of this operation. All three patients were alive with good QOL and two of them had no sign of recurrence 15 and 13 months after surgery, respectively. Pathological assessment of the extension of carcinomas in this series showed the presence of cancer cells in the nerve plexus at the root of the SPA. Thus, *en bloc* resection of the nerve plexus with the bifurcated ends of the CA was necessary for complete removal of cancer invasion. Complication-free and QOL-preserving surgery with prompt postoperative adjuvant chemotherapy would be a rational strategy for pancreatic carcinoma.

REFERENCES

1. **Wittekind C, Greene FL, Hutter RVP, et al.:** TNM Atlas (5th ed). Springer; 2004.
2. **Nakao A, Harada A, Nonami T, Kaneko T, Takagi H:** Clinical significance of carcinoma invasion of the extrapancreatic nerve plexus in pancreatic cancer. Pancreas 1996; 12(4):357-361.
3. **Sobin LH, MK G, Ch W:** TNM Classification of malignant tumours (7th ed.), 2009.
4. **Appleby LH:** The coeliac axis in the expansion of the operation for gastric carcinoma. Cancer 1953; 6(4):704-707.
5. **Mayumi T, Nimura Y, Kamiya J, et al.:** Distal pancreatectomy with en bloc resection of the celiac artery for carcinoma of the body and tail of the pancreas. Int J Pancreatol 1997; 22(1):15-21.
6. **Liu B:** Modified Appleby operation in treatment of distal pancreatic cancer. Hepatobiliary Pancreat Dis Int 2003; 2(4):622-625.
7. **Hirano S, Kondo S, Hara T, et al.:** Distal pancreatectomy with en bloc celiac axis resection for locally advanced pancreatic body cancer: long-term results. Ann Surg 2007; 246(1):46-51.
8. **Hishinuma S, Ogata Y, Tomikawa M, Ozawa I:** Stomach-preserving distal pancreatectomy with combined resection of the celiac artery: radical procedure for locally advanced cancer of the pancreatic body. J Gastrointest Surg 2007; 11(6):743-749.
9. **Morera-Ocon FJ, Carcel-Carcel I, Ballestin Vicente J, et al.:** Some reflexions on the modified Appleby procedure. JOP 2009; 10(6):674-678.
10. **Wu X, Tao R, Chongpeng P:** Distal pancreatectomy combined with celiac axis resection: an alternative approach for some advanced diatal pancreatic cancer. Ann Surg Oncol 2010; 17:1359-1366.
11. **Kosuge T, Makuuchi M, Takayama T, et al.:** Thickening at the root of the superior mesenteric artery on sonography: evidence of vascular involvement in patients with cancer of the pancreas. AJR Am J Roentgenol 1991; 156(1):69-72.
12. **Strasberg SM, Drebin JA, Linehan D:** Radical antegrade modular pancreatosplenectomy. Surgery 2003; 133(5):521-527.
13. **Kondo S, Katoh H, Hirano S, et al.:** Results of radical distal pancreatectomy with en bloc resection of the celiac artery for locally advanced cancer of the pancreatic body. Langenbecks Arch Surg 2003; 388(2):101-106.
14. **Kondo S, Katoh H, Hirano S, et al.:** Ischemic gastropathy after distal pancreatectomy with celiac axis resection. Surg Today 2004; 34(4):337-340.
15. **El-Eishi HI, Ayoub SF, El-Khalek MA:** The arterial supply of the human stomach. Acta Anat (Basel) 1973; 86(3):565-580.

Clin Exp Metastasis (2012) 29:101–110
DOI 10.1007/s10585-011-9433-5

RESEARCH PAPER

CXCR4/CXCL12 expression profile is associated with tumor microenvironment and clinical outcome of liver metastases of colorectal cancer

Nozomu Sakai · Hiroyuki Yoshidome · Takashi Shida · Fumio Kimura · Hiroaki Shimizu · Masayuki Ohtsuka · Dan Takeuchi · Masahiro Sakakibara · Masaru Miyazaki

Received: 6 December 2010 / Accepted: 1 November 2011 / Published online: 11 November 2011

Abstract Interaction between CXCR4 and CXCL12 plays a role in tumor progression. The present study examined CXCR4, CXCL12 and CD133 expression in liver metastases of colorectal cancer (CLM) and determined whether the expression profiles affect the tumor microenvironment and thus progression, and whether they could serve as a prognostic marker for survival. Liver metastases of colorectal cancer collected from 92 patients were evaluated by CXCR4, CXCL12 and CD133 immunohistochemistry and clinicopathological data were analyzed. The expression profile of CXCR4 was determined in the colorectal cancer cell line, SW48. The expression of cytoplasmic CXCR4 was higher in 36 (39%) patients than that indicated by CXCR4 staining intensity of hepatocytes. High levels of nuclear CXCR4 expression in 23 (25%) patients significantly correlated with CXCL12 expression in hepatocytes. Nuclear CXCR4 expression was increased in the cancer cells after exposure to CXCL12. Univariate and multivariate analyses demonstrated that the high levels of nuclear CXCR4 and CXCL12 expression in hepatocytes were significantly better prognostic factors for overall and hepatic disease-free survival in patients with CLM. The expression of CXCR4 and CXCL12 in CLM may have an interactive effect that could alter the tumor microenvironment. CXCR4 expression in metastatic liver tumors together with the upregulation of CXCL12 in hepatocytes may help to predict the clinical outcomes of patients with CLM after hepatectomy.

Keywords Colorectal metastases · Hepatectomy · Chemokine · Angiogenesis · CD133

Abbreviations

CLM	Liver metastases of colorectal cancer
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
FC	Fibrous pseudocapsule

Introduction

Colorectal carcinoma is a leading cause of cancer-related death. Regardless of curative resection to treat colorectal carcinoma, distant metastases develop in a significant number of patients [1]. The liver is a metastatic site of colorectal carcinoma, which is often associated with a poor prognosis [2, 3]. The "seed and soil theory" has been postulated to explain the directional migration and invasion of cancer cells into specific organs [4]. The chemokine network might play a role in the induction of organ-specific metastases [5]. Chemokines and their receptors were originally identified as chemoattractive interactions between immune cells and sites of inflammation [6]. Cancer cells also use chemokine networks to modulate the host microenvironment and facilitate cancer progression [7–10]. Several chemokine receptors and ligands function in tumor progression and invasiveness in several malignancies [11–15]. CXCR4 is an established inducer of colorectal cancer progression that correlates with clinical outcomes of

Nozomu Sakai and Hiroyuki Yoshidome contributed equally to this study.

N. Sakai · H. Yoshidome (✉) · T. Shida · F. Kimura · H. Shimizu · M. Ohtsuka · D. Takeuchi · M. Sakakibara · M. Miyazaki
Department of General Surgery, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
e-mail: h-yoshidome@umin.ac.jp

stage III/IV disease defined by the UICC classification [13]. The CXCR4 ligand, CXCL12/stromal-derived factor-1 (SDF-1), is also associated with tumor progression [16]. Kim et al. found significantly higher CXCR4 expression in liver metastases than in primary colorectal cancer, indicating that CXCL12 is abundantly expressed at metastatic sites [17]. In addition, CXCL12 accumulates in CD133-positive tumor cells that might in part, be derived from cancer stem cells [18, 19]. Therefore, interaction between CXCR4 and CXCL12 expression might cause tumor proliferation, migration, and invasion.

Chemokine receptors generally appear in the cell membrane and cytoplasm, thus leading to downstream signal transduction. CXCR4 is expressed in both the cytoplasm and nucleus of tumor cells [20, 21]. The cytoplasmic expression of CXCR4 is associated with a poor clinical outcome [12], but the nuclear expression of CXCR4 has not been fully elucidated. Furthermore, CXCR4 expression might be altered by the tumor microenvironment [8, 11, 22]. We postulated that different CXCR4/CXCL12 expression profiles in tumor cells and the tumor microenvironment interact and affect each other, thus determining the progression of colorectal liver metastases. In addition, other reports have described that the timing and numbers of liver metastases, lymph node metastasis of the primary tumor, hilar lymph node metastasis and the absence of a fibrous capsule on the metastatic tumor are prognostic factors for survival [23–25]. However, a correlation between the CXCR4/CXCL12 expression and the clinicopathological features of colorectal liver metastases has not been fully elucidated.

The present study examines CXCR4/CXCL12 expression in CLM to determine whether their expression profiles influence the progression of CLM, survival rates and hepatic recurrence.

Patients and methods

Patients and tissue samples

We retrospectively reviewed the medical records of 92 Japanese patients with CLM from among those who underwent curative hepatic resection (R0) at the Department of General Surgery at Chiba University (Chiba, Japan) between 1999 and 2007. Written informed consent was obtained from all of the patients to review their records in accordance with the ethical standards of the Helsinki Declaration of 1975. We defined synchronous metastases as those diagnosed before, or at the time of colorectal surgery. The patients were followed until death or November 30, 2010. The median follow-up time was 38 months (range 8–115 months). The clinicopathological characteristics of the patients are shown in Table 1.

Hematoxylin and eosin (HE) staining and immunohistochemistry

Tissue sections (4 μm thick) prepared from formalin-fixed paraffin-embedded tissue blocks were stained with HE and immunohistochemically assessed using either the CSA II kit for CXCR4 and CD133 or the ENVISION+ kit for CXCL12/SDF-1 (DAKO Cytomation, Carpinteria, CA, USA) according to the manufacturer's instructions. Antigens in all analyses were retrieved by microwave heating. The series and dilutions were as follows: (a) anti-mouse, anti-rat and anti-human CXCR4 rabbit polyclonal antibody (ab2074, Abcam, Cambridge, UK), 1:25; (b) anti-human/mouse CXCL12 antibody (MAB350, R&D Systems, Minneapolis, MN, USA), 1:20; (c) anti-mouse, rat and human CD133 rabbit polyclonal antibody (ab19898, Abcam, Cambridge, UK), 1:500. Appropriate positive controls containing the antigens of interest were simultaneously processed. The primary antibody was omitted from negative controls. The tissue sections were washed in water and counterstained with Mayer's hematoxylin. All immunohistochemical analyses were performed in duplicate.

Evaluation of CXCR4, CXCL12, and CD133

Immunoreactivity for CXCR4 was semi-quantified by assessing staining intensity. Cytoplasmic CXCR4 expression was classified as low or high relative to the staining intensity of hepatocytes. Immunoreactivity for CXCL12 was semi-quantified by assessing the staining intensity and ratio (%) of positive cells. CXCL12 expression was scored as 0, no staining; 1, occasional weak staining; 2, moderate staining and 3, intense staining. Positive staining of over 10% of tumor cells was required for scores of 2 and 3 (high expression). CXCL12 expression was classified as low (scores 0 and 1) or high (scores 2 and 3). High-power fields (200×) of 10 random areas within the tumor were evaluated. CD133 immunoreactivity was semi-quantified by assessing the staining intensity and ratios (%) of positively stained cells. CD133 expression was determined as 0, no staining; 1, <5% of tumor cells positively stained and 2, ≥5% of tumor cells positively stained. Amounts of CD133 expression were classified as low (scores 0 and 1) or high (score 2). High-power (200×) fields of 10 random areas within tumors were evaluated. The two investigators (TS and MO) who evaluated the immunohistochemical findings were blinded to the clinicopathological features and prognoses of the patients. The expression of these factors was evaluated both at tumor sites and in hepatocytes.

Table 1 Clinicopathological characteristics of patients with colorectal liver metastases ($n = 92$)

Clinicopathological characteristics	Number of patients
Age	
≥60 years	56
<60 years	36
Gender	
Male	58
Female	34
Lymph node metastasis of the primary lesion	
Positive	58
Negative	34
Timing of metastasis	
Synchronous	44
Metachronous	48
Tumor size	
≥50 mm	26
<50 mm	66
Fibrous pseudo-capsule	
Positive	15
Negative	77
Number of tumors	
Solitary	23
Multiple	69
Primary lesion site	
Colon	53
Rectum	39
Degree of differentiation	
Well, moderately	85
Poorly, mucinous	7

Colorectal cancer cell line and culture medium

The colorectal cancer cell line, SW48 (American Type Culture Collection, Manassas, VA, USA) was cultured in Leibovitz's L-15 (Sigma Aldrich, St. Louis, MO, USA) medium supplemented with 1% penicillin and streptomycin and 10% fetal bovine serum (FBS) [26].

CXCR4 expression determined by fluorescence microscopy

SW48 cells (5×10^5/well in 800 µl) were seeded in four-well slide chambers (LabTek® II Chamber Slide™ System, Nalge Nunc International, Rochester, NY, USA) 24 h and then serum-starved (0.5% FBS) overnight. The cells were stimulated with CXCL12 (200 ng/ml: R&D Systems,) at 37°C for 24 h, fixed in 4% paraformaldehyde at room temperature for 10 min, and then incubated at room temperature for 10 min with 0.3% Triton X-100 and 1.0% bovine serum albumin in PBS. Fixed and permeabilized cells were stained with rabbit anti-human CXCR4 antibody for 60 min at room temperature, followed by anti-rabbit Alexa Fluor® 488 (Invitrogen, Carlsbad, CA, USA) and DAPI (Invitrogen, Carlsbad, CA, USA) for 30 min at room temperature. CXCR4 internalization was then analyzed by fluorescence microscopy.

Statistical analysis

Data were compared using the rank sum test. Associations between discrete variables were assessed using the Fisher exact probability test. Recurrence or death was estimated using the Kaplan–Meier method and statistical significance was examined using the log-rank test. Multivariate analysis was determined using the Cox proportional hazards model. Data were analyzed using SigmaStat 3.0 and SPSS 11.5 software. All data are expressed as means ± SD. Results were considered significant at $P < 0.05$.

Results

CXCR4 expression in metastatic liver tumors

We immunohistochemically examined whether CXCR4 expression in tumors increases in patients with colorectal liver metastases as follows. CXCR4 expression (staining intensity) was compared to that of hepatocytes in specimens of colorectal liver metastases from 92 patients (Fig. 1). The patients were assigned to groups according to the staining intensity of CXCR4 relative to that of hepatocytes. Levels of CXCR4 expression in the cytoplasm of tumor cells were high in 36 (39%) and low in 56 (61%) of the 92 patients. The CXCR4 was also expressed in the nuclei of tumor cells in some patients with colorectal liver metastases. Levels of nuclear CXCR4 expression were high in 23 (25%) and low in 69 (75%) of the 92 patients examined (Fig. 1a, b and c).

CXCR4 expression in metastatic liver tumors and patients' characteristics

We analyzed the characteristics of the patients to determine whether CXCR4 expression in metastatic liver tumors correlates with clinicopathological parameters. Levels of CXCR4 expression in metastatic liver tumors and clinicopathological parameters are shown in Table 2. A fibrous pseudo-capsule defined by HE staining surrounding the tumors of some patients with colorectal liver metastases [24] adversely correlated with high, but not low levels of CXCR4 expression ($P = 0.040$), whereas other clinicopathological parameters did not significantly differ between the two groups (Table 2).

CXCL12 expression in metastatic liver tumors and patient characteristics

We immunohistochemically determined CXCL12 expression in CLM from 92 patients (Fig. 1d, e, and f). Levels of cytoplasmic CXCL12 expression in tumor cells were high in 51 (55%) and low in 41 (45%) of the 92 patients examined, whereas correlations between clinicopathological parameters and CXCL12 expression did not significantly differ between these two groups (Table 2). CXCL12 was also expressed in hepatocytes surrounding the tumors at high and low levels in 68 (74%) and 24 (26%), respectively, of the 92 patients examined (Fig. 1d, e, and f).

Relationship between CXCR4 and CXCL12 expression

Correlations between cytoplasmic CXCL12 and CXCR4 expression in metastatic liver tumors did not significantly differ between the two groups ($P = 0.206$; Table 2). Whereas, the correlations between nuclear CXCR4 expression in tumor cells and CXCL12 expression in hepatocytes significantly differed ($P = 0.030$; Table 2).

Fluorescence microscopy of CXCR4 expression in the SW48 colon cancer cell line

Fluorescence microscopy revealed membrane and cytoplasmic CXCR4 expression in colon cancer cells after 24 h of culture in mock medium (Fig. 2) and increased nuclear expression of CXCR4 after 24 h of exposure to CXCL12. This was confirmed by merged light blue signals of DAPI and Alexa 488 (CXCR4) in nuclei (Fig. 2).

CD133 expression in metastatic liver tumors

An immunohistochemical analysis of tumors revealed high and low CD133 expression in 28 (30%) and 64 (70%) of the 92 patients examined.

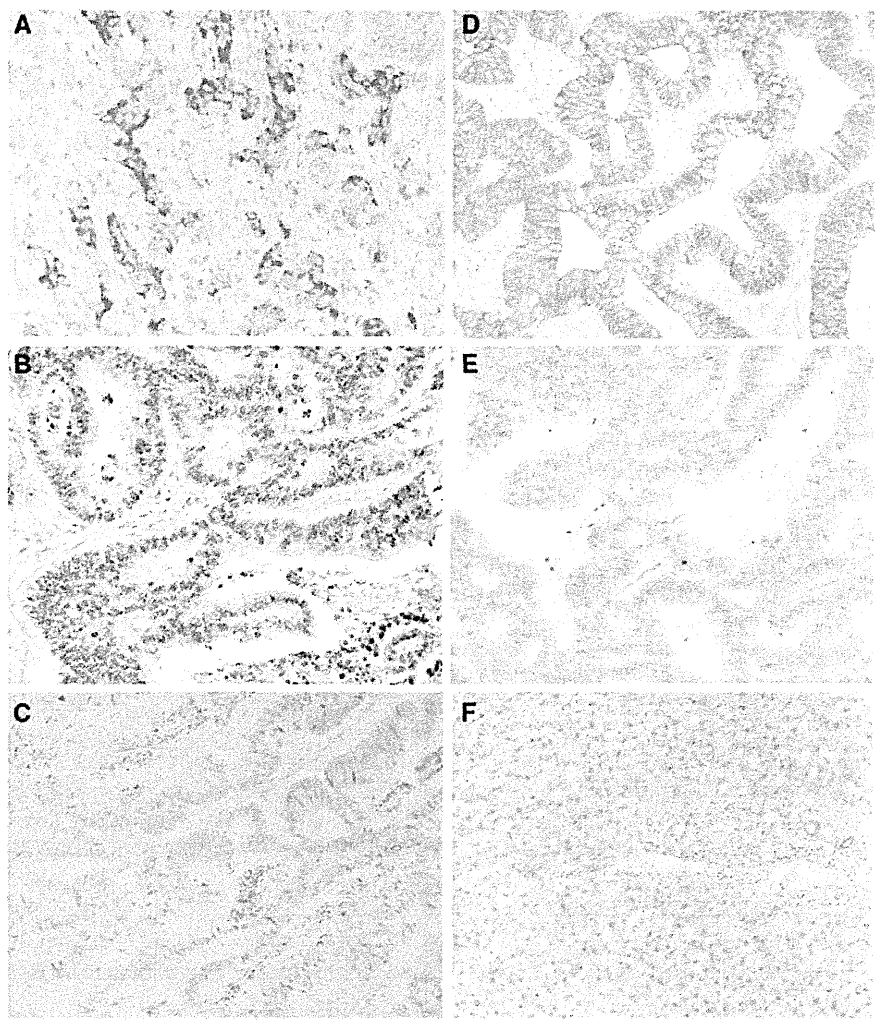

Fig. 1 Immunohistochemical assessment of expression of CXCR4 **a–c** in metastatic liver tumors and of CXCL12 **d–f** in tumors and hepatocytes. **a** Cytoplasmic, **b** nuclear and **c** absent CXCR4 expression. **d** High, **e** low and **f** positive CXCL12 expression in hepatocytes. Original magnification ×200

Table 2 Clinicopathological characteristics are associated with CXCR4 and CXCL12 expression

Clinicopathological characteristics	CXCR4 expression (cytoplasm)			CXCL12 expression		
	High ($n = 36$)	Low ($n = 56$)	P value*	High ($n = 51$)	Low ($n = 41$)	P value*
Age (≥60 years)	26	30	0.084	28	28	0.21
Gender (Male)	22	36	0.83	29	29	0.20
Lymph node metastasis of primary lesion (+)	21	37	0.51	34	24	0.52
Timing of metastasis (synchronous)	18	26	0.83	22	22	0.40
Tumor size (≥50 mm)	11	15	0.81	13	13	0.64
Fibrous capsule (+)	2	13	**0.040**	9	6	0.78
Number of tumors (≥2)	9	14	>0.9	12	11	0.81
Primary lesion site (Colon)	17	36	0.13	30	23	0.83
Degree of differentiation (well, moderately)	33	52	>0.9	47	38	>0.9

CXCL12 expression (Tumor)	CXCR4 expression (tumor)		P value*
	High ($n = 36$)	Low ($n = 56$)	
High ($n = 51$)	23	28	0.206
Low ($n = 41$)	13	28	

CXCL12 expression (Liver)	CXCR4 expression (nucleus)		P value*
	High ($n = 23$)	Low ($n = 69$)	
High ($n = 68$)	21	47	0.030
Low ($n = 24$)	2	22	

*Fisher probability test

Bold values indicate statistically significance

CXCR4 and CXCL12 expression correlated with survival

We analyzed overall survival using the Kaplan–Meier method to determine whether CXCR4 and CXCL12 expression in metastatic liver tumors is related to overall survival after hepatic resection. The results showed a significantly lower overall survival rate in patients with high cytoplasmic and no nuclear CXCR4 expression in tumor cells than in any other groups (Fig. 3a). The overall survival rates were significantly higher in patients with high levels of nuclear CXCR4 expression than in those with none (Fig. 3b), and in those with positive, than negative CXCL12 expression in hepatocytes (Fig. 3c). These rates were significantly lower in patients with positive, than with negative CD133 expression in tumors (Fig. 3d).

We examined whether the CXCR4/CXCL12 expression is a significant prognostic factor for overall and hepatic disease-free survival after hepatic resection using univariate and multivariate analyses. Univariate analysis demonstrated that the number of metastases, primary lymph node involvement, nuclear CXCR4 expression in tumor cells, CXCL12 expression in hepatocytes, and high levels of CD133 expression in tumors were significant prognostic factors for overall survival (Table 3). The number of metastases, tumor size, timing of metastases and CXCL12 expression in hepatocytes were significant prognostic factors for hepatic disease-free survival (Table 3). Multivariate analysis showed that positive primary lymph node involvement, low nuclear CXCR4 expression in tumor cells and low CXCL12 expression in hepatocytes were significant independent prognostic factors for overall survival, and that the timing of metastases, low levels of nuclear CXCR4 expression in tumor cells and low CXCL12 expression in hepatocytes were significant independent prognostic factors for hepatic disease-free survival in patients with colorectal liver metastases (Table 3).

Discussion

This study demonstrated that the expression profiles of CXCR4/CXCL12 correlate with clinical outcomes and might be altered by the tumor microenvironment, thus leading to a change in tumor behavior in CLM. The expression of CXCR4/CXCL12 in terms of chemotactic properties and tumor progression in several malignancies has been described, but expression profiles were analyzed as an intrinsic activity in the tumor environment like that of colorectal carcinoma. In addition, the relationship between

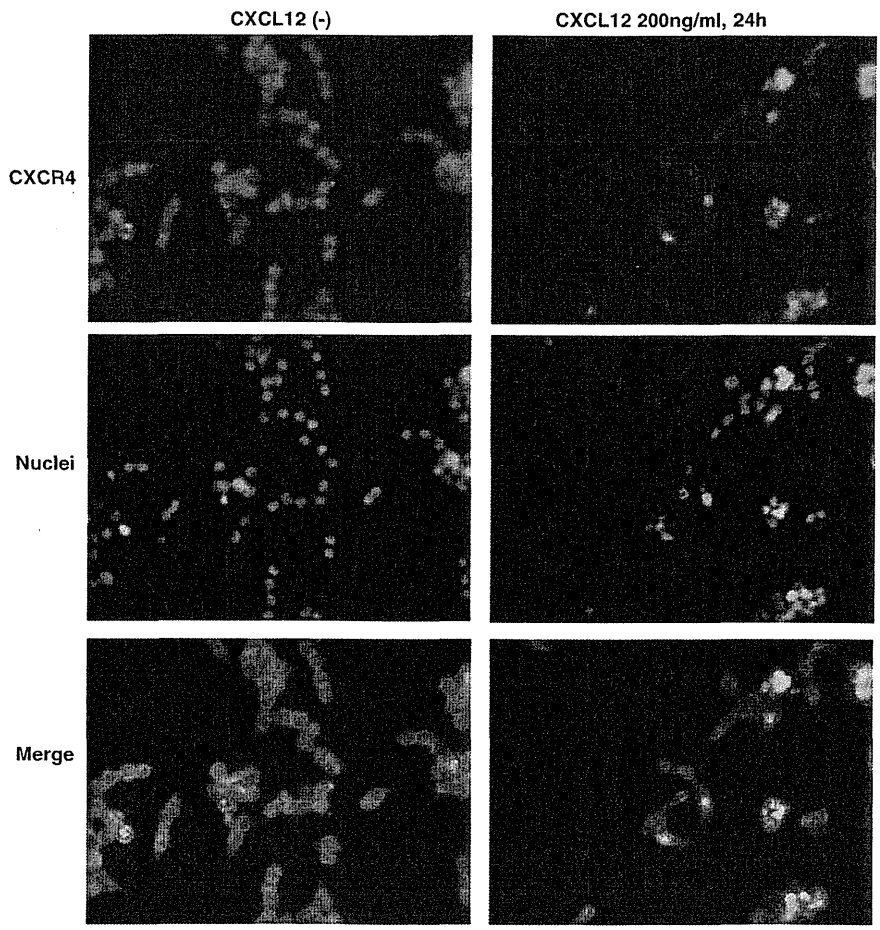

Fig. 2 Nuclear localization of CXCR4 after CXCL12 stimulation in SW48 colon cancer cells. SW48 cells were stimulated with 200 ng/ml CXCL12 for 24 h and then immunostained and observed by fluorescence microscopy. *Green*, membrane and cytoplasmic CXCR4; *deep blue*, nucleus. Some CXCR4 have colocalized in the nucleus (light blue) of stimulated SW48 cells. (Color figure online)

CXCR4/CXCL12 expression and prognosis has not been investigated in a large number of colorectal liver metastases.

The metastatic mechanisms of epithelial-to-mesenchymal transition (EMT) and tumor microenvironment have recently become widely recognized [27]. Chemokines and their receptors were originally identified by the ability to generate chemoattractive interactions between immune cells and sites of inflammation [6]. Recent observations have revealed that cancer cells utilize chemokine networks to modulate the host microenvironment in favor of cancer progression [28]. Several chemokines and their receptors such as CXCR2, CXCR3, CXCR4, CXCR7, CCR2, CCR6, and CCR7 are involved in colorectal cancer [12–15, 29–31]. Interactions between CXCR4 and CXCL12 are considered to play a role in the progression of colon cancer [13, 32]. The EMT is also mediated by the CXCR4/CXCL12 system [33, 34]. These findings suggested that CXCR4 plays a role in tumor progression and metastasis.

Cytoplasmic CXCR4 expression correlates with tumor recurrence and clinical outcomes in several cancers including colon cancer [12, 17, 35]. Circulating cytoplasmic CXCR4-positive tumor cells might be responsible for extravasation and the organ-specific metastasis induced by CXCL12 gradients [29]. The liver is one metastatic site that results from interaction between CXCL12 and CXCR4. The cytoplasmic expression and role of CXCR4 are controversial. The present study found that cytoplasmic CXCR4 expression did not reach statistical difference for poor overall survival and that it did not correlate with CXCL12 expression in metastatic colorectal cancer cells. Therefore, the expression and role of cytoplasmic CXCR4 probably differs between metastatic and primary sites, which would be consistent with the findings of Shim et al. [22].

Some types of cancers express nuclear CXCR4, but its precise function has not been fully elucidated [20, 21, 36–39]. Nuclear CXCR4 expression correlates with a better prognosis in non-small cell lung cancer [20]. Consistent with this finding, nuclear CXCR4 expression closely correlated with better overall and hepatic disease-free survival in the present study. Upregulated nuclear CXCR4 expression might counteract the effect of the cytoplasmic CXCR4 expression that is associated with a poor prognosis. On the other hand, nuclear CXCR4 expression is associated with a poor prognosis in primary colon cancer [21, 38]. Like the

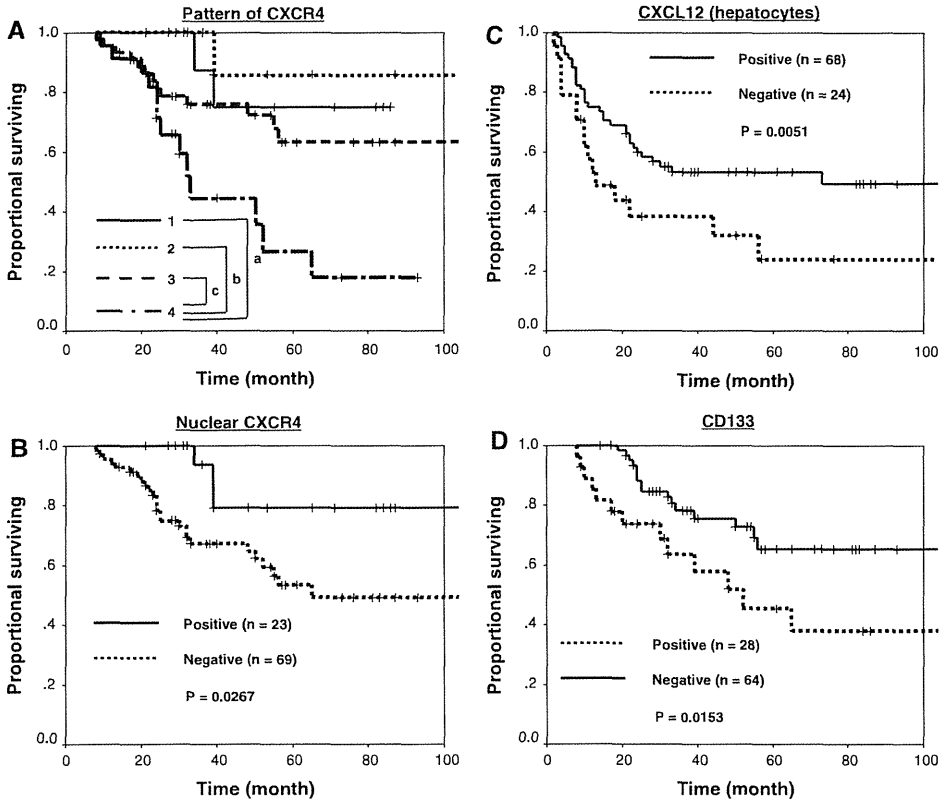

Fig. 3 Overall survival rates stratified by expression of **a** cytoplasmic and nuclear **b** CXCR4. *CY* cytoplasmic expression, *NC* nuclear expression. 1, CY (−) + NC (+); 2, CY (+) + NC (+); 3, CY (−) + NC (−); 4, CY (+) + NC (−). Expression profiles significantly differed (a, $P = 0.0022$; b, $P = 0.0113$; c, $P = 0.0116$). Overall survival rates stratified by expression of **b** nuclear CXCR4, **c** hepatocyte CXCL12, and **d** CD133

role of epidermal growth factor receptor (EGFR) in association with cyclin D1, internalized CXCR4 might act as a transcription factor [38, 40] that could induce tumor progression accompanied by NF-κB activation [41]. However, our data contradict this notion. Although the precise molecular mechanism and role of nuclear CXCR4 expression remain unknown, one theory has been proposed [11, 22, 42–44]. Upregulated CXCL12 in hepatocytes was an independent significant prognosis factor for both overall and hepatic disease-free survival. The results of our fluorescence microscopy study of CXCR4 expression in cancer cell line are consistent with the finding that increased CXCL12 levels cause nuclear CXCR4 internalization in cancer cell lines in vitro [44], and suggest that the tumor microenvironment alters CXCR4 expression profiles in cancer cells. Spano et al. demonstrated that interaction between CXCL12 and CXCR4 might be disrupted when CXCR4 is expressed in the nucleus [20]. Inhibiting CXCR4 and CXCL12 interaction is likely to reduce metastases [45]. These findings suggest that further metastases into organs other than the liver might not be induced by the tumor microenvironment and if so, curative hepatic resection for metastases would improve survival. Nuclear CXCR4 and hepatocyte CXCL12 expression correlated in the current study, suggesting that consistently upregulated CXCL12 expression in the liver alters the microenvironment, reduces cytomembrane CXCR4 expression and leads to CXCR4 internalization. Thus, the tumor microenvironment might be altered in a specific manner after liver metastasis becomes established.

CXCR7 has recently been identified as another receptor for CXCL12 [29]. CXCR4 and CXCL12 expression did not correlate in tumor cells, suggesting that CXCR7 is associated with metastases of colorectal cancer. Although we have no further information about CXCR7 expression, further investigation of interactions between CXCL12 and CXCR7 might be warranted. CXCL12/CXCR4 expression accumulates in CD133 positive tumor cells that might, in part, be derived from cancer stem cells [18, 19]. CD133 expression is an independent prognostic marker of poor survival in colorectal cancer [46]. The present study found that increased CD133 expression tended to indicate poor overall survival, suggesting that that it plays a specific role in the progression of colorectal liver metastases.

Table 3 Univariate and multivariate analysis of predictive factors for overall and hepatic disease-free survival

Factors	Overall survival		Hepatic disease-free survival	
	3-year (MST; months)	*P* value*	3-year (MST; month)	*P* value*
Timing of metastases				
Synchronous ($n = 44$)	65% (65)	0.17	28% (18)	0.0003
Metachronous ($n = 48$)	65%		68%	
Tumor size				
<50 mm ($n = 71$)	76%	0.13	56% (73)	0.0087
≥50 mm ($n = 21$)	67% (52)		26% (17)	
Primary lymph node involvement				
Negative($n = 34$)	89%	0.0128	60%	0.13
Positive ($n = 58$)	66% (56)		43% (24)	
Number of metastases				
≤4 ($n = 74$)	81%	0.0084	55% (73)	0.0044
≥5 ($n = 18$)	48% (32)			
Cytoplasm CXCR4 expression				
Low ($n = 56$)	78%	0.14	51% (56)	0.76
High ($n = 36$)	67%		45% (30)	
Nuclear CXCR4 expression				
Negative ($n = 69$)	67%	0.0267	43% (23)	0.064
Positive ($n = 23$)	93%		68%	
Hepatocyte CXCL12 expression				
Negative ($n = 24$)	49% (25)	0.0051	38% (13)	0.0268
Positive ($n = 68$)	80%		53% (73)	
CD133 expression				
Low ($n = 64$)	78%	0.0153	49% (33)	0.78
High ($n = 28$)	64% (52)		49% (30)	

Factors	95% confidence intervals			
	Relative risk	Lower	Upper	*P* value**
Overall survival				
Timing of metastases (synchronous)	1.27	0.52	3.09	0.60
Tumor size (≥50 mm)	0.94	0.37	2.37	0.89
Primary lymph node involvement (positive)	3.87	1.40	10.7	**0.009**
Number of metastases (≥5)	2.05	0.75	5.59	0.16
Cytoplasm CXCR4 expression (low)	0.43	0.18	1.02	0.056
Nuclear CXCR4 expression (low)	4.05	1.19	13.8	**0.025**
Hepatocyte CXCL12 expression (low)	2.75	1.17	6.55	**0.022**
CD133 expression (high)	2.03	0.94	4.37	0.070
Hepatic disease-free survival				
Timing of metastases (synchronous)	2.54	1.34	4.83	**0.004**
Tumor size (≥50 mm)	1.82	0.92	3.58	0.083
Primary lymph node involvement (positive)	1.73	0.90	3.33	0.099
Number of metastases (≥5)	1.38	0.64	2.98	0.41
Cytoplasm CXCR4 expression (low)	0.89	0.49	1.63	0.71
Nuclear CXCR4 expression (low)	2.40	1.08	5.33	**0.031**
Hepatocyte CXCL12 expression (low)	2.09	1.10	3.98	**0.025**
CD133 expression (high)	1.01	0.53	1.92	>0.9

MST median survival time (months)

* Log-rank test, ** Cox proportional hazards model

Bold values indicate statistically significance

The present study identified a correlation between CXCR4 expression and a fibrous pseudocapsule (FC) surrounding the tumor. According to a previous report, survival is better among patients with, than without FC formation [24]. However, the mechanism for such formation remains obscure. A previous and the present study found a significantly lower microvessel density in patients with, than without FC (data not shown). The tumor environment inside FC might be hypoxic, which would reduce CXCR4 expression [44] and a hypoxic tumor environment beneath FC might further inhibit cytoplasmic CXCR4 expression and tumor progression. Notably, one study has shown that hepatic stellate cells express CXCR4 [47]. Although the mechanism of FC formation has not been clarified, CXCL12 upregulation in the liver probably induces the activation of hepatic stellate cells which produce a fibrotic matrix, thus forming FC.

High levels of nuclear CXCR4 and hepatocyte CXCL12 expression are associated with overall and hepatic disease-free survival compared with other reported clinical factors [23–25]. Thus, the expression profiles of CXCR4 and CXCL12 might predict remnant liver cancer recurrence and a poor prognosis. In conclusion, CXCR4/CXCL12 may play crucial roles in the biologically aggressive behavior of colorectal liver metastasis, and CXCR4 expression in metastatic liver tumors together with CXCL12 upregulation in hepatocytes may be useful to predict the clinical outcomes of patients with CLM after hepatectomy. Further studies are required to define the role of nuclear CXCR4 expression in patients with colorectal liver metastasis.

Acknowledgments This work was supported in part by grants from the Japan Society for the Promotion of Science (Grant# 19591577, 22591500).

References

1. Rosen M, Chan L, Beart RW Jr et al (1998) Follow-up of colorectal cancer: a meta-analysis. Dis Colon Rectum 41:1116–1126
2. Nordlinger B, Guiguet M, Vaillant JC et al (1996) Surgical resection of colorectal carcinoma metastases to the liver. A prognostic scoring system to improve case selection, based on 1568 patients. Association Française de Chirurgie. Cancer 77:1254–1262
3. Fong Y, Cohen AM, Fortner JG et al (1997) Liver resection for colorectal metastases. J Clin Oncol 15:938–946
4. Paget S (1889) The distribution of secondary growth in cancer. Lancet 1:571–573
5. Kakinuma T, Hwang ST (2006) Chemokines, chemokine receptors, and cancer metastasis. J Leukoc Biol 79:639–651
6. Oppenheim JJ, Zachariae CO, Mukaida N et al (1991) Properties of the novel proinflammatory supergene "intercrine" cytokine family. Annu Rev Immunol 9:617–648
7. Murphy PM (2001) Chemokines and the molecular basis of cancer metastasis. N Engl J Med 345:833–835
8. Burger JA, Kipps TJ (2006) CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. Blood 107:1761–1767
9. Strieter RM, Burdick MD, Mestas J et al (2006) Cancer CXC chemokine networks and tumour angiogenesis. Eur J Cancer 42:768–778
10. Zlotnik A (2006) Chemokines and cancer. Int J Cancer 119: 2026–2029
11. Koshiba T, Hosotani R, Miyamoto Y et al (2000) Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: a possible role for tumor progression. Clin Cancer Res 6:3530–3535
12. Kim J, Mori T, Chen SL et al (2006) Chemokine receptor CXCR4 expression in patients with melanoma and colorectal cancer liver metastases and the association with disease outcome. Ann Surg 244:113–120
13. Schimanski CC, Schwald S, Simiantonaki N et al (2005) Effect of chemokine receptors CXCR4 and CCR7 on the metastatic behavior of human colorectal cancer. Clin Cancer Res 11:1743–1750
14. Ghadjar P, Coupland SE, Na IK et al (2006) Chemokine receptor CCR6 expression level and liver metastases in colorectal cancer. J Clin Oncol 24:1910–1916
15. Yoshidome H, Kohno H, Shida T et al (2009) Significance of monocyte chemoattractant protein-1 in angiogenesis and survival in colorectal liver metastases. Int J Oncol 34:923–930
16. Kollmar O, Rupertus K, Scheuer C et al (2007) Stromal cell-derived factor-1 promotes cell migration and tumor growth of colorectal metastasis. Neoplasia 9:862–870
17. Kim J, Takeuchi H, Lam ST et al (2005) Chemokine receptor CXCR4 expression in colorectal cancer patients increases the risk for recurrence and for poor survival. J Clin Oncol 23:2744–2753
18. Miki J, Furusato B, Li H et al (2007) Identification of putative stem cell markers, CD133 and CXCR4, in hTERT-immortalized primary nonmalignant and malignant tumor-derived human prostate epithelial cell lines and in prostate cancer specimens. Cancer Res 67:3153–3161
19. Lin EH, Hassan M, Li Y et al (2007) Elevated circulating endothelial progenitor marker CD133 messenger RNA levels predict colon cancer recurrence. Cancer 110:534–542
20. Spano JP, Andre F, Morat L et al (2004) Chemokine receptor CXCR4 and early-stage non-small cell lung cancer: pattern of expression and correlation with outcome. Ann Oncol 15:613–617
21. Yoshitake N, Fukui H, Yamagishi H et al (2008) Expression of SDF-1 alpha and nuclear CXCR4 predicts lymph node metastasis in colorectal cancer. Br J Cancer 98:1682–1689
22. Zhang L, Yeger H, Das B et al (2007) Tissue microenvironment modulates CXCR4 expression and tumor metastasis in neuroblastoma. Neoplasia 9:36–46
23. Minagawa M, Makuuchi M, Torzilli G et al (2000) Extension of the frontiers of surgical indications in the treatment of liver metastasis from colorectal cancer: long-term results. Ann Surg 231:487–499
24. Ambiru S, Miyazaki M, Isono T et al (1999) Hepatic resection for colorectal metastases: analysis of prognostic factors. Dis Colon Rectum 42:632–639
25. Fong Y, Fortner J, Sun RL et al (1999) Clinical score for predicting recurrence after hepatic resection for metastatic colorectal cancer: analysis of 1001 consecutive cases. Ann Surg 230: 309–318
26. Hamada K, Monnai M, Kawai K et al (2008) Liver metastasis models of colon cancer for evaluation of drug efficacy using NOD/Shi-scid IL2Rγnull (NOG) mice. Int J Oncol 32:153–159
27. Tse JC, Kalluri R (2007) Mechanisms of metastasis: epithelial-to-mesenchymal transition and contribution of tumor microenvironment. J Cell Biochem 101:816–829