in the sum of the products of two perpendicular diameters of all lesions for a minimum of 4 weeks. Progressive disease was defined as an increase of 25% or more in the sum of the products of two perpendicular diameters of all lesions, or the appearance of any new lesion. Progression-free survival time was defined as the time from the date of initial treatment to the first documentation of progression or death. Overall survival was measured from the date of initial treatment to date of death or the date of the last follow-up. Progression-free and overall survival times were calculated by the Kaplan–Meier method. Serum carcinoembryonic antigen (CEA) levels and serum carbohydrate antigen 19-9 (CA19-9) levels were measured at least every 8 weeks by a radioimmunometric assay using the Centocor radioimmunoassay kit (Centocor Inc., Malvern, PA, USA).

RESULTS

Patient characteristics

Twenty-one patients were enrolled in this study from May 2004 and November 2005 at the National Cancer Center Hospital, Tokyo, and the National Cancer Center Hospital East, Kashiwa, Chiba, Japan. The characteristics of the patients are listed in Table 2. The median age was 59 years (range: 51–74). Karnofsky performance status was 100 in 12 patients (57%), 90 in 8 (38%) and 80 in one (5%). The median maximum tumour size was 37 mm (range: 25–60), and the median planning target volume was 265 cm^3 (range: 153–408). The causes of the unresectable PCs were invasion of the celiac trunk in nine patients, invasion of the superior mesenteric artery in eight patients and invasion of both regions in four patients. Patients were treated with S-1 and concurrent radiation over four dose levels, as listed in Table 1. After completion of chemoradiotherapy, 20 patients (95%) received gemcitabine alone for their cancer until disease progression, and one patient received the other treatment at another hospital.

Table 2 Patient characteristics

Characteristics	Number of patients	%
Age (years)		
Median	59	
Range	51–74	
Gender		
Male	9	43
Female	12	57
Kamofsky performance status		
100	12	57
90	8	38
80	1	5
Tumour location		
Head	13	62
Body-tail	8	38
Maximum tumour size (mm)		
Median	37	
Range	25–60	
CEA (ng/ml)		
Median	4.5	
Range	1.0–75.0	
CA19-9 (U/ml)		
Median	759	
Range	1–6,300	

CEA = carcinoembryonic antigen; CA19–9 = carbohydrate antigen 19–9.

Toxicity

The toxicities observed in the 21 enrolled patients are listed in Table 3. With regard to overall haematological toxicity, grade 3 neutropenia was observed in only one patient at the dose of level 1, and other grades 3–4 toxicities were not observed. For non-haematological toxicity, grade 3 anorexia and nausea (three patients), grade 3 vomiting (one patient) and grade 3 haemorrhagic gastritis (one patient) occurred at level 3, and grade 3 AST elevation was observed in a patient at level 4. As a late toxicity, duodenal ulcer with epigastralgia was observed in one patient at level 3 (S-1 70 mg m^{-2}) 8 months after chemoradiotherapy, requiring embolisation of the gastroduodenal artery to treat severe bleeding from the ulcer and a 2-month hospital stay. No other grades 3–4 nonhaematological toxicities or treatment-related deaths occurred in this study. Treatment was suspended in four patients (level 2, one; level 3, two; level 4, one patient) because of obstructive jaundice (two patients) or grade 3 anorexia (two patients); the durations of S-1 dose withholding were 3, 12, 2 and 13 days, respectively. One patient with grade 3 anorexia (level 3) was unable to resume this treatment. The compliance rate of the patients taking S-1 was as high as 99% (1170/1176 doses).

There was no occurrence of DLT at the dose of levels 1 or 2, but two of six patients who received a level 3 dose experienced DLT; one of these patients required suspension of treatment for more than 12 days due to grade 3 anorexia, nausea and vomiting after the third fraction of chemoradiotherapy, and a second developed grade 3 haemorrhagic gastritis after completion of 13 fractions. However, no DLT at a dose of level 4 was observed, and S-1 at 80 mg m^{-2} with concurrent radiotherapy was considered to be well-tolerated.

Five patients (level 2, two; level 3, two; level 4, one) of the 21 who were enrolled had to abandon this treatment. Two patients at level 2 developed massive ascites and infarction of the cerebellum, respectively, during chemoradiotherapy. The cause of the massive ascites was disease progression, as cancer cells were confirmed in the ascitic fluid. The cerebellar infarction was considered to have been unrelated to the treatment, because the patient had a history of the same problem. Two patients at level 3 had to discontinue the treatment because of DLT according to the protocol, and one patient at level 4 decided to stop the treatment, despite lack of severe toxicity, at her own request.

Efficacy

All the patients were included in the response evaluation. Four patients (levels 1 and 2, 0; level 3, one; level 4, three) achieved a partial response, giving an overall response rate of 19% (95% confidence interval, 5–42%). Four patients (19%) showed a minor response, and nine (43%) and three patients (14%) had no change and progressive disease, respectively. Tumor response could not be evaluated in one patient (5%), because she was transferred to another hospital to seek some other treatment for her PC. None of the patients' conditions improved to resectable or operable diseases after the completion of treatment. After the start of chemoradiotherapy, the serum CA19-9 level was reduced by more than 50% compared to the pretreatment level in 14 (88%) of 16 patients who had shown a pretreatment level of 100 U/ml or greater, and the serum CEA level was reduced by more than 50% relative to the pretreatment level in three (100%) of three patients who had a pretreatment level of 10 ng ml^{-1} or greater. Eighteen of the 21 patients had disease progression at the time of analysis. The pattern of disease progression was distant metastases in 11 (52%), deterioration of general condition in five (24%) and locoregional recurrence in two patients (10%). The median progression-free survival time for all patients was 8.9 months (Figure 1). At the time of analysis, 13 patients had died due to tumour progression. The median survival time and 1-year survival rate for patients as a whole were 11.0 months and 42.9%, respectively (Figure 1).

Table 3 Toxicity

	Number of patients											
	Level 1 ($n=3$)			Level 2 ($n=5$)			Level 3 ($n=6$)			Level 4 ($n=7$)		
Grade	1,2	3	4	1,2	3	4	1,2	3	4	1,2	3	4
Leucocytes	3	0	0	3	0	0	3	0	0	6	0	0
Neutrophiles	1	1	0	1	0	0	2	0	0	3	0	0
Haemoglobin	0	0	0	2	0	0	1	0	0	4	0	0
Platelets	0	0	0	1	0	0	1	0	0	2	0	0
Anorexia	2	0	0	3	0	0	1	3	0	5	0	0
Nausea	0	0	0	2	0	0	1	3	0	6	0	0
Vomiting	1	0	0	0	0	0	2	1	0	3	0	0
Diarrhoea	1	0	0	0	0	0	0	0	0	0	0	0
Mucositis	0	0	0	0	0	0	0	0	0	0	0	0
Fatigue	2	0	0	2	0	0	2	0	0	2	0	0
Gastritis	0	0	0	0	0	0	0	1	0	0	0	0
Duodenal ulcer	0	0	0	0	0	0	0	1	0	0	0	0
Bilirubin	1	0	0	0	0	0	0	0	0	0	0	0
Hypoalbuminaemia	1	0	0	1	0	0	3	0	0	5	0	0
AST	1	0	0	1	0	0	4	0	0	2	0	0
ALT	1	0	0	0	0	0	3	0	0	1	1	0
Alkaline phosphatase	0	0	0	0	0	0	1	0	0	2	0	0
Creatinine	0	0	0	0	0	0	1	0	0	0	0	0

AST = aspartate aminotransferase; ALT = alanine aminotransferase.

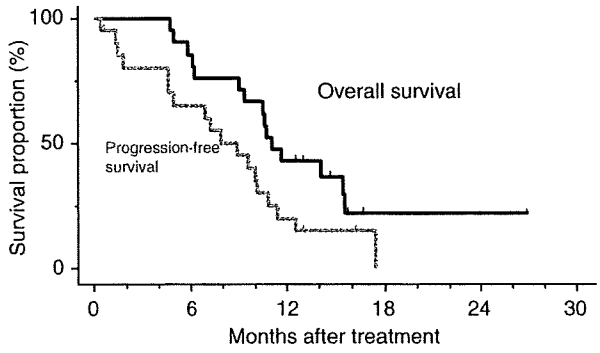

Figure 1 Overall survival and progression-free survival curves of 21 patients who received S-1 with concurrent radiotherapy for locally advanced pancreatic cancer. Tick marks indicate censored cases.

DISCUSSION

On the basis of results of previous randomised controlled trials (Moertel *et al*, 1969, 1981; Gastrointestinal Tumor Study Group, 1988), the combination of 5-FU therapy and radiotherapy has become a frequently employed treatment for locally advanced PC (Willett *et al*, 2005; Yip *et al*, 2006). Because of the modest survival benefit of 5-FU-based chemoradiotherapy, numerous investigators are pursuing phase I and II trials of radiotherapy with new chemotherapeutic agents such as gemcitabine, paclitaxel, capecitabine, bevacizumab, gefitinib and erlotinib (Blackstock *et al*, 2003; Okusaka *et al*, 2004; Rich *et al*, 2004; Crane *et al*, 2006; Czito *et al*, 2006). However, no marked improvement of survival has been observed. S-1 is an oral fluoropyrimidine derivative that has demonstrated excellent efficacy with mild toxicity in patients with metastatic PC (Furuse *et al*, 2005). It is considered to be beneficial because of its convenience of being administered by the oral route. In addition, combined S-1 and radiotherapy has been demonstrated to exert a synergistic effect against 5-FU-resistant cancer xenografts (Harada *et al*, 2004; Nakata *et al*, 2006). Therefore, a clinical trial of concurrent radiotherapy with S-1 therapy for locally advanced PC was designed to intensify the treatment efficacy and improve the convenience of administration.

In this study, a limited radiation field, of which the planning target volume included only the gross tumour volume without prophylactic nodal irradiation, was adopted to minimise the volume of normal tissue treated, because our retrospective study showed that a larger planning target volume for irradiation was the significant predictor of severe acute gastrointestinal toxicity in patients treated with chemoradiotherapy (Ito *et al*, 2006). A similar radiation field has been attempted in recent reported trials of chemoradiotherapy to decrease the degree of gastrointestinal toxicity (Muler *et al*, 2004; Crane *et al*, 2006). Gastrointestinal toxicities, such as anorexia, nausea and vomiting, are major troublesome adverse events during chemoradiotherapy, necessitating intravenous fluid infusion and sometimes discontinuation of chemoradiotherapy (Talamonti *et al*, 2000; Crane *et al*, 2002; McGinn and Zalupski, 2003; Okusaka *et al*, 2004). In the present study, some gastrointestinal toxicities were observed, but were easily managed. Moreover, the limited radiation field used in this study did not result in excess failures in the border of radiation field, because locoregional recurrence was observed in only two patients of this series.

In this study, DLT was observed in only two patients at level 3 (S-1 70 mg m^{-2}). The DLT in the first patient was grade 3 anorexia, nausea and vomiting, requiring suspension of treatment for longer than 12 days, and the second DLT was grade 3 haemorrhagic gastritis. Other than DLT toxicity, acute grades 3–4 toxicities during chemoradiotherapy were observed in only three patients: grade 3 neutropenia, grade 3 anorexia and nausea, and grade 3 AST elevation in one patient each. As a late toxicity, duodenal ulcer was observed 8 months after treatment in one patient at level 3, but no other late toxicity occurred. Accordingly, S-1 at a daily dose of 80 mg m^{-2} (level 4) was considered to be well tolerated, and this dose was deemed recommendable.

In patients with locally advanced PC who are receiving chemoradiotherapy, it is important to enhance local control while simultaneously reducing the risk of distant metastases. In concurrent gemcitabine-based chemoradiotherapy, both full-dose gemcitabine and standard-dose radiotherapy are difficult to administer because of their associated toxicities (Crane *et al*, 2002; Blackstock *et al*, 2003; McGinn and Zalupski, 2003; Okusaka

et al, 2004). In contrast, in the present trial, the combination of full-dose S-1 ($80\,mg\,m^{-2}$) and standard-dose radiotherapy (50.4 Gy/28 fractions) was easy to administer and had favourable toxicity profiles. Therefore, this regimen might have a dual benefit of counteracting systemic tumour spread as well as acting as a potent radiosensitizer for local control. With regard to the antitumour activity of this treatment, four (19%) of the 21 patients achieved a partial response, and the response rate at the recommended dose was 43% (3/7). The progression-free survival time (median: 8.9 months) and overall survival time (median: 11.0 months) were also favourable as a phase I trial. In this study, many patients (95%) received gemcitabine alone after completion of this regimen. Such adjuvant gemcitabine therapy might influence the efficacy of treatment, although the extent of its impact on tumour response and survival has not been fully elucidated in patients with locally advanced PC. Since both the efficacy and toxicity profile of this regimen appear to be attractive, a phase II trial is required to clarify the antitumour activity, survival and toxicity of S-1 $80\,mg\,m^{-2}\,day^{-1}$ with concurrent radiation therapy for locally advanced PC.

In conclusion, the recommended dose of S-1 with concurrent radiotherapy is $80\,mg\,m^{-2}\,day^{-1}$ on the day of irradiation, and this regimen has a mild toxicity profile while delivering substantial antitumour activity for patients with locally advanced PC. Orally administered S-1 may offer an easy alternative to intravenous 5-FU without impairing the quality of life. A phase II trial of S-1 at the optimal dose of $80\,mg\,m^{-2}\,day^{-1}$ with concurrent radiation in patients with locally advanced PC is now underway in a multi-institutional setting.

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REFERENCES

Blackstock AW, Tepper JE, Niedwiecki D, Hollis DR, Mayer RJ, Tempero MA (2003) Cancer and leukemia group B (CALGB) 89805: phase II chemoradiation trial using gemcitabine in patients with locoregional adenocarcinoma of the pancreas. *Int J Gastrointest Cancer* **34**: 107–116

Crane CH, Abbruzzese JL, Evans DB, Wolff RA, Ballo MT, Delclos M, Milas L, Mason K, Charnsangavej C, Pisters PW, Lee JE, Lenzi R, Vauthey JN, Wong AB, Phan T, Nguyen Q, Janjan NA (2002) Is the therapeutic index better with gemcitabine-based chemoradiation than with 5-fluorouracil-based chemoradiation in locally advanced pancreatic cancer? *Int J Radiat Oncol Biol Phys* **52**: 1293–1302

Crane CH, Ellis LM, Abbruzzese JL, Amos C, Xiong HQ, Ho L, Evans DB, Tamm EP, Ng C, Pisters PW, Charnsangavej C, Delclos ME, O'Reilly M, Lee JE, Wolff RA (2006) Phase I trial evaluating the safety of bevacizumab with concurrent radiotherapy and capecitabine in locally advanced pancreatic cancer. *J Clin Oncol* **24**: 1145–1151

Czito BG, Willett CG, Bendell JC, Morse MA, Tyler DS, Fernando NH, Mantyh CR, Blobe GC, Honeycutt W, Yu D, Clary BM, Pappas TN, Ludwig KA, Hurwitz HI (2006) Increased toxicity with gefitinib, capecitabine, and radiation therapy in pancreatic and rectal cancer: phase I trial results. *J Clin Oncol* **24**: 656–662

Furuse J, Okusaka T, Funakoshi A, Boku N, Yamao K, Ohkawa A, Saito H (2005) A phase II study of S-1 in patients with metastatic pancreatic cancer. *Proc Am Soc Clin Oncol (abstract)* **25**: 104

Gastrointestinal Tumor Study Group (1988) Treatment of locally unresectable carcinoma of the pancreas: comparison of combined-modality therapy (chemotherapy plus radiotherapy) to chemotherapy alone. *J Natl Cancer Inst* **80**: 751–755

Harada K, Kawaguchi S, Supriatno, Onoue T, Yoshida H, Sato M (2004) Combined effects of the oral fluoropyrimidine anticancer agent, S-1 and radiation on human oral cancer cells. *Oral Oncol* **40**: 713–719

Hirata K, Horikoshi N, Aiba K, Okazaki M, Denno R, Sasaki K, Nakano Y, Ishizuka H, Yamada Y, Uno S, Taguchi T, Shirasaka T (1999) Pharmacokinetic study of S-1, a novel oral fluorouracil antitumor drug. *Clin Cancer Res* **5**: 2000–2005

Inuyama Y, Kida A, Tsukuda M, Kohno N, Satake B (2001) Late phase II study of S-1 in patients with advanced head and neck cancer. *Gan To Kagaku Ryoho* **28**: 1381–1390

Ishii H, Okada S, Tokuuye K, Nose H, Okusaka T, Yoshimori M, Nagahama H, Sumi M, Kagami Y, Ikeda H (1997) Protracted 5-fluorouracil infusion with concurrent radiotherapy as a treatment for locally advanced pancreatic carcinoma. *Cancer* **79**: 1516–1520

Ito Y, Okusaka T, Kagami Y, Ueno H, Ikeda M, Sumi M, Imai A, Fujimoto N, Ikeda H (2006) Evaluation of acute intestinal toxicity in relation to the volume of irradiated small bowel in patients treated with concurrent weekly gemcitabine and radiotherapy for locally advanced pancreatic cancer. *Anticancer Res* **26**: 3755–3759

Japan Society for Cancer Therapy (1993) Criteria for the evaluation of the clinical effects of solid cancer chemotherapy. *J Jpn Soc Cancer Ther* **28**: 101–130

Kawahara M, Furuse K, Segawa Y, Yoshimori K, Matsui K, Kudoh S, Hasegawa K, Niitani H (2001) Phase II study of S-1, a novel oral fluorouracil, in advanced non-small-cell lung cancer. *Br J Cancer* **85**: 939–943

McGinn CJ, Zalupski MM (2003) Radiation therapy with once-weekly gemcitabine in pancreatic cancer: current status of clinical trials. *Int J Radiat Oncol Biol Phys* **56**: 10–15

Moertel CG, Childs DS, Reitemeier RJ, Colby MY, Holbrook MA (1969) Combined 5-fluorourail and supervoltage radiation therapy of locally unresectable gastrointestinal cancer. *Lancet* **2**: 865–867

Moertel CG, Frytak S, Hahn RG, O'Connell MJ, Reitemeier RJ, Rubin J, Schutt AJ, Weiland LH, Childs DS, Holbrook MA, Lavin PT, Livstone E, Spiro H, Knowlton A, Kalser M, Barkin J, Lessner H, Mann-Kaplan R, Ramming K, Douglas Jr HO, Thomas P, Nave H, Bateman J, Lokich J, Brooks J, Chaffey J, Corson JM, Zamcheck N, Novak JW (1981) Therapy of locally unresectable pancreatic carcinoma: a randomized comparison of high dose (6000 rads) radiation alone, moderate dose radiation (4000 rads + 5-fluorouracil), and high dose radiation + 5-fluorouracil. *Cancer* **48**: 1705–1710

Muler JH, McGinn CJ, Normolle D, Lawrence T, Brown D, Hejna G, Zalupski MM (2004) Phase I trial using a time-to-event continual reassessment strategy for dose escalation of cisplatin combined with gemcitabine and radiation therapy in pancreatic cancer. *J Clin Oncol* **22**: 238–243

Nakata E, Fukushima M, Takai Y, Nemoto K, Ogawa Y, Nomiya T, Nakamura Y, Milas L, Yamada S (2006) S-1, an oral fluoropyrimidine, enhances radiation response of DLD-1/FU human colon cancer xenografts resistant to 5-FU. *Oncol Rep* **16**: 465–471

Ohtsu A, Baba H, Sakata Y, Mitachi Y, Horikoshi N, Sugimachi K, Taguchi T (2000) Phase II study of S-1, a novel oral fluoropyrimidine derivative, in patients with metastatic colorectal carcinoma S-1. Cooperative Colorectal Carcinoma Study Group. *Br J Cancer* **83**: 141–145

Okusaka T, Ito Y, Ueno H, Ikeda M, Takezako Y, Morizane C, Kagami Y, Ikeda H (2004) Phase II study of radiotherapy combined with gemcitabine for locally advanced pancreatic cancer. *Br J Cancer* **91**: 673–677

Rich T, Harris J, Abrams R, Erickson B, Doherty M, Paradelo J, Small Jr W, Safran H, Wanebo HJ (2004) Phase II study of external irradiation and weekly paclitaxel for nonmetastatic, unresectable pancreatic cancer: RTOG-98-12. *Am J Clin Oncol* **27**: 51–56

Sakata Y, Ohtsu A, Horikoshi N, Sugimachi K, Mitachi Y, Taguchi T (1998) Late phase II study of novel oral fluoropyrimidine anticancer drug S-1 (1 M tegafur–0·4 M gimestat–1 M otastat potassium) in advanced gastric cancer patients.. *Eur J Cancer* **34**: 1715–1720

Shirasaka T, Shimamato Y, Ohshimo H, Yamaguchi M, Kato T, Yonekura K, Fukushima M (1996a) Development of a novel form of an oral 5-fluorouracil derivative (S-1) directed to the potentiation of the tumor selective cytotoxicity of 5-fluorouracil by two biochemical modulators. *Anticancer Drugs* **7**: 548–557

Shirasaka T, Nakano K, Takechi T, Satake H, Uchida J, Fujioka A, Saito H, Okabe H, Oyama K, Takeda S, Unemi N, Fukushima M (1996b) Antitumor activity of 1 M tegafur–0.4 M 5-chloro-2,4-dihydroxypyridine–1 M potassium oxonate (S-1) against human colon carcinoma orthotopically implanted into nude rats. *Cancer Res* **56**: 2602–2606

Talamonti MS, Catalano PJ, Vaughn DJ, Whittington R, Beauchamp RD, Berlin J, Benson III AB (2000) Eastern cooperative oncology group phase I trial of protracted venous infusion fluorouracil plus weekly gemcitabine with concurrent radiation therapy in patients with locally advanced pancreas cancer: a regimen with unexpected early toxicity. *J Clin Oncol* **18**: 3384–3389

Ueno H, Okusaka T, Ikeda M, Takezako Y, Morizane C 2005 An early phase II study of S-1 in patients with metastatic pancreatic cancer. *Oncology* **68**: 171–178

Willett CG, Czito BG, Bendell JC, Ryan DP (2005) Locally advanced pancreatic cancer. *J Clin Oncol* **23**: 4538–4544

Yip D, Karapetis C, Strickland A, Steer CB, Goldstein D (2006) Chemotherapy and radiotherapy for inoperable advanced pancreatic cancer. *Cochrane Database Syst Rev.* 2006 Jul 19;3:CD002093

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Adjuvant imatinib treatment improves recurrence-free survival in patients with high-risk gastrointestinal stromal tumours (GIST)

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Palliative imatinib treatment has dramatically improved survival in patients with malignant gastrointestinal stromal tumours, particularly in patients with tumours harbouring activating *KIT* mutations. To evaluate the effectiveness of adjuvant imatinib after radical surgery, a consecutive series of patients with high-risk tumours ($n = 23$) was compared with historic controls ($n = 48$) who were treated with surgery alone. The mean follow-up period was over 3 years in both groups. Only 1 out of 23 patients (4%) in the adjuvant treatment group developed recurrent disease compared to 32 out of 48 patients (67%) in the control group. This preliminary study indicates that 1 year of adjuvant treatment with imatinib dramatically improves recurrence-free survival. Confirmation of these findings awaits the results of ongoing randomised studies.

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Keywords: adjuvant treatment; gastrointestinal stromal tumour (GIST); imatinib; *KIT*; mutation; *PDGFRA*

The survival of patients with metastatic and inoperable malignant gastrointestinal stromal tumours (GIST), particularly those whose tumours have *KIT* exon 11 mutations, has improved dramatically since the introduction of imatinib mesylate into treatment protocols (Verweij *et al*, 2004). The role of adjuvant imatinib treatment in GIST, however, is unclear and is currently being investigated in ongoing trials. Four such studies, including patients have undergone radical (R0) surgery, are currently being conducted with different inclusion criteria in terms of malignant potential according to the consensus grading system of Fletcher *et al* (2002). ACOSOG Z9000 addresses treatment with imatinib (400 mg day^{-1} p.o.(orally)) for 1 year in patients with high-risk GIST with no control arm, while ACOSOG Z9001 compares imatinib treatment with placebo in patients with tumours $\geqslant 3$ cm (low, intermediate, and high-risk tumours). EORTC 62024 is designed for patients with intermediate- and high-risk GIST treated with imatinib (400 mg day^{-1} p.o.(orally)) *vs* placebo for 2 years. Finally, SSG XVIII includes high-risk GIST treated with imatinib (400 mg day^{-1} p.o.(orally)) for either 1 or 3 years.

The purpose of this study was to report our experience with adjuvant imatinib, while awaiting the results of ongoing multicentre trials. Our study consists of a single-centre, consecutive pilot series of 23 patients with high-risk GIST who have been treated with adjuvant imatinib (400 mg day^{-1}) for 1 year after R0 resection. These cases are compared with historical controls from a previous population-based series (Nilsson *et al*, 2005; Bumming *et al*, 2006) with matched risk scores with respect to tumour size and maximal proliferative activity with Ki67 antibodies.

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MATERIALS AND METHODS

The pilot adjuvant imatinib study group consisted of 23 consecutive patients (11 women and 12 men; mean age 56 years, range 21–82 years) with high-risk GIST diagnosed between February 2001 and June 2005. The mean tumour size was 9.4 cm (s.d. = 7.7, range 2–35 cm), and the mean Ki67 max% (maximum percentage of cells positive with Ki67 immunostains) was 7.0 (s.d. 5.0, range 2–10%). These patients received adjuvant imatinib (400 mg day^{-1} p.o.(orally)) for 12 months after R0 resection. The mean follow-up after onset of imatinib treatment was 40 months (s.d. = 14, range 18–62 months). Mutational analyses of *KIT* exons 9, 11, 13, and 17 and *PDGFRA* exons 12 and 18 were performed with dHPLC and bidirectional direct sequencing in both patient groups (Andersson *et al*, 2006).

The majority of patients (19 out of 23, 83%) receiving adjuvant imatinib had tumours with mutations in *KIT* or *PDGFRA*. Seventeen patients had tumours with *KIT* exon 11 mutations (eight deletions, five missense mutations, and four duplications). One patient had a GIST with duplication in *KIT* exon 9 and one patient's tumour had deletion in *PDGFRA* exon 18. Four patients had tumours that lacked *KIT* and *PDGFRA* mutations.

There were 48 matched (with regard to tumour size and Ki67 max %) historical controls of high-risk GIST with R0 resections, including 25 women and 23 men with a mean age of 67 years (s.d. 13, range 25–87 years). Mean tumour size was 12.3 cm (s.d. = 7, range 3.5–33 cm), and mean Ki67 max% was 11.7 (s.d. = 11.8,

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How I do it

Wrapping of skeletonized and divided vessels using the falciform ligament in distal pancreatectomy

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Abstract

Background: A pancreatic fistula is a major cause of morbidity in patients undergoing distal pancreatectomy (DP). A pancreatic fistula may expose skeletonized or divided vessels directly to pancreatic juice, creating a setting for vessel erosion and delayed intra-abdominal hemorrhage (DIH). With the aim of protecting vessels near the pancreatic stump from potential pancreatic fistulas, we have adopted a surgical option by which these vessels are wrapped using a pedicled falciform ligament.

Methods: After completing DP, the pedicled falciform ligament is spread out widely on major vessels exposed during resection near the pancreatic stump, and fixed to the surrounding retroperitoneal connective tissue. These procedures allow the complete separation of these vessels from the pancreatic stump. We reviewed the cases of 8 patients who underwent DP including these procedures.

Results: The mobilization of the falciform ligament and the wrapping of the vessels were successfully performed without any complications. Although 2 patients (14.5%) developed pancreatic fistulas, DIH did not occur in any of the patients.

Conclusions: The wrapping of the skeletonized and divided vessels using a pedicled falciform ligament is simple and easy, and may be an effective prophylactic measure against DIH following DP.

Keywords: Pancreatic fistula; Delayed intra-abdominal hemorrhage; Distal pancreatectomy; Falciform ligament

Pancreatic fistula is a common complication of distal pancreatectomy (DP) [1]. The incidence of pancreatic fistula after DP ranges from 5% to 60% [2–15]. Even recent advances in medical and surgical care of DP cannot completely eliminate the possibility of pancreatic fistula development [3,6,8,13,14,16]. A pancreatic fistula exposes skeletonized or divided vessels directly to pancreatic juice and/or intraperitoneal abscess, creating a setting for vessel erosion and lethal delayed intra-abdominal hemorrhage (DIH) [17,18]. The incidence of DIH after DP ranges from 2% to 4% [7,19]. Taken together, these reports suggest that the protection of these vessels from pancreatic fistulas may be envisaged as a prophylactic measure against DIH after DP. With the aim of protecting vessels near the pancreatic stump from a potential pancreatic fistula, we have adopted a surgical option by which these vessels are wrapped using the pedicled falciform ligament. This surgical option is simple and easy, and appears to minimize the incidence of DIH after DP. Herein, we present our novel procedure and the preliminary results of 8 patients who underwent DP employing this option.

Patients and Methods

Surgical techniques for mobilization of the falciform ligament and the wrapping of vessels

A pedicled falciform ligament is easily and rapidly obtained during a midline abdominal incision. After incising the linea alba, the preperitoneal fat is bluntly dissected to the right prior to incising the peritoneum. The falciform ligament is mobilized by dividing it near the umbilicus and incising its anterior peritoneal reflections along the posterior right rectus sheath. An additional length can be obtained by continuing the anterior incision cephalad to the undersurface of the diaphragm and to the triangular ligament, then incising the posterior peritoneal reflections cephalad to the

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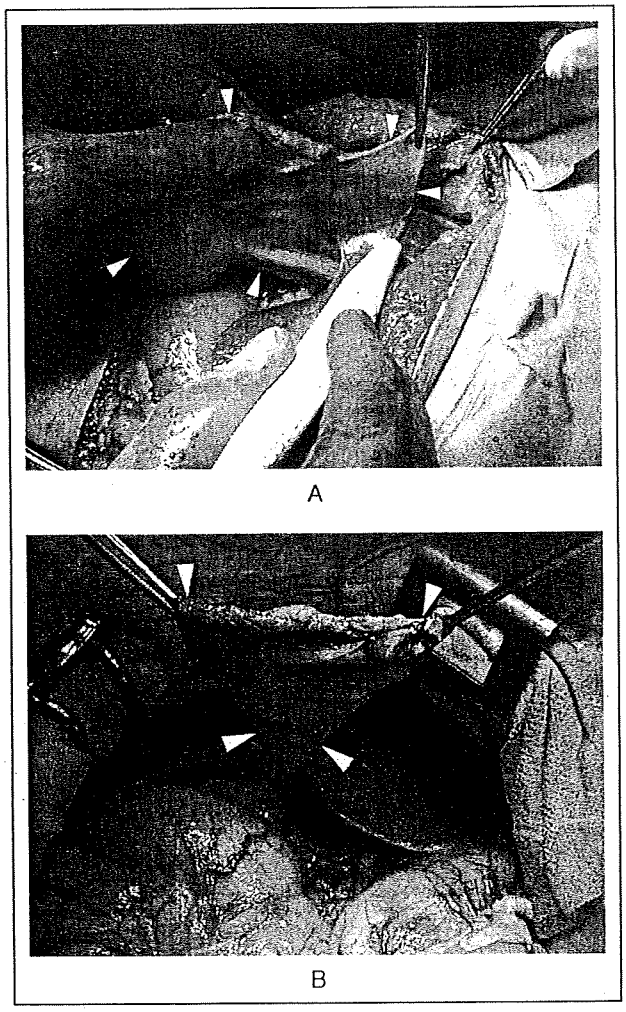

Fig. 1. (A) The mobilization of the falciform ligament (*arrowheads*). (B) The pedicled falciform ligament (*arrowheads*).

anterior surface of the liver to the triangular ligaments (Fig. 1A) [20]. The pedicled falciform ligament (Fig. 1B) will usually reach the space of the pancreatic stump and the splanchnic vessels exposed during resection. After completing DP, the pedicled falciform ligament is perineally brought through a newly opened hole in the lesser omentum to the pancreatic stump area. Then it is spread out widely on the vessels near the pancreatic stump, such as the common hepatic artery, superior mesenteric vein, portal vein, and stumps of the splenic artery/vein (Fig. 2A), and fixed with interrupted 3-0 silk sutures to the surrounding retroperitoneal connective tissue (Fig. 2B). These procedures allow the complete separation of the vessels from the pancreatic stump (Fig. 2B).

Patients and DP procedures

Between September 2003 and November 2005, eight patients underwent DP employing the wrapping of the vessels using the pedicled falciform ligament. The patients were 4 men and 4 women with a mean age of 68 years (range 41 to 76 years). The indications for DP included ductal adenocarcinoma (n = 2), intraductal papillary mucinous adenocarcinoma (n = 1), and intraductal papillary mucinous adenoma (n = 5). A concomitant splenectomy was carried out in all 8 patients.

During DP, lymph nodes around the common hepatic artery were dissected. The splenic artery and vein were divided at their origin. Therefore, the skeletonized common hepatic artery, stumps of the splenic artery/vein and, occasionally, the superior mesenteric vein and/or portal vein were exposed near the pancreatic stump. The pancreas was transected at the level of the superior mesenteric vein. Five patients had a stapled closure of the pancreatic stump. Stapled transection was achieved using an Auto Suture ENDO-UNIVERSAL-clip-instrument (United States Surgical Corporation, Norwalk, CT). In the other 3 patients, during the pancreatic transection, the pancreatic parenchyma was divided using an electrocautery or a surgical scalpel (electrocautery: 2, scalpel: 1). In these cases, the main pancreatic

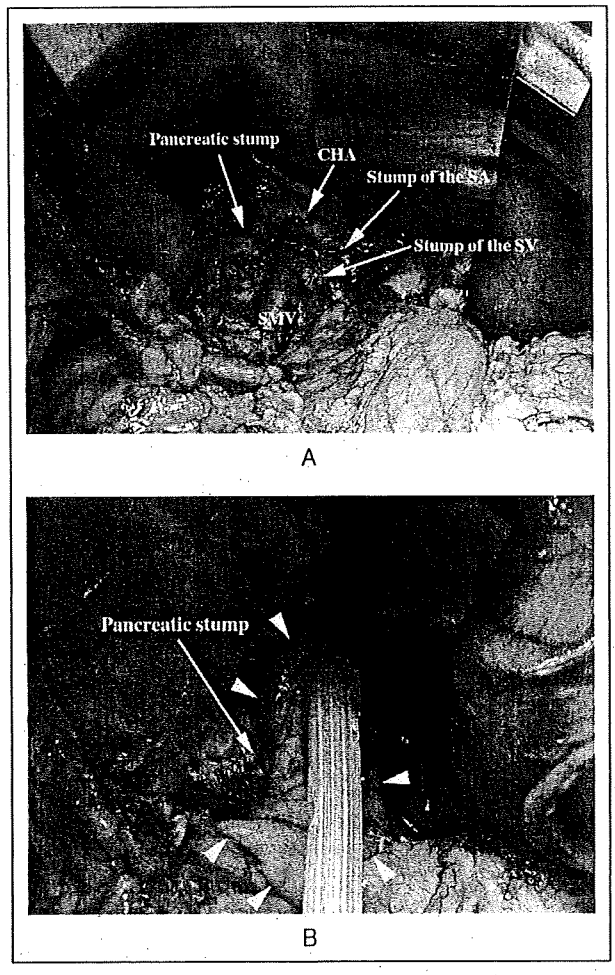

Fig. 2. (A) The wrapping of the vessels using the pedicled falciform ligament. Surgical view before the wrapping of the pedicled falciform ligament. The common hepatic artery, stump of the splenic artery, stump of the splenic vein, superior mesenteric vein, and portal vein are exposed near the pancreatic stump. CHA, common hepatic artery; SA, splenic artery; SV, splenic vein; SMV, superior mesenteric vein. (B) Surgical view after the wrapping of the pedicled falciform ligament (*arrowheads*). This procedure allows the complete separation of the vessels from the pancreatic stump. A closed suction drain (silicone rubber drain) was placed adjacent to the pancreatic stump and fixed to the pedicled falciform ligament.

duct was ligated with a nonabsorbable suture, and the stump of the pancreas was left open. Fibrin glue was not applied to the stump in any of the patients. A closed suction drain was placed adjacent to the pancreatic stump and fixed with an absorbable suture to the falciform ligament (Fig. 2B). Octreotide was not administered postoperatively.

A pancreatic fistula is defined as a biochemical leakage in the presence of clinical sequelae, such as the saponification of drainage fluid, fever or leucocytosis, intra-abdominal abscess formation, and the need for percutaneous drainage or reoperation [15]. A biochemical leakage is defined as an amylase level in drainage fluid that is more than 4-fold the upper limit of the normal serum amylase level on postoperative day 3 or 5 [15].

Results

The mobilization of the falciform ligament and the wrapping of the vessels were successfully performed without any complications. At the end of the operation, none of the patients experienced ischemia in the pedicled falciform ligament. The mean operating time and blood loss were 228 (range 181 to 344) minutes and 218 (range 55 to 511) mL, respectively.

Pancreatic fistula occurred in 2 patients (14.5%), both associated with bacterial infection. The pancreatic fistulas resolved spontaneously by 32 days after conservative treatments including pancreatic rest and/or intermittent local irrigation with saline via the drain. These were then closed with no additional serious conditions related to the fistula, such as DIH, pseudocyst formation, and sepsis. The drain was removed on postoperative day 7 in patients with no evidence of pancreatic fistula. Complications associated with the drain removal did not occur in any case. There were no postoperative deaths.

Comments

Most pancreatic fistulas after DP usually resolve spontaneously, albeit over a long period, after conservative treatments including pancreatic rest (no oral intake with total parenteral nutrition or octreotide administration) and adequate drainage (drains placed during the initial operation or postoperatively via the percutaneous approach) [21–23]. However, some patients with pancreatic fistulas develop complications, such as intraperitoneal abscess, sepsis, and lethal DIH [7]. Although the incidence (2% to 4%) of DIH after DP [7,19] is lower than that (2% to 8%) after pancreatoduodenectomy [17,24–28], preventing DIH associated with pancreatic fistula is an important step toward improving the short-term outcome after DP.

The prevention of DIH is also a major concern in pancreatoduodenectomy. The preferred surgical option for the prevention of DIH after pancreatoduodenectomy is the protection of the skeletonized or divided vessels from intra-abdominal complications, such as pancreatic fistula and intra-abdominal infection [18,24,29,30]. Indeed, the placement of the omental flap between these vessels and the pancreaticojejunostomy is successful for reducing the incidence of DIH after pancreatoduodenectomy (0% to 1%) [18,24]. These data support the idea that the protection of vessels could also be a measure against DIH following DP. For this purpose, the use of the omental flap in DP may be as good as those previously reported for pancreatoduodenectomy [18,24,29,30]. However, an adequate omentum is not available in some patients [20]. Complications of the use of the omental flap, such as intestinal obstruction, the total necrosis of the flap, and infection, have been reported [24]. Therefore, we consider the falciform ligament an excellent alternative to the omentum for the protection of vessels.

The falciform ligament is the obliterated umbilical vein (ligamentum teres or round ligament) and its encompassing parietal peritoneum. The pedicled falciform ligament, when adequately mobilized, is a large (15 cm to 30 cm) autologous tissue that will usually reach any surgical area in the upper abdomen. Although its availability is not widely appreciated, it has been used in several abdominal operations including those for hepatic injury [20], perforated gastroduodenal ulcer [31], and hiatal herniorrhaphy [32]. The use of the falciform ligament in DP has not been described [33].

The presented surgical option is a simple and easy technique for the complete separation of the vessels from the pancreatic stump. It is suggested that the falciform ligament can prevent the diffusion of pancreatic juice with or without bacterial infection and protect the vessels. In this study, none of the patients developed DIH. However, it could not be confirmed whether the present surgical option itself prevented DIH. Further controlled randomized studies involving large numbers of patients are warranted to confirm the value of the present surgical option.

In conclusion, the present surgical option (the wrapping of the vessels using the pedicled falciform ligament) is technically easy, and we believe that this may prevent DIH caused by a pancreatic fistula following DP.

References

[1] Halloran CM, Ghaneh P, Bosonnet L, et al. Complications of pancreatic cancer resection. Dig Surg 2002;19:138–46.
[2] Montorsi M, Zago M, Mosca F, et al. Efficacy of octreotide in the prevention of pancreatic fistula after elective pancreatic resections: a prospective, controlled, randomized clinical trial. Surgery 1995; 117:26–31.
[3] Suzuki Y, Kuroda Y, Morita A, et al. Fibrin glue sealing for the prevention of pancreatic fistulas following distal pancreatectomy. Arch Surg 1995;130:952–5.
[4] Konishi T, Hiraishi M, Kubota K, et al. Segmental occlusion of the pancreatic duct with prolamine to prevent fistula formation after distal pancreatectomy. Ann Surg 1995;221:165–70.
[5] Brennan MF, Moccia RD, Klimstra D. Management of adenocarcinoma of the body and tail of the pancreas. Ann Surg 1996;223: 506–11.
[6] Suzuki Y, Fujino Y, Tanioka Y, et al. Randomized clinical trial of ultrasonic dissector or conventional division in distal pancreatectomy for non-fibrotic pancreas. Br J Surg 1999;86:608–11.
[7] Lillemoe KD, Kaushal S, Cameron JL, et al. Distal pancreatectomy: indications and outcomes in 235 patients. Ann Surg 1999;229: 693–700.
[8] Sugo H, Mikami Y, Matsumoto F, et al. Distal pancreatectomy using the harmonic scalpel. Surgery 2000;128:490–1.
[9] Balcom JH IV, Rattner DW, Warshaw AL, et al. Ten-tear experience with 733 pancreatic resections: changing indications, older patients, and decreasing length of hospitalization. Arch Surg 2001;136:391–7.
[10] Matsumoto T, Ishio T, Sasaki A, et al. Pancreatic resection with ultrasonically activated scalpel: preliminary observation. Hepatogastroenterology 2002;49:635–8.
[11] Sheehan MK, Beck K, Creech S, et al. Distal pancreatectomy: does the method of closure influence fistula formation? Am Surg 2002; 68:264–8.

[12] Fahy BN, Frey CF, Ho HS, et al. Morbidity, mortality, and technical factors of distal pancreatectomy. Am J Surg 2002;183:237–41.
[13] Takeuchi K, Tsuzuki Y, Ando T, et al. Distal pancreatectomy: is staple closure beneficial? Aust NZ J Surg 2003;73:922–5.
[14] Suc B, Msika S, Fingerhut A, et al. Temporary fibrin glue occulusion of the main pancreatic duct in the prevention of intraabdominal complications after pancreatic resection. Ann Surg 2003;237:57–65.
[15] Bilimoria KM, Cormier JN, Mun Y, et al. Pancreatic leak after left pancreatectomy is reduced following main pancreatic duct ligation. Br J Surg 2003;90:190–6.
[16] Li-Ling J, Irving M. Somatostatin and octreotide in the prevention of postoperative pancreatic complications and the treatment of enterocutaneous pancreatic fistulas: a systematic review of randomized controlled trials. Br J Surg 2001;88:190–9.
[17] Choi SH, Moon HJ, Heo JS, et al. Delayed hemorrhage after pancreaticoduodenectomy. J Am Coll Surg 2004;199:186–91.
[18] Kurosaki I, Hatakeyama K. Omental wrapping of skeletonized major vessels after pancreaticoduodenectomy. Int Surg 2004;89:90–4.
[19] Sledzianowski JF, Duffas JP, Muscari F, et al. Risk factors for mortality and intra-abdominal, orbidity after distal pancreatectomy. Surgery 2005;137:180–5.
[20] Fischer RP, Gervin As. The use of falciform ligament in the repair of hepatic injuries. Surg Gynecol Obstet 1985;161:383–4.
[21] Ridgeway MG, Stabile B. Surgical management and treatment of pancreatic fistulas. Surg Clin North Am 1996;76:1159–73.
[22] Bassi C, Falconi M, Pederzoli P. Pancreatic fistula. In: Howard JM, Idezuki Y, Ihse I, et al, editors. *Surgical Diseases of the Pancreas*. 3rd ed. Baltimore: Williams & Wilkins; 1998:827–34.
[23] Pederzoli P, Bassi C, Falconi M, et al. Conservative treatment of external pancreatic fistulas with parenteral nutrition alone or in combination with continuous intravenous infusion of somatostatin, glucagons or calcitonin. Surg Gynecol Obstet 1986;163:428–32.
[24] Maeda A, Ebata T, Kanemoto H, et al. Omental flap in pancreaticoduodenectomy for protection of splanchnic vessels. World J Surg 2005;29:1122–6.
[25] Yeo CJ, Cameron JL, Sohn TA, et al. Six hundred fifty consecutive pancreaticoduodenectomies in the 1990s: pathology, complications, and outcomes. Ann Surg 1997;226:248–57.
[26] Miedema BW, Sarr MG, van Heerden JA, et al. Complications following pancreaticoduodenectomy. Current management. Arch Surg 1992;127:945–9.
[27] Rumstadt B, Schwab M, Koeth P, et al. Hemorrhage after pancreatoduodenectomy. Ann Surg 1998;227:236–41.
[28] Yoshida T, Matsumoto T, Morii Y, et al. Delayed massive intraperitoneal hemorrhage after pancreatoduodenectomy. Int Surg 1998;83:131–5.
[29] Moriura S, Ikeda S, Ikezawa T, et al. The inclusion of an omental flap in pancrfeatoduodenectomy. Surg Today 1994;24:940–1.
[30] Seyama Y, Kubota K, Kobayashi T, et al. Two-staged pancreatoduodenectomy with external drainage of pancreatic juice and omental graft technique. J Am Coll Surg 1998;187:103–5.
[31] Costalat G, Alquier Y. Combined laparoscopic and endoscopic treatment of perforated gastroduodenal ulcer using the ligamentum teres hepatis (LTH). Surg Endosc 1995;9:677–80.
[32] Van Helsdingen GCF. Hiatal herniorrhaphy with posterior gastropexy utilizing the ligamentum teres hepatis. Int Surg 1968;50:128–32.
[33] Baker RJ, Fischer SK, Lilloemoe KL, et al. *Mastery of Surgery*. Baltimore: Lippincott Williams & Wilkins; 2001:1313.

Combined vascular resection in operative resection for hilar cholangiocarcinoma: Does it work or not?

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Background. *It is still not clear how combined vascular resection affects the outcome of patients with hilar cholangiocarcinoma. Our aim was to evaluate implications of combined vascular resection in patients with hilar cholangiocarcinoma by analyzing the outcomes of all patients who underwent operative resection.*

Methods. *A total of 161 of 228 consecutive patients with hilar cholangiocarcinoma underwent bile duct resection with various types of hepatectomy (88%) and pancreaticoduodenectomy (4%). Combined vascular resection was carried out in 43 patients. Thirty-four patients had portal vein resection alone, 7 patients had both portal vein and hepatic artery resection, and 2 patients had right hepatic artery resection only. The outcomes were compared between the 3 groups: the portal vein resection alone (34), hepatic artery resection (9), and non-vascular resection (118).*

Results. *Histologically-positive tumor invasion to the portal vein beyond the adventitia was present in 80% of 44 patients undergoing combined portal vein resection. Operative mortality occurred in 11 (7%) patients. The survival rates of the non-vascular resection group were better than that of the portal vein resection alone and the hepatic artery resection groups: 1, 3, and 5 years after curative resection, 72%, 52%, and 41% versus 47%, 31%, and 25% ($P < .05$), and 17%, 0%, and 0% ($P < .0001$), respectively. Multivariate analysis showed 4 independent prognostic factors of adverse effect on survival after operation; operative curability, lymph node metastases, portal vein resection, and hepatic artery resection.*

Conclusions. *Although both portal vein and hepatic artery resection are independent poor prognostic factors after curative operative resection of locally advanced hilar cholangiocarcinoma, portal vein resection is acceptable from an operative risk perspective and might improve the prognosis in the selected patients, however, combined hepatic artery resection can not be justified. (Surgery 2007;141:581-8.)*

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HILAR CHOLANGIOCARCINOMA remains difficult to resect with curative intent despite the recent evolution and improvement of preoperative diagnostic imaging and operative techniques in hepatic resection. The resection rate is reported to be less than 40%, and curative resection with negative operative margin has been carried out in 15% to 83% of resected patients.[1] The importance of a negative operative margin for long-term survival has been confirmed by the results of several studies reported previously.[2-4] The reasons for irresectability in most patients with hilar cholangiocarcinoma are local extensive invasion to major vessels, such as the hepatic artery and the portal vein, and distant metastases, including peritoneal dissemination, liver metastases, distant lymph nodal metastases, and extra-abdominal metastases. The anatomic features of the hepatic hilus often make it easy for hilar cholangiocarcinoma to invade major vessels, such as those mentioned above. Therefore, hilar cholangiocarcinoma occasionally requires combined vascular resection and reconstruction, to obtain,

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negative resection margin due to involvement of hilar vasculatures, despite the fact that most patients with hilar cholangiocarcinoma are resected with unilateral hepatic lobectomy.

Several previous reports have described combined vascular resection in patients with hilar cholangiocarcinoma.[4-8] There is no general consensus, however, on the criteria for resectability of hilar cholangiocarcinoma involving the portal vein and the hepatic artery that requires combined vascular resection and reconstruction for the purpose of obtaining a cancer-free resection margin. Jarnagin et al[9] reported that encasement or occlusion of the main portal vein proximal to its bifurcation is one of criteria for irresectability in patients with cholangiocarcinoma. We have resected hilar cholangiocarcinoma involving the portal vein and the hepatic artery aggressively with combined vascular resection. In this study, our aim was to evaluate whether or not combined vascular resection of hilar cholangiocarcinoma is beneficial.

PATIENTS AND METHODS

Patients. Between january 1981 and March 2004, 204 patients with hilar cholangiocarcinoma were evaluated for resection at our institution; 187 patients underwent exploration for the purpose of potentially curative resection of whom 161 were resected. Pathologic tumor staging was carried out according to the UICC classification.[10]

Preoperative clinical assessment, as well as a laboratory and imaging workup including ultrasonography, magnetic resonance cholangiopancreatography (MRCP), cholangiography through the percutaneous transhepatic or endoscopic retrograde approaches, contrast-enhanced computed tomography (CT), and selective angiography, were carried out to establish the nature and extent of the disease and to define ductal and vascular anatomic details. All patients also underwent other generalized preoperative tests to assess their operative fitness. Preoperative portal embolization was carried out 14 to 22 days before operation in 16 patients in whom the remnant liver volume after operative resection was expected to be less than 40% of the expected whole liver volume from 1994 onward. The portal venous branch was embolized via the ileocolic vein, after mini-laparotomy under lumbar anesthesia. Of 161 resected patients, 149 underwent preoperative biliary drainage via a percutaneous transhepatic or an endoscopic retrograde transpapillary route for relief of obstructive jaundice and for correct evaluation of biliary lesion. Our criteria of irresectability defined by local, tumor-related factors were as follows: (1) tumor extension to bilateral secondary biliary radicles, except the caudate bile duct branches; (2) tumor extension to bilateral secondary portal vein branches; (3) tumor extension to bilateral secondary hepatic artery branches; and (4) expected remnant liver volume less than 30% of the whole liver volume We also evaluated age, gender, preoperative biliary drainage, and preoperative serum total bilirubin concentrations. Resected neoplasms were evaluated histologically as to operative margin, tumor size, tumor differentiation, nodal metastases, lymphatic vessel invasion, venous invasion, and T stage. In the patients with combined vascular resection, the extent of tumor invasion to the walls of the portal vein and the hepatic artery were also evaluated histologically. The 161 resected patients were stratified into 3 groups for evaluation of the efficacy of combined vascular resection in treatment of hilar cholangiocarcinoma: a portal vein resection alone group (n = 34), a hepatic artery resection group (n = 9), and a non-vascular resection group (n = 118). Postoperative complications and survival were analyzed in each of the 3 groups. In the same period, we managed 67 patients with unresectable hilar cholangiocarcinoma. The criteria for irresectability were, as described above, local tumor related factors and distant metastases. Unresectable patients underwent biliary drainage by percutaneous transhepatic or endoscopic nasobiliary routes, and were treated by biliary stenting with metallic or plastic endoprosthesis. In this series over a 24-year period, the management plan has not been changed except for the introduction of preoperative portal vein embolization from 1994 and of parenchyma-preserving hepatectomy[4] from 1990.

Operative procedures. Operative procedures were selected according to tumor extent in the bile duct, portal vein, and hepatic artery as determined by preoperative and intraoperative evaluation (Table I). Curative resection was defined as histologically negative operative margins at the hepatic stump of the bile duct, duodenal stump of the bile duct, and excisional surface. As reported previously,[4,11] parenchyma-preserving segment I hepatectomy and resection of segments I and IV were selected to limit resection as much as possible to what was necessary for curative purposes, especially in patients with comorbid medical conditions indicating increased operative risk (for example, advanced age, diabetes mellitus, liver dysfunction, and combined pancreaticoduodenectomy). Combined vascular resection was carried out in 43 of 161 resected patients; 34 patients had portal resection, 7 patients both portal vein and hepatic artery resection, and 2 patients only right hepatic artery

Table I. Operative procedures for hilar cholangiocarcinoma

	Patients (n)	*%*
Hilar bile duct resection	20	12
Hepatectomy	141	88
S_1^*-resection	14	9
$S_1 + S_4$ resection	10	6
Central bisegmentectomy	1	0.6
Extended left hepatectomy	52	32
Left trisegmentectomy	7	4
Extended right hepatectomy	50	31
Right trisegmentectomy	7	4
Total	161	100

*S, Hepatic segments were described according to the classification of Couinaud.

Table II. Vascular reconstruction in combined vascular resection

Vessels	*Patients (n)*
Portal vein	41
End to end	39
Autologous vein graft	2
Hepatic vein and inferior vena cava	5
Primary closure	3
Autologous vein graft	2
Hepatic artery	9
Reconstruction	
Left hepatic artery	3
Right hepatic artery	3
No reconstruction	
Right anterior hepatic artery	1
Right hepatic artery	1
Left hepatic artery	1

resection (Table II). The decision for combined vascular resection was made by the intra-operative macroscopic findings of tumor invasion to the vessels in conjunction with the preoperative imaging. The portal vein was reconstructed in an end-to-end fashion in 39 patients (95%), and autologous vein grafts, using a left renal vein,[12] in 2 patients. The hepatic artery was reconstructed in 6 patients in an end-to-end fashion.

Pancreaticoduodenectomy was carried out in 7 patients, including 4 patients in non-vascular resection group, 2 patients in portal vein resection group, and 1 patient in both portal vein and hepatic artery resection group. All operative procedures included resection of the extrahepatic duct and gallbladder with bilioenteric anastomosis using a Roux-en-Y jejunal loop. Biliary stent tubes were placed for bilioenteric anastomosis through a retrograde transhepatic route at the time of operation, and removed 3 to 4 weeks postoperatively. No adjuvant chemotherapy was given to patients who underwent resection. The 13 patients in whom the bile duct stump was a positive underwent external radiation treatment. No aggressive chemotherapy was given during any of the observation periods to patients who had undergone resection. The patency of reconstructed blood vessels was evaluated by Doppler ultrasonography and contrast-enhanced CT during the short-term and long-term follow-up.

Statistical analysis. Statistical analysis of patient survival was carried out according to the Kaplan-Meier method. Comparison of patient survival in the different groups was carried out using the log-rank test. Survival analyses were conducted according to various procedures and in regard to tumor characteristics, and operative and postoperative deaths. Pairwise comparisons among the 3 groups in regard to patient characteristics, operative features, operative curability, histopathologic features, operative morbidity, and mortality rates were analyzed by Mann-Whitney U and χ^2 tests for continuous and discontinuous variables, respectively. We used log-rank tests in univariate analyses to determine whether there were significant differences between subgroups. Multivariate regression analysis of factors related to outcomes was carried out using the Cox proportional hazard model. Significance was established at P less than .05. Statistical calculations were carried out with the use of SPSS software (SPSS, Inc., Chicago, Ill).

RESULTS

Patient characteristics and operative features. Various patient characteristics and operative features (including age, gender, preoperative biliary drainage, preoperative serum bilirubin concentration, hepatic resection, portal vein resection, pancreaticoduodenectomy, and intra-operative blood loss) were compared among the 3 groups (Table III). There were no significantly different factors among the 3 groups, except for portal vein resection. Histopathologic features of resected neoplasms, tumor size, tumor differentiation, lymph node metastases, lymphatic vessel invasion, venous invasion, perineural invasion, and T stage were examined (Table IV). There were no remarkable differences in these histopathologic features among the 3 groups, but several groups had a relatively small number of patients.

Histologic studies of cancer invasion to resected vessels. There was positive tumor invasion into the wall of the portal vein beyond the adventitia in 80% of all portal veins resected from 44 patients, includ-

Table III. Patient characteristics and surgical procedures for 161 resected patients with hilar cholangiocarcinoma

	Vascular resection			
	Portal vein alone	*Hepatic artery*	*None*	*Total*
Patients (n)	34	9	118	161
Age	64 ± 9	59 ± 9	65 ± 11	64 ± 10
Gender (male:female)	18:16	7:2	77:41	102:59
Biliary drainage	32	9	108	149
Serum bilirubin (mg/dl)	1.7 ± 1.6	2.6 ± 1.1	2.1 ± 1.4	2.1 ± 1.4
Hepatic resection	33	9	99	141
Right sided	20	2	35	57
Left sided	12	6	41	59
Central	1	1	24	25
Bile duct resection alone	1	0	19	20
Portal vein resection	34:0	7:2	0:119	41:121
Pancreatico duodenectomy	2	1	4	7
Blood loss (ml)	1,975 ± 1,474	1,726 ± 1,253	1,523 ± 1,147	1,590 ± 1,214

Table IV. Histopathologic features of resected neoplasms in 161 patients with hilar cholangiocarcinoma

	Vascular resection			
	Portal vein alone	*Hepatic artery*	*None*	*Total*
Number of patients	34	9	118	1161
Curative resection (%)	19 (56)	6 (67)	77 (65)	102 (63)
Tumor size (mm)	27 ± 10	35 ± 13	26 ± 10	26 ± 10
Tumor differentiation (well:mod:poor)*	7:21:6	1:6:2	47:49:22	55:76:30
Nodal metastasis	17	7	53	77
Lymphatic vessel invasion	32	8	104	144
Venous invasion	29	9	102	140
Perineural invasion	33	9	102	144
T_{is+1}:T_2:T_{3+4}†	0:8:24	0:1:8	7:88:25	7:97:57

*Well, moderate, and poor differentiation.
†T-staging system according to the UICC-TNM classification.

ing invasion into the adventitia in 10 patients (24%), into the media in 19 (46%), and into the intima in 4 (10%). Four of 9 patients cases (44%) had undergoing hepatic artery resection had positive tumor invasion to the adventitia. In the other 5 patients, tumor invasion did not reach the adventitia of the hepatic arterial wall, despite obvious arterial encasement in both preoperative and intraoperative findings. In 8 patients with no invasion of the portal vein, lymph node involvement was found in 5, and in 33 patients with invasion of the portal vein, nodal metastases were present in 18 (55%). There was no remarkable difference between the 2 groups.

Operative morbidity and mortality. The most serious postoperative complication encountered in this series was hyperbilirubinemia (serum T-bil >10 mg/dl) in 26 patients (16%) as shown in Table V. This serious complication resulted in lethal liver failure, accounting for 9 of 11 hospital deaths. The other common complications were pleural effusion and anastomosis breakdown of hepaticojejunostomy. None of the 41 patients with portal vein reconstruction had abnormal patency of the portal vein at any time postoperatively. Two of 9 patients after hepatic artery reconstruction developed late obstruction of reconstructed hepatic artery. Operative mortality, including hospital deaths, was 7% in the 161 resected patients. The mortality rate after the hepatic artery resection was greater than that in the non-vascular resection group ($P < .001$), but was not significantly different from that in the portal vein resection alone group, although the number were small.

Univariate and multivariate analysis of prognostic factors. Univariate survival analysis identified curability, lymph node metastases, venous invasion, perineural invasion, portal vein resection, hepatic

Table V. Operative morbidity and mortality after resection in hilar cholangiocarcinoma

	Vascular resection			
	Portal vein alone (n = 34)	*Hepatic artery* (n = 9)*	*None (n = 118)*	*Total (n = 161)*
Morbidity rate (%)	13 (38)	7 (78)	42 (36)	62 (39)
Hyperbilirubinemia	4	4	18	26
Anastomosis breakdown	3	3	14	20
Pleural effusion	7	3	14	24
Rupture of pseudoaneurysm	1	1	5	7
Mortality rate (%)	3	3	5	11(7)
Operative death	0	1	3	4
Hospital death	3	3	5	11

*Including combined resection of portal vein and hepatic artery in 7 patients.

artery resection, and hepatic resection as factors with a statistically significant prognostic influence (Table VI). Age, gender, lymphatic vessel invasion, tumor size, histologic differentiation, extended hepatectomy, and adjuvant postoperative irradiation therapy did not significantly affect prognosis after resection. Multivariate analysis suggested 4 independent prognostic factors that influenced survival after resection: curability, lymph node metastases, portal vein resection, and hepatic artery resection (Table VII). Hepatectomy was not a significant independent factor.

Survival. Survival rates in the non-vascular resection group were 63%, 39%, and 30%, 1, 3, and 5 years after resection, and were significantly better than in these who had undergone resection of the portal vein alone 50%, 19%, and 16%, and of the hepatic artery 11%, 0%, and 0%, respectively. Survival rates in the non-resection group were 15% and 0% at 1 and 2 years. There was a significant difference in survival rates between the portal vein resection alone group and the non-resection group ($P < .001$), but no significant difference between the hepatic artery resection group and the non-resection group but acknowledging the small number of patients after hepatic artery resection alone (Fig 1).

Among all patients who had undergone curative resection, survival rates of the portal vein resection alone group, the hepatic artery resection group, and the non-vascular resection group were 47%, 31%, and 25%, 17%, 0%, and 0%, and 72%, 52%, and 41% at 1, 3, 5 years postoperatively, respectively. Median survival was for 340, 213, and 1,157 days, respectively (Fig 2). Furthermore, among patients with hilar cholangiocarcinoma without lymph nodes metastases, survival rates after curative resection were 63%, 33%, and 33%, 0%, 0%, and 0% and 86%, 72%, and 57% at 1, 3, 5 years postoperatively in the portal vein resection alone, hepatic artery resection and non-vascular resection groups, respectively. Median survival in the 3 groups was for 555, 213, and 2,260 days, respectively (Fig 3). The outcome of the 33 patients with histologically positive invasion to the portal vein was not different from that of 8 patients with histologically negative invasion to the portal vein; 10% versus 25% ($P = .886$). Similarly no remarkable difference of the outcome were present in the series excluding the patients with operative or non-cancer-related deaths postoperatively; 12% versus 40% ($P = .395$) but both these latter comparisons involve few patients making the statistical comparisons not powerful. There was no obvious difference of the recurrent patterns such as local recurrence, carcinomatous peritonitis, and hepatic and extra-abdominal metastases between the groups of portal vein resection or vascular resection and no-vascular resection.

DISCUSSION

Combined vascular resection for hilar cholangiocarcinoma has been reported previously by Tsuzuki et al,[13] Sakaguchi et al,[14] and Fortner et al[15] in small series of patients. Klempnauer et al[2] first reported portal vein resection in a large series of 39 patients, and 1 patient who underwent hepatic artery resection, of 151 patients undergoing resection for hilar cholangiocarcinoma. The operative mortality rate of 17% among patients with combined vascular resection for hilar cholangiocarcinoma was high, and their aggressive approach resulted in a 5-year survival rate of 10%. Similar results, reflecting the increased risk of combined vascular resection for hilar cholangiocarcinoma, were presented in our previous report[16] and by Neuhaus et al.[6] Operative mortality in the latter 2 reports was 16% and 17%, respectively. Operative

Table VI. Univariate analysis of overall survival in patients with hilar cholangiocarcinoma*

Factor	*Survival rate (%)*			*Median survival (d)*	P *value*
	1 y	3 y	5 y		
Curability					
Curative (n = 59)	64	42	36	910	.0001
Non-curative (n = 102)	46	10	0	345	.0001
Nodal involvement					
Positive (n = 82)	37	13	9	305	.0001
Negative (n = 79)	79	55	42	1,185	.0001
Venous invasion					
Positive (n = 139)	59	26	21	485	.0235
Negative (n = 16)	76	61	49	1,670	.0235
Perineural invasion					
Positive (n = 140)	55	29	23	484	.0494
Negative (n = 21)	75	59	40	1,455	.0494
Portal vein resection					
Positive (n = 41)	42	16	13	325	.0006
Negative (n = 120)	63	39	30	711	.0006
Hepatic artery resection					
Positive (n = 9)	11	11	0	213	.0005
Negative (n = 152)	60	35	26	550	.0005
Hepatectomy					
Positive (n = 140)	59	35	27	606	.0282
Negative (n = 21)	39	16	11	345	.0282

*There was no significant difference of survival after surgery in age, gender, lymphatic vessel invasion, tumor size, histologic differentiation, extended hepatectomy, and adjuvant postoperative irradiation therapy.

Table VII. Multivariate analysis of overall survival in patients with hilar cholangiocarcinoma

Factor	*Relative risk*	95% CI		P *value*
		Lower	*Upper*	
Curability	2.332	1.532	3.549	<.0001
Nodal involvement	2.779	1.797	4.289	<.0001
Venous invasion	1.116	0.549	2.229	.7770
Perineural invasion	1.221	0.598	2.496	.5834
Portal vein resection	1.570	1.031	2.391	.0354
Hepatic artery resection	2.293	1.052	5.000	.0369
Hepatectomy	0.803	0.465	1.389	.4333

CI, confidence interval.

mortality in combined vascular resection seemed to be affected by whether the portal vein or the hepatic artery was resected. Most reports have addressed portal vein resection such as Klempnauer et al[2] and Neuhaus et al.[6] Series that Miyazaki et al[16] and Gerhards et al[5] have reported included several patients with hepatic artery resection with 8 of 25 and 9 of 12 cases of vascular resection, respectively. Hepatic artery resection may bring about a higher operative mortality rate than portal vein resection, because hepatic artery resection has been combined with portal vein resection in most patients. Combined resection involving the portal vein and the hepatic artery may obligate longer periods of liver ischemia for vascular reconstruction, which may lead to more severe ischemic damage to the remnant liver after major hepatectomy and may result in lethal liver failure. Therefore, the portal vein resection alone group and the hepatic artery resection group should each be analyzed separately to assess how combined vascular resection can affect morbidity and mortality in operative treatment of hilar cholangiocarcinoma, and whether or not these aggressive operative approaches can bring about beneficial effects in prognosis. For this reason, our study was stratified into non-vascular resection, portal vein resection alone, and hepatic artery resection groups, to evaluate

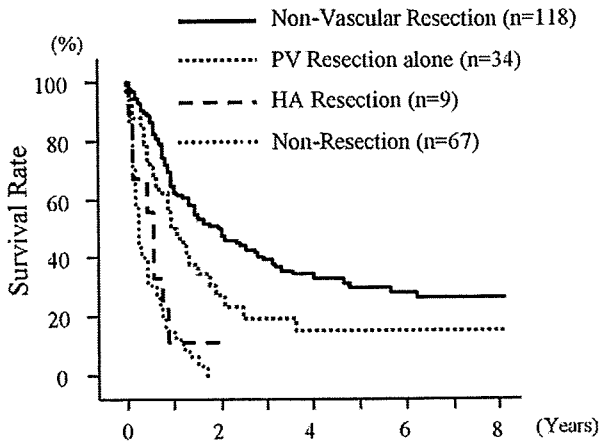

Fig 1. Survival after operative resection in all resected 161 patients. The survival rates in the non-vascular resection group and the portal vein (PV) resection alone was greater than in the non-resection group ($P < .0001$). The survival rate in the hepatic artery (HA) resection group was not different from that in the non-resection group. The survival rate in the non-vascular resection group was better than that in the portal vein (PV) resection alone ($P < .05$).

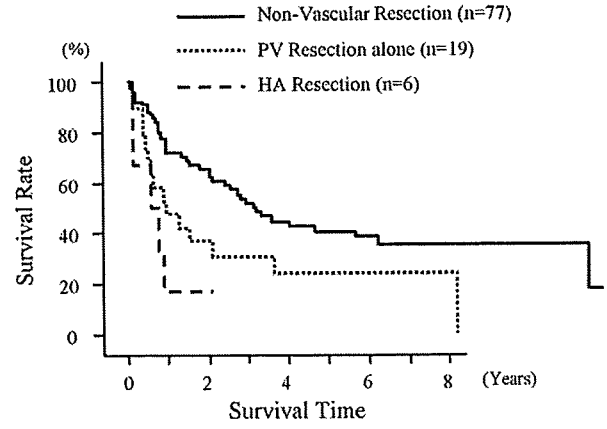

Fig 2. Survival after curative resection. Survival in the non-vascular resection group was better than in the portal vein (PV) resection group ($P < .05$).

more clearly the clinical implications of combined vascular resection for advanced hilar cholangiocarcinoma.

The 9% operative mortality rate in the portal vein resection alone group in the current study was not different significantly from the 4% rate in the non-vascular resection group. This operative mortality after combined vascular resection might be reduced by recent advances in operative technique, especially with the recent introduction of preoperative portal vein embolization and of parenchyma preserving hepatectomy.[4] Indeed, we have had no

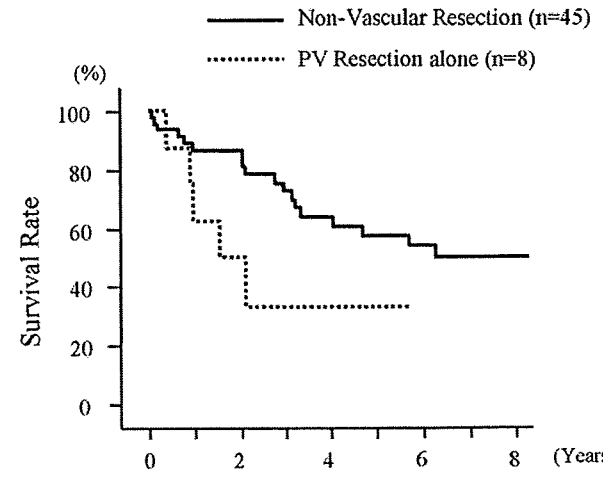

Fig 3. Survival after curative resection in hilar cholangiocarcinoma without lymph node metastases. There was no significant difference between survival rates of the non-vascular resection group and of the portal vein (PV) resection alone group.

operative deaths during the last 3 years in our institution by use of these advances; however the 33% operative mortality rate in the hepatic artery resection group was significantly greater than in the non-vascular resection group, but not significantly different from that in the portal vein resection alone group, although the numbers are small. Ebata et al[7] and Munoz et al[17] have reported a similar mortality rates of about 10%, in their portal vein resection groups of 52 patients each. There are few reports of operative mortality rates after hepatic artery resection. Madariaga et al[18] and Gerhards et al[5] have reported very high mortality rates of 5/9 and 5/9 respectively, after hepatic artery resection for hilar cholangiocarcinoma. Despite recent advances in operative techniques for hepatic artery reconstruction, as shown with results in liver transplantation several reasons might account for these high mortality rates after hepatic artery resection for hilar cholangiocarcinoma, such as a greater duration of liver ischemia due to simultaneous portal vein resection in most cases, and pre-existing liver dysfunction due to obstructive jaundice and persistent cholangitis.

The histologic features in the resected vessels were interesting, because cancer invasion into the adventitia was present in 80% of the resected portal veins and 40% of the resected hepatic arteries. In our series, combined vascular resection was carried our when cancer invasion to the vessels was diagnosed on the basis of both preoperative imaging findings and intraoperative macroscopic findings. Similar results, with no histologic invasion in 31%

of resected portal veins, have been reported by Ebata et al.[7] In contrast, caution should be exercised in when planning combined hepatic artery resection, because cancer invasion into adventitia of the hepatic artery occurs only about half of the patients despite clinically findings of apparent cancer invasion, as shown in our series; nevertheless, if the cancer encases the artery, resection for cure will require hepatic artery resection anyway.

In our series, combined vascular resection was carried out only in the patients with suspicion of cancer invasion to the vessels. We did not carry out an "en bloc" vascular resection such as reported by Neuhaus et al[6] who reported benefits of an "en bloc" portal vein resection in extended right hepatectomy. We are not sure whether this issue affects the results or not. As indicated by our results of multivariate analysis, both portal vein resection and hepatic artery resection are independent prognostic factors after operative resection in addition to both lymph node metastases and curative resection. Most studies reported previously[4,7,18] have indicated that combined vascular resection of the portal vein with hepatic artery resection was independently a factor in poor prognosis (in studies with both small and larger numbers of patients). Our study showed clearly that resections of the portal vein alone and of the hepatic artery were an independent prognostic factor after resection. In contrast, survival after both types of vascular resection differed from that of the non-vascular resection group and of the non-resection group. Portal vein resection alone seemed to confer a beneficial effect on prognosis of patients with hilar cholangiocarcinoma involving the portal vein, without increasing operative risk, however, combining hepatic artery resection with the portal vein resection was not of benefit in terms of survival, in comparison with the outcomes of unresectable patients. From our results, it seems that combined hepatic artery resection had no beneficial effect on prognosis and led to an increase in operative morbidity and mortality. In contrast, portal vein resection can be used aggressively in advanced cases of hilar cholangiocarcinoma in patients without lymph node metastases.

In conclusion, although portal vein resection is acceptable from a operative risk perspective and seems to improve the outcome in the selected patients with locally advanced hilar cholangiocarcinoma, hepatic artery resection could not be justified.

REFERENCES

1. Jarnagin WR. Cholangiocarcinoma of the extrahepatic bile ducts. Semin Surg Oncol 2000;19:156-76.
2. Klempnauer J, Ridder GJ, Werner M, Weimann A, Pichlmayr R. What constitutes long term survival after surgery for hilar cholangiocarcinoma? Cancer 1997;79:26-34.
3. Iwatsuki S, Todo S, Marsh JW, Madariaga JR, Lee RG, Dvorchik I, et al. Treatment of hilar cholangiocarcinoma (Klatskin Tumors) with hepatic resection or transplantation. J Am Coll Surg 1998;187:358-64.
4. Miyazaki M, Ito H, Nakagawa K, Ambiru S, Shimizu H, Okaya T, et al. Parenchyma-preserving hepatectomy in the surgical treatment of hilar cholangiocarcinoma. J Am Coll Surg 1999;189:575-83.
5. Gerhards MF, Gulik TM, Wit LT, Obertop H, Gouma DJ. Evaluation of morbidity and mortality after resection for hilar cholangiocarcinoma-a single center experience. Surgery 2000;127:395-404.
6. Neuhaus P, Jonas S, Bechstein WO, Lohmann R, Radke C, Kling N, et al. Extended resections for hilar cholangiocarcinoma. Ann Surg 1999;230:808-19.
7. Ebata T, Nagino M, Kamiya J, Uesaka K, Nagasaka T, Nimura Y. Hepatectomy with portal vein resection for hilar cholangiocarcinoma. Ann Surg 2003;238:720-7.
8. Munoz L, Roayaie S, Maman D, et al. Hilar cholangiocarcinoma involving the portal vein bifurcation: long-term results after resection. J Hepatobiliary Pancreat Surg 2002; 9:237-41.
9. Jarnagin WR, Fong Y, DeMatteo RP, et al. Staging, resectability, and outcome in 225 patients with hilar cholangiocarcinoma. Ann Surg 2001;234:507-19.
10. International Union Against Cancer (UICC). TNM classification of malignant tumors, 6th ed. New York: Wiley-Liss; 2002.
11. Miyazaki M, Ito H, Nakagawa K, et al. Segments I and IV resection as a new approach for hepatic hilar cholangiocarcinoma. Am J Surg 1998;185:229-31.
12. Miyazaki M, Itoh H, Kaiho T, et al. Portal vein reconstruction at the hepatic hilus using a left renal vein graft. J Am Coll Surg 1995;180:497-8.
13. Tsuzuki T, Ogata Y, Iida S, Nakamura I, Takenaka Y, Yoshii H. Carcinoma of the bifurcation of the hepatic ducts. Arch Surg 1983;118:1147-51.
14. Sakaguchi S, Nakamura S. Surgery of the portal vein in resection of cancer of the hepatic hilus. Surgery 1986; 99:344-9.
15. Fortner JG, Vitelli CE, Maclean BJ. Proximal extrahepatic bile duct tumors. Arch Surg 1989;124:1275-9.
16. Miyazaki M, Ito H, Nakagawa K, et al. Aggressive surgical approaches to hilar cholangiocarcinoma: hepatic or local resection? Surgery 1998;123:131-6.
17. Munoz L, Roayaie S, Maman D, et al. Hilar cholangiocarcinoma involving the portal vein bifurcation: long-term results after resection. J Hepatobiliary Pancreat Surg 2002; 9:237-41.
18. Madariaga JR, Iwatsuki S, Todo S, Lee RG, Irish W, Starzl TE. Liver resection for hilar and peripheral cholangiocarcinomas: a study of 62 cases. Ann Surg 1998;227:70-9.

Rapamycin, a specific inhibitor of the mammalian target of rapamycin, suppresses lymphangiogenesis and lymphatic metastasis

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Tumor lymphangiogenesis is now known to play a causal role in lymph node metastasis, and thus its inhibition would have great significance for the prevention of lymph node metastasis in cancer therapy. VEGF-C has recently been identified as a key molecule that involved in tumor lymphangiogenesis and lymphatic metastasis. However, the expressional regulation of VEGF-C is not fully understood. We investigated the role of mTOR, which is a downstream kinase of the phosphatidylinositol 3-kinase/Akt pathway, and the MAPK family (MEK1/2, p38, and JNK) in the regulation of VEGF-C and VEGF-A expression in B13LM cells, a lymphatic metastasis-prone pancreatic tumor cell line. We also investigated the antilymphangiogenic effect of rapamycin, a specific inhibitor of mTOR *in vivo* using male BALB/c nu/nu mice. VEGF-C expression was inhibited by the inhibitors for mTOR, p38, and JNK, but not by the inhibitor for MEK1/2, whereas VEGF-A expression was inhibited by all four of these inhibitors. The serum starvation-induced expression of VEGF-C was inhibited by rapamycin, whereas that of VEGF-A was incompletely inhibited. The metastatic experiment *in vivo* demonstrated that the number and the area of lymphatic vessels in the primary tumors were significantly decreased by rapamycin. Finally, the lymph node metastasis was significantly suppressed in rapamycin-treated mice. Our results suggest that mTOR, p38, and JNK play important roles in VEGF-C expression, and that rapamycin has an antilymphangiogentic effect and exerts the expected inhibition of lymphatic metastasis. (*Cancer Sci* 2007; 98: 726–733)

Metastatic dissemination is the final process in the progression of malignant tumors. Therefore, prevention of metastasis must be an ultimate goal for the treatment of malignant tumors. Spread of a malignant tumor from its primary site occurs by three main routes: vascular, lymphatic and transcoelomic. Among them, metastasis to the regional lymph nodes is often the earliest appearing metastasis, which significantly affects the prognosis of patients. In conjunction with recent advances in our understanding of the lymphatic system, accumulated experimental data have shown that tumor-induced lymphangiogenesis is an important mechanism promoting lymphatic metastasis.[1,2–4] A series of studies that investigated the relationship between the lymphatic vessel density in tumors and lymph node metastasis demonstrated that high lymphatic vessel density correlated with frequent lymph node metastasis[5–10] and with poor survival in multiple tumor types, including breast cancer, head and neck squamous cell carcinoma, and melanoma.[5,7,8]

VEGF-C has recently been identified as a key regulator in lymphangiogenesis.[11,12] VEGF-C has a VEGF-homologs region in the N-terminal and binds VEGFR-3, an fms-like tyrosine kinase receptor.[13] VEGF-C also binds to VEGFR-2 and can activate angiogenesis, but the higher affinity of VEGF-C for VEGFR-3 than for VEGFR-2 suggests that VEGF-C is a biologically relevant ligand of VEGFR-3.[14–16] Overexpression of VEGF-C in breast cancer cells promotes tumor lymphangiogenesis and increased lymph node metastasis.[1] The correlation between the expression of VEGF-C in tumor cells and lymph node metastasis is significant for a variety of tumor types, and the VEGF-C level in the primary tumor positively correlates with poor prognosis of patients.[17–24] Thus, the inhibition of VEGF-C expression in tumor cells could be a potential strategy for preventing lymph node metastasis.

The expressional regulation of VEGF-A has been well investigated. Hypoxia induces VEGF-A expression in an Akt-dependent pathway with downstream activation of HIF-1.[25] The MAPK family also mediates the signal transduction of VEGF-A expression in response to cell stresses.[26] Extracellular stimulation by growth factors has been shown to induce VEGF-C[27] and VEGF-A[28] at similar levels. However, the signal transduction involved in the expressional regulation of VEGF-C has not been fully established. PI3K and the MAPK family are involved in the signal transduction pathways of IGF-1-induced VEGF-C expression in lung carcinoma cells.[29] In contrast, IGF-1R signaling negatively regulates VEGF expression in prostatic cancer cells under conditions of androgen depletion.

Rapamycin is a lipophilic macrolide antibiotic that was initially developed as a fungicide and immunosuppressant.[30] Rapamycin acts as a specific inhibitor of mTOR, a serine/threonine kinase, that appears to be downstream of the PI3K/Akt signal pathway.[31] A complex of rapamycin and the FKBP-12 binds to mTOR and inhibits its activity.[32] The signaling through mTOR regulates phosphorylation and activation of its two major downstream components, p70S6K and eIF4E-binding protein 1 (4E-BP1).[33,34] Phosphorylation of p70S6K allows translation of ribosomal proteins.[35] Phosphorylation of 4E-BP1 regulates cap-dependent translation by enabling the formation of an active eIF4E complex.[36] mTOR plays a pivotal role in regulating the transcription initiation of many genes related to the process of oncogenic transformation and cancer progression.[37,38]

It has been noted that rapamycin and its derivatives exert a potent antitumor action on a variety of solid tumors.[39–42] The antiangiogenic effect of rapamycin is one of the mechanisms responsible for suppressing the tumor progression.[31,42] The mechanism responsible for the antiangiogenic effect of rapamycin is the inhibition of VEGF-A expression that is regulated by the Akt/mTOR pathway.[43] However, neither the effect of

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Abbreviations: 4E-BP1, initiation factor 4E-binding protein 1; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; eIF4E, initiation factor 4E; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HIF, hypoxia-inducible factor; FKBP, FK506-binding protein; IGF, insulin-like growth factor; IGF-1R, insulin-like growth factor receptor-1; PBS, phosphate buffered saline; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; RT-PCR, reverse transcriptase polymerase chain reaction; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; TGF-β tumor growth factor-β VEGF, vascular endothelial growth factor.

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rapamycin in regulating VEGF-C expression nor the effect of rapamycin on tumor-associated lymphangiogenesis has been determined. We investigated the role of signal pathways, including the mTOR pathway and MAPK pathways, in regulating the VEGF-C expression in tumor cells, and also investigated whether rapamycin reduces lymphatic metastasis using lymphatic-metastasis-prone murine pancreatic tumor cells. The main purpose of this study was to determine the effect of rapamycin on lymphangiogenesis and lymph node metastasis.

Materials and Methods

Mice. Male BALB/C *nu/nu* mice, 4 weeks old, were obtained from Japan SLC, Hamamatsu, Japan. All mice were maintained under specific pathogen-free conditions at the Center for Animal Experimentation, Chiba University Graduate School of Medicine. Regular laboratory food and tap water were made available *ad libitum*. All animal experiments were carried out under the guidelines of Chiba University.

Cell lines and culture conditions. Lymphatic metastasis-prone cells were established by *in vivo* selection of cells of a rat pancreatic tumor cell line, AR42J-B13, which were kindly provided by Professor Kojima (Gummma University, Maebashi, Japan). AR42J-B13 cells were inoculated subcutaneously into nude mice. After 3 weeks, mice were killed and the metastatic lymph nodes were resected. The resected lymph nodes were cut into small fragments *in vitro*. The fragmented tissues were then incubated with collagenase (1 mg/mL)(Wako, Osaka, Japan) for 24 h and washed with PBS. Harvested cells were cultured *in vitro* and re-inoculated into the nude mice for the second round of selection. The lymphatic metastasis-prone cell line, which was designated as B13LM, was obtained by repeating the procedure 10 times. B13LM cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) containing penicillin (50 unit/mL), streptomycin (50 μg/mL) (Invitrogen, Carlsbad, CA, USA), L-glutamine (2 mM), and 10% FBS (Biological Industries, Kibbutz Beit Haemek, Israel) at 37°C in 5%CO_2. For serum starvation, B13LM cells were cultured in the medium without FBS for 24 h.

Inhibition of signal transduction kinases. B13LM cells were cultured with serial concentrations of inhibitors for signal transduction kinases. Rapamycin (Cell Signaling Technology, Beverly, MA, USA) was added to the medium at a concentration of 0 nM, 1 nM, 10 nM, or 100 nM. U0126 (an inhibitor of MEK1/2) (Calbiochem, La Jolla, CA, USA), SB202190 (an inhibitor of p38) (Calbiochem), and SP600125 (an inhibitor of JNK) (Calbiochem) were added to the medium at a concentration of 0 μM, 1 μM, or 25 μM. Cells were treated by each inhibitor for 48 h and then prepared for Western blot or quantitative RT-PCR analysis.

Enzyme-linked immunosorbent assay. Concentrations of VEGF-C in the medium of the cultured B13LM cells were determined by ELISA (Bender MedSystems, Burlingame, CA, USA). The immunoassays were carried out and analyzed according to the manufacturer's instructions. B13LM cells were cultured in the medium with rapamycin (100 nM), then conditioned medium was used for ELISA. All samples and standards were run in duplicate.

Quantitative RT-PCR analysis. Total RNAs from B13LM cells were isolated using an RNeasy Mini kit (Quiagen, Tokyo, Japan) according to the manufacturer's instructions. One microgram of total RNA was subjected to a reverse transcription reaction, using a Ready To Go T-primed 1st strand cDNA synthesis kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). The cDNA from 33 ng of total RNA was used as a template. VEGF-A and VEGF-C mRNA levels were quantified by means of a LightCycler (Roche Diagnostics, Mannheim, Germany), using the double-strand-specific dye SYBE Green I and a HybProbe LightCycler RNA amplification kit specifically adapted for one-step RT-PCR in glass capillaries with a LightCycler instrument (Roche Diagnostics). The primer sequences used in this study were as follows: for rat VEGF-A, 5′-TATATCTTCAAGCCGTCCTG-3′ (forward) and 5′-TTGGTCTGCATTCACATCTG-3′ (reverse); rat VEGF-C, 5′-TGTCCAGCAAACTACGTGTG-3′ (forward) and 5′-ACTGGCAGGTGTCTTCATCC-3′ (reverse); rat β-actin, 5′-CTCCAGGATCTCACGCTCTA-3′ (forward) and 5′-AGAAGAAGCTGGGAAGAGAC-3′ (reverse). The cycling conditions were as follows: initial reverse transcription at 61°C for 30 min, denaturation at 95°C for 30 s, and 45 cycles of denaturation at 95°C for 1 s, annealing at 60°C for 15 s, and elongation at 65°C for 1 min with a ramp of 5°C/s (with fluorescence acquisition at the end of each elongation stage). The expression level of each mRNA was adjusted using the level of β-actin mRNA, and expressed as the ratio to β-actin mRNA.

Western blot analysis. B13LM cells were cultured in fully supplemented medium for 24 h, then cultured for 48 h with medium containing kinase inhibitors or cultured for 1 h, 2 h, 4 h, and 12 h without FBS. After this conditioning period, cells were homogenized in lysis buffer (1 mL RIPA buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM (EDTA), 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS], 100 μL Protease Inhibitor Cocktail [Sigma], and 10 μL Phosphatase Inhibitor Cocktail [Sigma]) and put on ice for 2 h. Protein concentration of each sample was determined by using a Bio-Rad Protein Assay kit (Bio-Rad, Laboratories, Hercules, CA, USA) according to manufacturer's instructions. Lysates containing 50 μg of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes (Nihon Eido, Tokyo, Japan). Nonspecific reactions were blocked for 4 h with TBS-T (10 mM Tris [pH 7.4], 100 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk. Then membranes were incubated overnight at 4°C with each antibody. After being washed with TBS-T containing non-fat dry milk, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibodies. The protein blots were visualized by chemiluminescence using ECL (Amersham).

Antibodies against Akt, phosphor-Akt (Ser473), eIF4E, phospho-eIF4E (Ser209), 4E-BP1, phospho-4E-BP1 (Thr70), p70S6K, phospho-p70S6K (Thr421/Ser424), mTOR, phospho-mTOR (Ser2448) were purchased from Cell Signaling Technology. Antibodies against VEGF-A and VEGF-C were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Experimental metastatic model. To establish subcutaneous tumors in mice, 5.0×10^6 B13LM cells were injected subcutaneously into the left inferior limb. Tumors were allowed to grow for 7 days and the establishment of subcutaneous tumors was confirmed before rapamycin treatment. Rapamycin (1.5 mg/kg per day)($n = 10$) or vehicle alone ($n = 11$) was intraperitoneally administrated to the mice every day from 8 days after the injection of tumor cells. The tumor volume ([major axis] × [minor axis]^2 × π/6) was measured every other day. Three weeks from the start of treatment, mice were killed. The subcutaneous tumors were removed and prepared for histological analysis. Lymph node metastasis was investigated, and the occurrence of metastasis was confirmed by microscope.

Histological and immunohistochemical analysis. Formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin and these sections were also used for immunohistochemical analysis. Immunostaining was carried out using the labeled streptoavidin-biotin-peroxidase (Dako Cytomation, Kyoto, Japan) and microwave antigen retrieval techniques. Goat polyclonal anti-LYVE-1 (1:100) was obtained from Santa Cruz Biotechnology. Diaminobenzidine tetrahydrochloride substrate was used to visualize the positive staining.

Evaluation of lymphatic vessels. Evaluation of lymphatic vessels in the vicinity of the subcutaneous tumors was carried out using

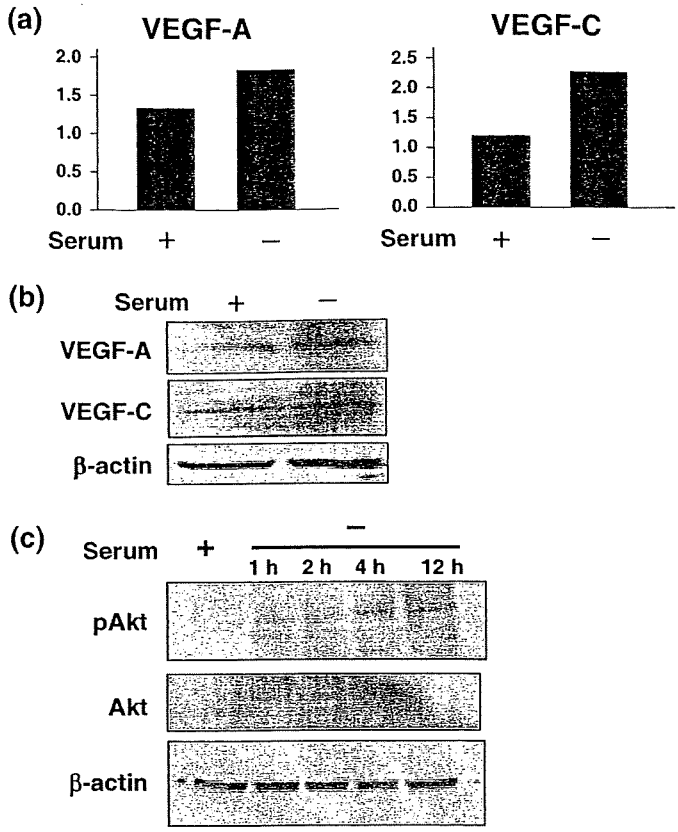

Fig. 1. The expression of vascular endothelial growth factor-A (VEGF-A) and VEGF-C in B13LM cells was evaluated by quantitative reverse transcription-polymerase chain reaction (RT-PCR) (a) and Western blot analysis (a representative data of two experiments) (b). Constitutive expression of VEGF-A and VEGF-C was observed in B13LM cells under a culture condition with 10% fetal bovine serum (FBS). The expression of VEGF-A and VEGF-C was up-regulated by culture in serum-free medium for 24 h (c) Western blot analysis of Akt and phosphor-Akt in B13LM cells. Proteins were prepared form B13LM cells that were cultured with 10% FBS (serum +) or without FBS for 1 h, 2 h, 4 h and 12 h (serum –). Phosphorylation of Akt (pAkt) was increased in serum-free medium.

LYVE-1 stains and computer-assisted quantitative analysis. Sections were scanned at low magnification, and 10 hot spot areas with the greatest numbers of positively stained vessels were identified in each section. Each hot spot was examined in turn at 400 × magnification and captured using an AxioCam MRc5 digital camera system (Carl Zeiss, Tokyo, Japan). The number of lymphatic vessels was counted, and the mean value for the 10 hot spot areas was determined for each section. The area of lymphatic vessel lumen was measured using an AxioVision 4.4 image analysis system (Carl Zeiss) by tracing the lymphatic vessel walls on a computer monitor. The mean value of the 10 hot spot areas was calculated and used as the value for each section.

Statistical analysis. Data are given as the mean ± standard deviation in quantitative experiments. The significance of the data was determined by unpaired Student's *t*-test for the evaluation of lymphangiogenesis, by a repeated-measure ANOVA test for the evaluation of tumor size, and by χ^2 test for the evaluation of lymph node metastasis. Values of P of less than 0.05 were considered statistically significant.

Results

Expression of VEGF-A and VEGF-C in B13LM cells. To investigate the role of the mTOR pathway in tumor lymphangiogenesis, we used cells of a lymphatic-metastasis prone tumor cell line, B13LM. We investigated the expression of VEGF-A and VEGF-C in B13LM cells by quantitative RT-PCR and Western blot analysis. B13LM cells constitutively expressed both VEGF-A and VEGF-C under normal culture conditions. To evaluate whether the expressional regulation of VEGF in response to cell stress remained intact in B13LM cells, we examined the expression of VEGF-A and VEGF-C under serum-starved conditions. The expressions of both VEGF-A and VEGF-C were up-regulated when the cells were cultured in serum-free medium for 24 h (Fig. 1a,b). Phosphorylation of Akt was increased in B13LM cells by the withdrawal of FBS from the cultured medium (Fig. 1c). Increased phosphorylation of Akt under the serum-starved conditions suggested that the PI3K/Akt pathway, which is upstream of mTOR, was activated by serum starvation in the B13LM cells.

Inhibition of the mTOR pathway in B13LM cells by rapamycin. We evaluated the inhibitory effect of rapamycin, a specific inhibitor of mTOR, in B13LM cells by analyzing the phosphorylation state of mTOR and the target molecules of mTOR. mTOR and its three downstream molecules, 4E-BP1, p70S6K, and eIF4E, were all phosphorylated in B13LM cells under the culture conditions containing 10% FBS, indicating the activity of the mTOR pathway in B13LM cells. Slight dephosphorylation of mTOR was observed in the B13LM cells treated with 1 nM of rapamycin, and mTOR appeared to be remarkably dephosphorylated in the B13LM cells treated with 100 nM of rapamycin. 4E-BP1, eIF4E and p70S6K were also dephosphorylated in B13LM cells treated with 1 nM of rapamycin. The reductions in the phosphorylation of these target molecules were considerable, although some phosphorylation remained (Fig. 2). These results indicated the efficacy of rapamycin for inhibiting the mTOR signal pathway in B13LM cells.

Inhibition of the expression of VEGF-C by rapamycin. We investigated whether the expressions of VEGF-A and VEGF-C were inhibited by rapamycin in B13LM cells *in vitro*. Dose-dependent reductions of VEGF-A and VEGF-C expression were observed when the cells were cultured with rapamycin for 48 h. The reduction of VEGF-C expression was more significant than that of VEGF-A expression (Fig. 3a). The secretion of VEGF-C was also reduced when B13LM cells were treated with rapamycin (Table 1). Next we investigated whether rapamycin can inhibit the serum starvation-induced induction of VEGFs. The mRNA expression of both VEGF-A and VEGF-C was increased under the normal culture conditions (Fig. 1a). Rapamycin repressed mRNA expression of both VEGF-A and VEGF-C of B13LM cells in normal culture conditions. Moreover, rapamycin repressed the serum starvation-induced expression of both of VEGF-A and VEGF-C, although the inhibition of VEGF-A mRNA was incomplete (Fig. 3b).

VEGF-C expression was inhibited by the inhibitors of p38 and JNK but not by the inhibitor of MEK1/2. We investigated the role of signal transduction pathways via three MAPK family members – MAPK kinase (MEK)1/2, p38, and c-Jun N-terminal kinase (JNK) – in the regulation of VEGF-C expression. We also investigated the regulation of VEGF-A expression by MAPK signaling, because MAPK signaling is known to be involved in the regulation of VEGF-A expression. The inhibitors of MEK1/2, p38, and JNK inhibited the expression of VEGF-A mRNA in a dose-dependent manner in B13LM cells. The inhibitors of p38 and JNK also inhibited the expression of VEGF-C mRNA, although a higher concentration of inhibitor was needed for effective inhibition of VEGF-C expression than for effective inhibition of VEGF-A. The inhibitory effect of the MEK1/2 inhibitor on the expression of VEGF-C mRNA was not clear (Fig. 4).

Rapamycin inhibited the intratumor lymphangiogenesis and the lymphatic metastasis in nude mice. We evaluated whether rapamycin plays a role in lymphangiogenesis and lymphatic metastasis

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