Table 4
Multivariate Cox Proportional Hazards Analysis for the Candidate Variables

	Overall Survival			Recurrence-Free Survival		
Prognostic Factor	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
CXCL12+ tumor budding grade	1.859	0.782-4.417	.160	2.099	0.998-4.415	.051
Tumor depth*	1.422	0.624-3.242	.402	1.406	0.702-2.814	.336
Lymphatic vessel invasion	0.990	0.305-3.215	.986	1.483	0.478-4.598	.495
Blood vessel invasion	1.278	0.505-3.238	.605	1.531	0.661-3.549	.320
Lymph node metastasis	3.987	1.302-12.212	.015	2.052	0.886-4.755	.094

HR, hazard ratio; CI, confidence interval.
* For description, see the footnotes for Table 1.

staining ($P < .001$), lymph node metastasis ($P = .022$), and lung metastasis ($P = .014$). In addition, it showed a tendency to be correlated with liver metastasis ($P = .052$) (Table 1). Univariate analysis using the Cox proportional hazards model revealed that the combination of CXCL12 expression and CXCL12+ tumor budding grade was a significant prognostic factor (Table 2). Multivariate analysis using the Cox proportional hazards model revealed that the combination of CXCL12 expression and CXCL12+ tumor budding grade was an independent and significant prognostic factor for overall survival, together with lymph node metastasis, and also an independent prognostic factor for recurrence **Table 5**.

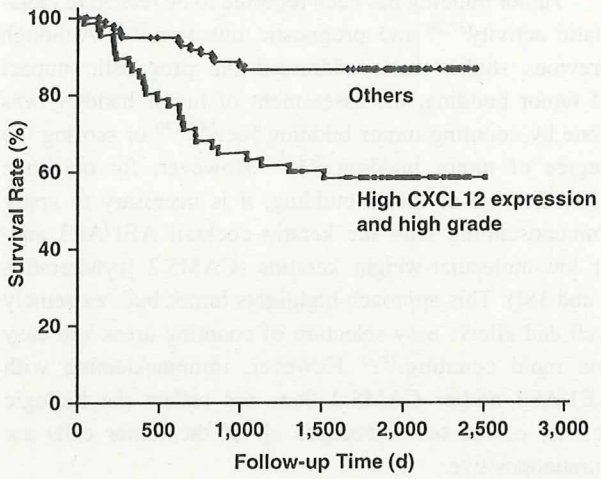

Figure 3 Kaplan-Meier survival curves of patients with colorectal carcinoma subdivided according to the combination of the proportion of CXCL12 expression and the grade of CXCL12+ tumor budding ($P < .001$; log-rank test).

Discussion

CXCL12 expression in colorectal cancer cells and at foci of tumor budding was found to be an independent predictive factor for cancer recurrence and poor survival. The present study indicated that CXCL12 expression in tumor cells was correlated with liver metastasis and was an independent prognostic factor together with lymph node metastasis. As it has been demonstrated that CXCL12 promotes tumor growth and malignancy,[21] colorectal cancers exhibiting high CXCL12 expression seem to show aggressive biologic behavior, with poor patient survival. To our knowledge, correlations between CXCL12 expression and long-term survival have been recognized in breast carcinoma,[21] ovarian

Table 5
Multivariate Cox Proportional Hazards Analysis for the Candidate Variables

	Overall Survival			Recurrence-Free Survival		
Prognostic Factor	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
CXCL12 expression and CXCL12+ tumor budding grade	2.48	1.065-5.776	.035	2.713	1.313-5.605	.007
Tumor depth*	1.261	0.556-2.860	.579	1.28	0.639-2.562	.486
Lymphatic vessel invasion	1.061	0.324-3.478	.922	1.571	0.504-4.892	.436
Blood vessel invasion	1.26	0.496-3.203	.627	1.51	0.650-3.504	.338
Lymph node metastasis	3.932	1.296-11.929	.016	2.056	0.895-4.721	.089

HR, hazard ratio; CI, confidence interval.
* For description, see the footnotes for Table 1.

DOI: 10.1309/AJCPK35VZJEWCUTL

carcinoma,[19] glioma,[20] esophageal carcinoma,[17] and gastric carcinoma.[18] Immunohistochemical staining of frozen breast cancer tissues has demonstrated CXCL12 mostly in tumor cells and stromal cells, and the level of CXCL12 transcription in the tumor is correlated significantly with overall survival and incidence-free survival.[21] In addition, in this study, we noted that CXCL12 expression was stronger at the invasive front than in other areas of colorectal cancer, reflecting the fact that the invasive front shows the most active interaction between cancer and stroma. We therefore further focused on CXCL12 expression at sites of tumor budding.

Tumor budding has been reported to be related to metastatic activity[27,28] and prognostic outcome.[26,29] Although previous studies have addressed the prognostic impact of tumor budding, the assessment of tumor budding was done by counting tumor budding foci[24,25,30] or scoring the degree of tumor budding.[29,31,32] However, for objective quantification of tumor budding, it is necessary to apply immunostaining with the keratin cocktail AE1/AE3 and/or low-molecular-weight keratins (CAM5.2 [cytokeratins 8 and 18]). This approach highlights tumor buds extremely well and allows easy selection of counting areas and easy and rapid counting.[24,31] However, immunostaining with AE1/AE3 and/or CAM5.2 does not reflect the biologic activity of the tumor because all of the tumor cells are immunopositive.

In this study, we were able to count the numbers of tumor budding foci more easily than by H&E staining by using immunohistochemical analysis for CXCL12. CXCL12 immunostaining was used not only to highlight tumor cells but also to examine their potential aggressiveness by counting the number that were CXCL12+. CXCL12+ tumor budding grade was also correlated with the depth of tumor invasion, tumor budding grade determined by H&E staining, lymph node metastasis, and lung metastasis. CXCL12+ tumor budding grade was also demonstrated to be a prognostic indicator for overall survival and recurrence, although multivariate analysis showed that it was not an independent factor.

One reason why CXCL12+ tumor budding grade was not an independent factor may have been that the grade was judged in only 1 area where the budding foci were most intense and, therefore, probably did not reflect the properties of the whole tumor. Therefore, we combined the grading of CXCL12 immunopositivity for tumor buds with the proportion of tumor cells expressing CXCL12. We found that patients whose tumors showed high CXCL12 expression and high grade had the worst outcome. The combination of CXCL12 expression and CXCL12+ tumor budding grade was further correlated with the depth of tumor invasion, tumor budding grade determined by H&E staining, lymph node metastasis, and lung metastasis and tended to be correlated with liver metastasis. Moreover, this combination was an independent and significant prognostic factor for overall survival, together with lymph node metastasis, and also an independent prognostic factor for recurrence.

In our study, only a few grade 3 and 4 cases (5 of 165 cases) were included. Therefore, it seems possible that tumor differentiation did not affect the results of analyses. In general, the majority of colorectal carcinomas are diagnosed as grade 1 or 2, and the majority of grade 3 or 4 cases are included among cases that are more advanced than stage II or III.

In the present study, CXCL12 was expressed distinctly in colorectal cancer cells, normal epithelium of the colon (especially in the middle to upper third of each crypt in the mucosal layer), and blood and lymphatic endothelial cells and weakly in fibroblasts. Previous reports indicated that CXCL12 was constitutively expressed in various organs, including lymph nodes, lung, liver, thymus, and stromal cells such as fibroblasts, endothelial cells, and osteoblasts in bone marrow.[4,6,12]

Multiple biologic activities of CXCL12 have also been described.[7-10] For example, in vitro studies have shown that CXCL12 can modulate tumor cell proliferation and migration.[11,33,34] CXCL12 probably stimulates the formation of capillary-like structures by human vascular endothelial cells.[9,35,36] In addition, hypoxia-dependent up-regulation of the chemokine receptor CXCR4 practically promotes breast cancer invasion and organ-specific metastasis.[37,38] Colorectal carcinoma cells, especially those at the invasive front, are likely to be situated in a hypoxic milieu.[39,40] Therefore, this may lead to further tumor invasion through up-regulation of CXCL12/CXCR4.

CXCL12 expression in colorectal cancer cells and the grading of CXCL12 immunopositivity at foci of tumor budding are each significant prognostic factors. However, our present results suggest that, in colorectal carcinoma, CXCL12 expression used in combination with tumor budding grade is a more powerful prognostic indicator than either factor alone.

From the [1]Pathology Division, National Cancer Center Research Institute, Tokyo, Japan; and [2]Surgery Division, National Cancer Center Hospital, Tokyo.

Supported in part by a Grant-in-Aid for the Third Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare of Japan and grant 19790275 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Address reprint requests to Dr Akishima-Fukasawa: Pathology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan.

DOI: 10.1309/AJCPK35VZJEWCUTL

References

1. Ottaiano A, Franco R, Aiello Talamanca A, et al. Overexpression of both CXC chemokine receptor 4 and vascular endothelial growth factor proteins predicts early distant relapse in stage II-III colorectal cancer patients. *Clin Cancer Res.* 2006;12:2795-2803.
2. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. *Nature.* 2001;411:375-379.
3. Ibe S, Qin Z, Schuler T, et al. Tumor rejection by disturbing tumor stroma cell interactions. *J Exp Med.* 2001;194:1549-1559.
4. Shirozu M, Nakano T, Inazawa J, et al. Structure and chromosomal localization of the human stromal cell-derived factor 1 (*SDF1*) gene. *Genomics.* 1995;28:495-500.
5. Nagasawa T, Kikutani H, Kishimoto T. Molecular cloning and structure of a pre-B-cell growth-stimulating factor. *Proc Natl Acad Sci U S A.* 1994;91:2305-2309.
6. Tashiro K, Tada H, Heilker R, et al. Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins. *Science.* 1993;261:600-603.
7. Fernandis AZ, Prasad A, Band H, et al. Regulation of CXCR4-mediated chemotaxis and chemoinvasion of breast cancer cells. *Oncogene.* 2004;23:157-167.
8. Nagasawa T, Tachibana K, Kishimoto T. A novel CXC chemokine PBSF/SDF-1 and its receptor CXCR4: their functions in development, hematopoiesis and HIV infection. *Semin Immunol.* 1998;10:179-185.
9. Salcedo R, Wasserman K, Young HA, et al. Vascular endothelial growth factor and basic fibroblast growth factor induce expression of CXCR4 on human endothelial cells: in vivo neovascularization induced by stromal-derived factor-1alpha. *Am J Pathol.* 1999;154:1125-1135.
10. Tachibana K, Hirota S, Iizasa H, et al. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature.* 1998;393:591-594.
11. Phillips RJ, Burdick MD, Lutz M, et al. The stromal derived factor-1/CXCL12-CXC chemokine receptor 4 biological axis in non-small cell lung cancer metastases. *Am J Respir Crit Care Med.* 2003;167:1676-1686.
12. Muller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature.* 2001;410:50-56.
13. Zeelenberg IS, Ruuls-Van Stalle L, Roos E. The chemokine receptor CXCR4 is required for outgrowth of colon carcinoma micrometastases. *Cancer Res.* 2003;63:3833-3839.
14. Schimanski CC, Schwald S, Simiantonaki N, et al. Effect of chemokine receptors CXCR4 and CCR7 on the metastatic behavior of human colorectal cancer. *Clin Cancer Res.* 2005;11:1743-1750.
15. Kaifi JT, Yekebas EF, Schurr P, et al. Tumor-cell homing to lymph nodes and bone marrow and CXCR4 expression in esophageal cancer. *J Natl Cancer Inst.* 2005;97:1840-1847.
16. Taichman RS, Cooper C, Keller ET, et al. Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. *Cancer Res.* 2002;62:1832-1837.
17. Sasaki K, Natsugoe S, Ishigami S, et al. Expression of CXCL12 and its receptor CXCR4 correlates with lymph node metastasis in submucosal esophageal cancer. *J Surg Oncol.* 2008;97:433-438.
18. Ishigami S, Natsugoe S, Okumura H, et al. Clinical implication of CXCL12 expression in gastric cancer. *Ann Surg Oncol.* 2007;14:3154-3158.
19. Jiang YP, Wu XH, Shi B, et al. Expression of chemokine CXCL12 and its receptor CXCR4 in human epithelial ovarian cancer: an independent prognostic factor for tumor progression. *Gynecol Oncol.* 2006;103:226-233.
20. Salmaggi A, Gelati M, Pollo B, et al. CXCL12 expression is predictive of a shorter time to tumor progression in low-grade glioma: a single-institution study in 50 patients. *J Neurooncol.* 2005;74:287-293.
21. Kang H, Watkins G, Parr C, et al. Stromal cell derived factor-1: its influence on invasiveness and migration of breast cancer cells in vitro, and its association with prognosis and survival in human breast cancer. *Breast Cancer Res.* 2005;7:R402-R410.
22. Sobin L, Wittekind C; for the International Union Against Cancer (UICC), eds. *TNM Classification of Malignant Tumours.* 6th ed. New York, NY: Wiley-Liss; 2002.
23. Hamilton SR, Aaltonen LA. *Pathology and Genetics of Tumours of the Digestive System.* Lyon, France: IARC Press; 2000. *World Health Organization Classification of Tumours.*
24. Prall F, Nizze H, Barten M. Tumour budding as prognostic factor in stage I/II colorectal carcinoma. *Histopathology.* 2005;47:17-24.
25. Ueno H, Price AB, Wilkinson KH, et al. A new prognostic staging system for rectal cancer. *Ann Surg.* 2004;240:832-839.
26. Ueno H, Murphy J, Jass JR, et al. Tumour "budding" as an index to estimate the potential of aggressiveness in rectal cancer. *Histopathology.* 2002;40:127-132.
27. Okuyama T, Oya M, Ishikawa H. Budding as a risk factor for lymph node metastasis in pT1 or pT2 well-differentiated colorectal adenocarcinoma. *Dis Colon Rectum.* 2002;45:628-634.
28. Goldstein NS, Hart J. Histologic features associated with lymph node metastasis in stage T1 and superficial T2 rectal adenocarcinomas in abdominoperineal resection specimens: identifying a subset of patients for whom treatment with adjuvant therapy or completion abdominoperineal resection should be considered after local excision. *Am J Clin Pathol.* 1999;111:51-58.
29. Hase K, Shatney C, Johnson D, et al. Prognostic value of tumor "budding" in patients with colorectal cancer. *Dis Colon Rectum.* 1993;36:627-635.
30. Ueno H, Mochizuki H, Hashiguchi Y, et al. Predictors of extrahepatic recurrence after resection of colorectal liver metastases. *Br J Surg.* 2004;91:327-333.
31. Kazama S, Watanabe T, Ajioka Y, et al. Tumour budding at the deepest invasive margin correlates with lymph node metastasis in submucosal colorectal cancer detected by anticytokeratin antibody CAM5.2. *Br J Cancer.* 2006;94:293-298.
32. Nakamura T, Mitomi H, Kikuchi S, et al. Evaluation of the usefulness of tumor budding on the prediction of metastasis to the lung and liver after curative excision of colorectal cancer. *Hepatogastroenterology.* 2005;52:1432-1435.
33. Brand S, Dambacher J, Beigel F, et al. CXCR4 and CXCL12 are inversely expressed in colorectal cancer cells and modulate cancer cell migration, invasion and MMP-9 activation. *Exp Cell Res.* 2005;310:117-130.
34. Scotton CJ, Wilson JL, Scott K, et al. Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer. *Cancer Res.* 2002;62:5930-5938.
35. Salcedo R, Oppenheim JJ. Role of chemokines in angiogenesis: CXCL12/SDF-1 and CXCR4 interaction, a key regulator of endothelial cell responses. *Microcirculation.* 2003;10:359-370.
36. Mirshahi F, Pourtau J, Li H, et al. SDF-1 activity on microvascular endothelial cells: consequences on angiogenesis in in vitro and in vivo models. *Thromb Res.* 2000;99:587-594.

DOI: 10.1309/AJCPK35VZJEWCUTL

37. Phillips RJ, Mestas J, Gharaee-Kermani M, et al. Epidermal growth factor and hypoxia-induced expression of CXC chemokine receptor 4 on non-small cell lung cancer cells is regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin signaling pathway and activation of hypoxia inducible factor-1alpha. *J Biol Chem.* 2005;280:22473-22481.
38. Matteucci E, Locati M, Desiderio MA. Hepatocyte growth factor enhances CXCR4 expression favoring breast cancer cell invasiveness. *Exp Cell Res.* 2005;310:176-185.
39. Sullivan R, Graham CH. Hypoxia-driven selection of the metastatic phenotype. *Cancer Metastasis Rev.* 2007;26:319-331.
40. Zhong H, De Marzo AM, Laughner E, et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res.* 1999;59:5830-5835.

DOI: 10.1309/AJCPK35VZJEWCUTL

Clinical Translational Research

Oncology

Oncology 2009;77:53–62
DOI: 10.1159/000226112

Received: September 24, 2008
Accepted after revision: February 16, 2009
Published online: June 25, 2009

Podoplanin Expression Identified in Stromal Fibroblasts as a Favorable Prognostic Marker in Patients with Colorectal Carcinoma

Takahiro Yamanashi[a, c] Yukihiro Nakanishi[a] Gen Fujii[a] Yuri Akishima-Fukasawa[a]
Yoshihiro Moriya[b] Yae Kanai[a] Masahiko Watanabe[c] Setsuo Hirohashi[a]

[a]Pathology and [b]Surgery Divisions, National Cancer Center Research Institute and Hospital, Tokyo, and
[c]Department of Surgery, Kitasato University School of Medicine, Kanagawa, Japan

Key Words
Podoplanin · Colorectal cancer · Prognosis · Immunohistochemistry · Clinicopathologic study

Abstract
Objective: The microenvironment of cancer plays a critical role in its progression. However, the molecular features of cancer-associated fibroblasts (CAFs) are less well understood than those of cancer cells. We investigated the clinicopathological significance of podoplanin expression in stromal fibroblasts in patients with colorectal cancer (CRC). ***Methods:*** We selected podoplanin as an upregulated marker in CAF from a DNA microarray experiment. Consequently, podoplanin was identified as an upregulated gene. Immunohistochemical podoplanin expression was investigated at the National Cancer Center Hospital, Tokyo, Japan, in 120 patients with advanced CRC, and its clinicopathological significance was examined. The biological function of podoplanin expression was also assessed by a coculture invasion assay with CRC cell lines such as HCT116 and HCT15. ***Results:*** Podoplanin expression was exclusively confined to stromal fibroblasts and absent in tumor cells. Podoplanin is absent in normal stroma except for lymphatic vessels. Staining was considered positive when over 30% of the cancer stroma was stained. Positive podoplanin expression was significantly correlated with a more distal tumor localization ($p = 0.013$) and a shallower depth of tumor invasion ($p = 0.011$). Univariate analysis revealed that negative podoplanin expression in stromal fibroblasts was significantly associated with reduced disease-specific survival ($p = 0.0017$) and disease-free survival ($p < 0.0001$). Multivariate analysis revealed that negative podoplanin expression ($p = 0.016$) and lymph node metastasis ($p = 0.027$) were significantly associated with disease-free survival. CRC cell invasion was augmented by coculture with CAFs that were treated with siRNA for podoplanin. ***Conclusions:*** Our results suggest that a positive podoplanin expression in stromal fibroblasts could have a protective role against CRC cell invasion and is a significant indicator of a good prognosis in patients with advanced CRC, supported by biological analysis showing that podoplanin expression in CAFs is associated with decreased CRC cell invasion.

Introduction

Previous reports have indicated that tumor progression is influenced and controlled by cellular interaction derived from a complex relationship between stromal, epithelial, and extracellular matrix components [1–5].

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0030–2414/09/0771–0053$26.00/0

Accessible online at:
www.karger.com/ocl

Yukihiro Nakanishi, MD, PhD
Department of Pathology, National Cancer Center Research Institute
5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045 (Japan)
Tel. +81 3 3542 2511, ext. 4206, Fax +81 3 3248 2463
E-Mail yukihironakanishi@hotmail.com

Studies of breast, prostate, colon cancer and melanoma have identified a 'reactive stroma' that is characterized by a modified extracellular matrix composition, increased microvessel density, and the presence of inflammatory cells and fibroblasts with an 'activated' phenotype [6–11]. These modified fibroblasts, often termed myofibroblasts or cancer-associated fibroblasts (CAFs), are considered to play a central role in the complex process of tumor-stroma interaction and consequent tumorigenesis [1–5]. Numerous studies have provided evidence for a cancer-promoting role of activated CAFs [1–5], which supposedly initiate and promote tumor progression through specific communications with cancer cells. On the other hand, some CAFs could have a protective role against colorectal cancer (CRC) cell invasion. Nevertheless, the signals that could explain the transition of a normal fibroblast into a CAF are not fully understood, and therefore it has been unclear how the stromal reaction in cancer tissue supports and regulates tumor progression.

Podoplanin is a 38-kDa mucin-type transmembrane glycoprotein with extensive O-glycosylation and a high content of sialic acid, and has been implicated in tumor progression [12–16]. Podoplanin homologs include OTS-8, RT140, gp38, canine gp40, human gp36, and murine PA2.26 [17]. Since podoplanin is expressed on lymphatic, but not on blood vessel endothelium, it is also widely used as a specific marker for lymphatic endothelial cells and lymphangiogenesis [18, 19]. It has been reported that podoplanin-deficient mice die at birth due to respiratory failure, exhibiting a phenotype of dilated, malfunctioning lymphatic vessels and lymphedema [20]. Experiments addressing this issue have revealed that podoplanin colocalizes with ezrin, ERM (ezrin-radixin-moesin)-protein, at the cellular membrane, and that podoplanin promotes relocalization of ezrin to filopodia-like structures and induces cell migration in the absence of epithelial-mesenchymal transition [21]. Additionally, there is evidence to suggest that podoplanin promotes platelet aggregation, and that it may also be involved in cancer cell migration, invasion, metastasis, and malignant progression [22, 23]. The expression of podoplanin is upregulated in a number of different tumor types, including squamous cell carcinoma [13, 24], malignant mesothelioma [25, 26], Kaposi's sarcoma and angiosarcoma [19], hemangioblastoma [27], testicular seminoma [28], and brain tumors [12, 29, 30]. However, the physiological function of podoplanin is still unknown. Also, the functional contribution of podoplanin to tumor progression has remained elusive. Only a few previous studies have investigated podoplanin in fibroblasts, where PA2.26 antigen, a podoplanin homolog, is involved in reactive processes during skin remodeling [31].

In the present study, we selected podoplanin as a good upregulated marker molecule in CAF from a DNA microarray experiment (data not shown). Podoplanin expression in advanced colorectal carcinoma was investigated immunohistochemically and its clinicopathological significance was examined. Furthermore, its function in fibroblasts was assessed by using a coculture invasion assay with CRC cell lines.

Patients and Methods

Patients and Samples for Immunohistochemistry

One hundred twenty formalin-fixed and paraffin-embedded blocks of CRC were drawn from the files of the National Cancer Center Hospital (NCCH), Tokyo, Japan. All cases were surgically resected between July 1, 1996, and January 1, 1998, and diagnosed as primary advanced CRC. The patients included 76 (63.3%) males and 44 (36.7%) females ranging in age from 31 to 86 years (median 60 years). The patients were restricted to consecutive cases diagnosed as stage II (n = 50, 41.7%) or stage III (n = 70, 58.3%) pathologically, in which all patients had undergone curative resection and none received pre- or postoperative adjuvant chemotherapy or radiation therapy. No patients were excluded from this study because of adjuvant therapy. Follow-up studies were complete in all patients, with a period ranging from 0.1 months to 6.6 years (median 5.2 years). Seven (14.0%) patients at stage II and 22 (31.4%) at stage III developed recurrences, and 3 (6.0%) patients at stage II and 16 (22.9%) at stage III died of CRC. Among the cases showing recurrences, liver metastasis was observed in 5 (10.0%) stage II cases and 12 (17.1%) stage III cases. Clinicopathological factors were all classified according to the TNM classification of the International Union against Cancer [32]. Histologic classification of tumors was made according to the World Health Organization International Histological Classification of Tumors [33]. Among the study cases, 41 (34.2%) were classified as well differentiated adenocarcinoma, 75 (62.5%) as moderately differentiated adenocarcinoma, 3 (2.5%) as poorly differentiated adenocarcinoma, and 1 (0.8%) as mucinous adenocarcinoma.

Immunohistochemistry

Four-micrometer-thick sections of tissue samples of the 120 CRCs were stained for the selected molecule, podoplanin, which was identified as being overexpressed in CAFs. Sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Staining was done at room temperature as follows: all sections were quenched with 3% hydrogen peroxide solution in alcohol for 20 min to block endogenous peroxidase activity. After several washes in phosphate-buffered saline (PBS; Sigma, St. Louis, Mo., USA), the sections were heated in an autoclave to 121°C for 10 min in 0.01 M citrate buffer (pH 6.0) for antigen retrieval. Blocking was performed with 2% normal swine serum (NSS; Dako, Glostrup, Denmark) in PBS for 30 min, and then the sections were incubated with monoclonal antibody directed against human podoplanin (anti-D2–40; Dako) for 1 h at 1:50 dilution. After washing

in PBS, the sections were incubated with biotinylated antibody against mouse immunoglobulin G (Vector Laboratories, Burlingame, Calif., USA) for 30 min at 1:200 dilution, followed by streptavidin-conjugated horseradish peroxidase (Dako). Diaminobenzidine was used as a chromogen. Sections were counterstained with hematoxylin and coverslipped using Promounter (Meisei Electric Co., Bangkok, Thailand). For the negative control, 2% NSS was used instead of the primary antibody. Lymphatic vessels in normal stroma were used as a positive control for podoplanin immunopositivity.

Evaluation of Podoplanin Expression in CRCs

Immunostained sections of the 120 CRCs were evaluated using a light microscope by two observers (T.Y. and Y.A-F.) who were blinded to the patient characteristics. Podoplanin was evaluated according to the following criteria: staining was considered positive when staining equal to or stronger than that of lymphatic vessels was observed; staining was considered negative when it was absent or weaker than that of lymphatic vessels. Positive staining was divided into two groups: group A showing positive staining of 30% or more of the cancer stroma, and group B showing staining of less than 30% of the cancer stroma. Cases that were negative for podoplanin expression were classified into group B.

Statistical Analysis for Immunohistochemistry

Statistical tests were performed with StatView version 5.0 (SAS, Cary, N.C., USA). The relationships between immunohistochemical findings and clinicopathological factors were analyzed using Fisher's exact test, the χ^2 test, Student's t test, or the Mann-Whitney U test. Deaths from causes other than CRC were treated as censored cases. Overall survival, recurrence-free survival, and liver metastasis-free survival were measured from the date of surgery to the end of follow-up, recurrence, liver metastasis and death, respectively. Survival curves were made using the Kaplan-Meier method and compared using the log rank test. Both univariate and multivariate survival analyses were performed using the Cox proportional hazards regression model.

Cell Culture

The human fibroblast cell line CCD-112CoN (CRL-1541) derived from normal colon tissue and the human CRC cell lines HT29, HCT116, and HCT15 were obtained from the American Type Culture Collection. CCD-112CoN cells were maintained in Eagle's Minimal Essential Medium (Gibco, Carlsbad, Calif., USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 500 units/ml penicillin-streptomycin-fungizone (Gibco), 2 mmol/l L-glutamine (Gibco), and 1 mmol/l sodium pyruvate (Gibco). All CRC cell lines were maintained in RPMI-1640 medium (Sigma) supplemented with 10% FBS and antibiotics. All cell lines were cultured under conditions of 5% CO_2 in air at 37°C. CCD112CoN fibroblasts were used between the 25th and 30th passages for the experiments.

Preparation of Cancer-Conditioned Medium

Conditioned medium derived from cancer cell lines was used for induction of podoplanin in CAFs. After HT29 had been plated and allowed to attach to 75-cm^2 tissue culture dishes (Corning, Corning, N.Y., USA) for 24 h at subconfluency, the cells were rinsed twice with PBS and then incubated for another 72 h. The conditioned medium (CM) derived from HT29 (CM-HT29) was harvested, centrifuged at 200 *g* for 10 min to remove cell debris, and passed through a 0.2-μm filter (Millipore, Billerica, Mass., USA). Until use, the CM was stored at –20°C, which did not alter its biological activity (data not shown).

Design of siRNA and Its Use for Transfection

A siRNA duplex (sense, 5′-CAACAGUGUAACAGGCAUUdTdT-3′) specific for human podoplanin (GenBank accession No. NM_006474) was designed at Takara Bio Inc. (Shiga, Japan). Nonspecific control siRNA duplex with the same GC content as podoplanin siRNA (sense, 5′-AUACAGUGACAGCAACGUUdTdT-3′) was also purchased. Fibroblast cell line CCD-112CoN, in which podoplanin is not constitutively expressed in low-serum medium, was plated on 75-cm^2 tissue culture dishes in a subconfluent state. To create fibroblast cells expressing podoplanin, CCD-112CoN was allowed to adhere for 3 h in Eagle's Minimal Essential Medium with 10% FBS, washed twice with PBS and incubated in CM-HT29 with 10% FBS for 48 h. The cells were then plated on 6-well tissue culture plates at a density of 1.25×10^5 cells per well in CM-HT29 with 10% FBS. After overnight incubation, the cells were transfected with podoplanin siRNA or control siRNA at a final concentration of 100 nmol/l using LipofectAMINE 2000 (Invitrogen, Carlsbad, Calif., USA). For optimal transfection, a reduced serum medium (Opti-MEM; Gibco) was used to dilute the siRNA duplexes and LipofectAMINE 2000 according to the manufacturer's recommendations. Cells were used for Western blot analysis and Matrigel invasion assays after 72 h.

Western Blot Analysis

Cells were lysed in Tris-buffered saline-based lysis buffer (Tris-buffered saline, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium lauryl sulfate, protease inhibitor), and protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, Calif., USA). Equal amounts of proteins (10 μg) from the whole-cell lysates were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked in TPBS (0.1% Tween 20 in PBS) solution with 5% nonfat dry milk for 1 h, then incubated with monoclonal antibody directed against human podoplanin (anti-D2–40; Dako) for 2 h at 1:100 dilution in blocking solution. Subsequently, the membranes were washed with TPBS, followed by incubation with horseradish-peroxidase-conjugated anti-mouse IgG antibody (GE Healthcare, Amersham, UK) for 1 h at 1:1,000 dilution. Immunoreactive proteins were detected using an enhanced chemiluminescence kit (GE Healthcare). β-Actin monoclonal antibody (Sigma) at 1:2,000 dilution was used as protein loading control.

Matrigel Invasion Assay

The transfected fibroblasts were cocultured with the invasive CRC cell lines (either HT116 or HT15) and analyzed using the BD BioCoat Matrigel Invasion Assay System (24-well BioCoat Matrigel invasion chambers; BD Bioscience, San Jose, Calif., USA) in accordance with the manufacturer's recommendations with minor modification. Briefly, after 24 h of transfection, the fibroblasts were plated on the upper chamber of the transwell insert (8-μm pores) in the presence or absence of CM-HT29 with 10% FBS at a density of 1×10^4 cells, and the lower wells contained the same medium. After overnight incubation, either HCT116 or

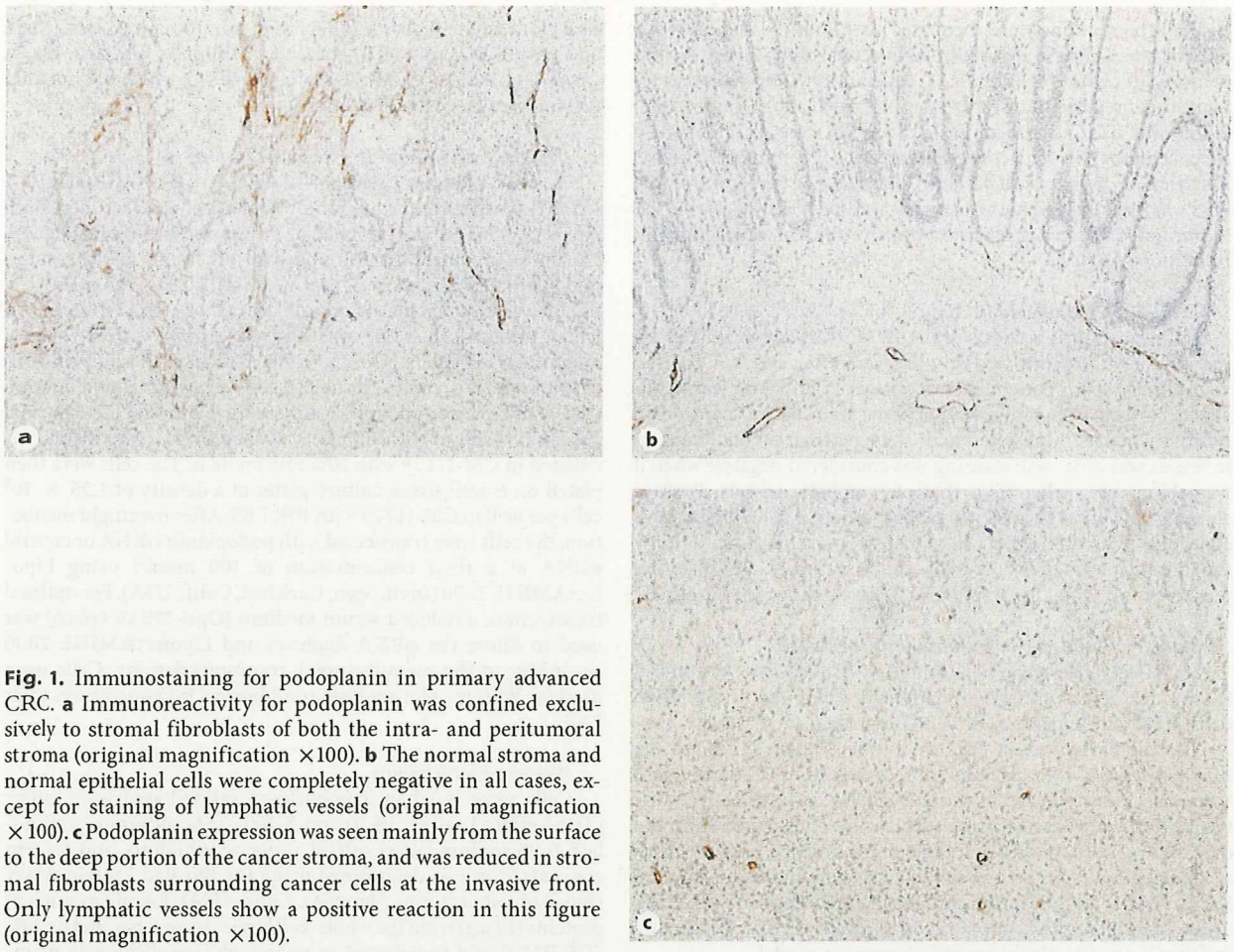

Fig. 1. Immunostaining for podoplanin in primary advanced CRC. **a** Immunoreactivity for podoplanin was confined exclusively to stromal fibroblasts of both the intra- and peritumoral stroma (original magnification ×100). **b** The normal stroma and normal epithelial cells were completely negative in all cases, except for staining of lymphatic vessels (original magnification ×100). **c** Podoplanin expression was seen mainly from the surface to the deep portion of the cancer stroma, and was reduced in stromal fibroblasts surrounding cancer cells at the invasive front. Only lymphatic vessels show a positive reaction in this figure (original magnification ×100).

HCT15 cells were seeded onto each of the upper chambers in CM-HT29 with 1% FBS at a density of 2×10^5 cells, where the fibroblasts were in a confluent state. Carcinoma cells were allowed to migrate through both the transfected fibroblasts and the Matrigel matrix membrane for 24 h at 37°C. After incubation, the nonmigrated cells in the upper chamber were gently removed with a cotton swab, and the carcinoma cells that had invaded through the Matrigel-coated inserts were stained with Diff-Quik (Sysmex, Kobe, Japan). The number of carcinoma cells on the lower side in 5 randomly chosen areas per membrane was counted under a light microscope at ×100 magnification. Means were based on the numbers obtained from the 5 randomly chosen areas for each treatment condition. All assays were performed in triplicate, and the differences in the counts of cells that had invaded among the carcinoma cells that had been cocultured with fibroblasts transfected with either podoplanin siRNA or control siRNA were analyzed using Student's t test. Statistical tests were two-sided at a 5% level of significance.

Results

Podoplanin Expression in CRC

Immunoreactivity for podoplanin was located in the cytoplasm and cell membrane of fibroblasts surrounding carcinoma cells. Immunoreactivity for podoplanin was confined exclusively to the stromal fibroblasts of both the intra- and the peritumoral stroma, and absent in stromal cells surrounding cancer cells budding from the tumor nests at the invasive front. Podoplanin expression was seen in stromal fibroblasts located mainly from the superficial to the deep area of the tumor, sparing the invasive front (fig. 1a–c), whereas normal stroma was completely negative in all cases except for lymphatic vessels (fig. 1). Podoplanin-positive staining was not observed in either normal epithelial cells or carcinoma cells. Fifty cases (41.7%) belonged to group A.

Table 1. Correlations between podoplanin expression and clinicopathological factors in patients with advanced CRC (stages II and III)

Variables	Podoplanin		p value
	group A (over 30)	group B (fewer than 30)	
Age, years	60.0 ± 11.7	61.3 ± 10.7	0.5105[1]
Gender			
Male	30 (25.0)	46 (38.3)	
Female	20 (16.7)	24 (20.0)	0.5219[2]
Tumor location			
Colon	23 (19.2)	48 (40.0)	
Rectum	27 (22.5)	22 (18.3)	0.0131[2]
Maximum diameter of the tumor			0.1565[3]
mm (median, range)	42, 15–107	49, 15–110	
Depth of invasion (pT)[4]			
T2	8 (6.7)	2 (1.7)	
T3	42 (35.0)	64 (53.3)	
T4	0	4 (3.3)	0.0106[2]
Lymph node metastasis (pN)			
Absence	25 (20.8)	25 (20.8)	
Presence	25 (20.8)	45 (37.5)	0.1176[2]
Histological grade[5]			
G1	19 (15.8)	22 (18.3)	
G2	30 (25.0)	45 (37.5)	
G3	1 (0.8)	3 (2.5)	0.634[2]
Lymphatic invasion			
Absence	12 (10.0)	13 (10.8)	
Presence	38 (31.7)	57 (47.5)	0.4704[2]
Venous invasion			
Absence	18 (15.0)	17 (14.2)	
Presence	32 (26.7)	53 (44.2)	0.164[2]

Figures in parentheses are percentages.
[1] Student's t test.
[2] χ^2 test or Fisher's exact test.
[3] Mann-Whitney U test.
[4] T2: tumor invades the muscularis propia; T3: tumor invades through the muscularis propia into the subserosa or peritoneal tissues; T4: tumor directly invades other organs or structures and/or perforates visceral peritoneum.
[5] G1: well-differentiated adenocarcinoma; G2: moderately differentiated adenocarcinoma; G3: poorly differentiated adenocarcinoma including signet ring cell adenocarcinoma and mucinous adenocarcinoma.

Prognostic Value of Podoplanin Expression in Patients with CRC

The correlations between podoplanin expression and clinicopathological factors are summarized in table 1. Group A was significantly correlated with a more distal tumor localization (p = 0.013) and a shallower depth of tumor invasion (p = 0.011) (table 1). There was no significant correlation between podoplanin expression, and age, gender, maximum tumor diameter, lymph node metastasis (pN), histological grade, lymphatic invasion, or venous invasion (table 1).

Patients in group A survived significantly longer than those who were negative, in terms of both disease-specific survival (DSS) and disease-free survival (DFS) (p = 0.0017 and p < 0.0001, respectively; fig. 2a, b). Patients in group A showed a significantly lower incidence of liver metastasis than patients in group B (p = 0.001; fig. 2c). For both DSS and DFS, univariate analysis using the Cox proportional hazards model revealed that podoplanin expression, depth of invasion (pT), and pN were significantly associated with prognosis (table 2), and that both podoplanin expression and pT were significantly associated with liver metastasis (table 2). Venous invasion factor tended to correlate with recurrence-free survival and liver metastasis-free survival (p = 0.086 and p = 0.051, respectively; table 2), but this tendency did not reach statistical significance. Multivariate analysis using the Cox proportional hazards model revealed that negative podoplanin expression and presence of pN were significantly associated with reduced DSS when adjusted for pT and venous invasion (p = 0.016 and p = 0.027, respectively; table 3). Multivariate analysis for both DFS and liver metastasis-free survival revealed that only podoplanin expression was associated with prognosis when adjusted for pT, pN, and venous invasion (p = 0.0023 and p = 0.020, respectively; table 3).

Results of Invasion Assay

To explore the biological role of podoplanin in fibroblasts, we used the RNA interference (RNAi) strategy to downregulate its expression. Increased podoplanin expression was observed in CAFs with CM-HT29, whereas it was not constitutively expressed in CCD112CoN cultured with low-serum medium. The podoplanin protein level in CAFs was substantially reduced within 72 h after transfection with 100 nmol/l podoplanin siRNA (fig. 3a). The result indicated that podoplanin siRNA effectively and specifically downregulated podoplanin protein expression in CAFs.

A tumor invasion assay was then performed using the Matrigel model to investigate whether reduced expression of podoplanin in CAFs increased the invasiveness of CRC cell lines in coculture. When CRC cell lines such as HCT116 and HCT15 were cocultured with CAFs transfected with control siRNA for podoplanin, a few invading cells were observed. However, when CRC cell lines were cocultured with CAFs transfected with siRNA1 for podo-

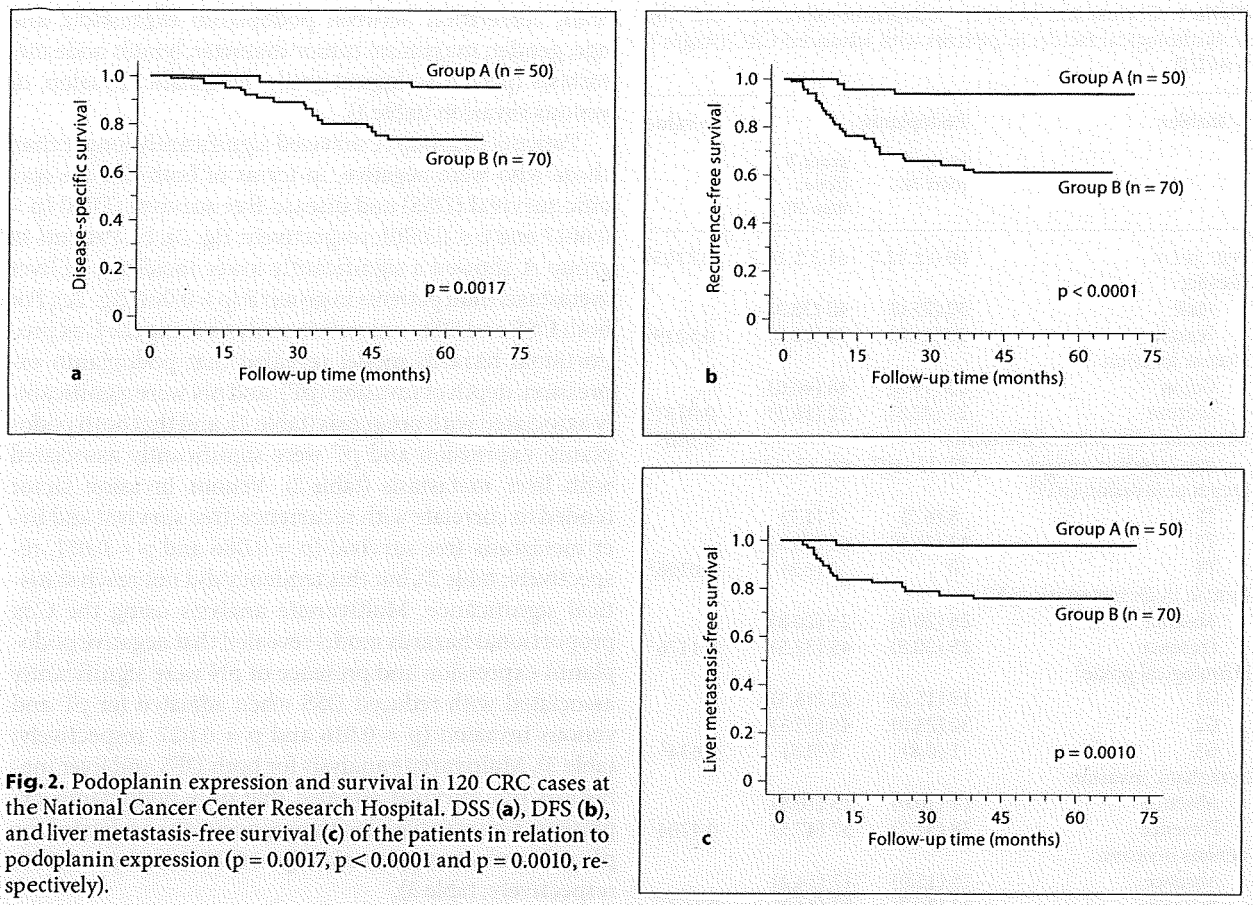

Fig. 2. Podoplanin expression and survival in 120 CRC cases at the National Cancer Center Research Hospital. DSS (**a**), DFS (**b**), and liver metastasis-free survival (**c**) of the patients in relation to podoplanin expression ($p = 0.0017$, $p < 0.0001$ and $p = 0.0010$, respectively).

Table 2. Univariate Cox proportional hazards analysis in patients with advanced CRC (stages II and III)

Prognostic factors	Disease-specific survival			Recurrence-free survival			Liver metastasis-free survival		
	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value
Expression of podoplanin (group A/group B)	0.135	0.031–0.586	0.0075	0.128	0.039–0.425	0.0008	0.075	0.010–0.563	0.0118
Age (≥60 years/<60 years)[1]	1.329	0.535–3.306	0.5401	0.711	0.342–1.479	0.3613	0.793	0.306–2.056	0.6328
Gender (male/female)	1.371	0.521–3.607	0.5231	0.847	0.404–1.773	0.6585	0.838	0.319–2.203	0.7205
Tumor location (colon/rectum)	1.210	0.476–3.074	0.6888	0.966	0.461–2.022	0.9259	1.652	0.582–4.690	0.3459
Maximum diameter of the tumor (≥45/<45 mm)[1]	0.703	0.283–1.747	0.4475	0.565	0.267–1.196	0.1353	0.836	0.322–2.166	0.7117
Depth of invasion (T2, T3/T4)	0.207	0.048–0.898	0.0354	0.180	0.054–0.599	0.0052	0.175	0.040–0.771	0.0212
Lymph node metastasis (absence/presence)	0.217	0.063–0.744	0.0151	0.390	0.166–0.913	0.0300	0.527	0.185–1.496	0.2285
Histological grade (G1/G2, G3)	0.634	0.228–1.761	0.3819	0.562	0.240–1.315	0.1836	1.278	0.486–3.357	0.6192
Lymphatic invasion (presence/absence)	1.087	0.361–3.277	0.8819	1.878	0.653–5.397	0.2421	2.189	0.500–9.574	0.2981
Venous invasion (presence/absence)	1.681	0.558–5.066	0.3561	2.325	0.887–6.097	0.0862	7.511	0.996–56.666	0.0505

HR = Hazard ratio; CI = confidence interval. [1] Two groups are divided by the median.

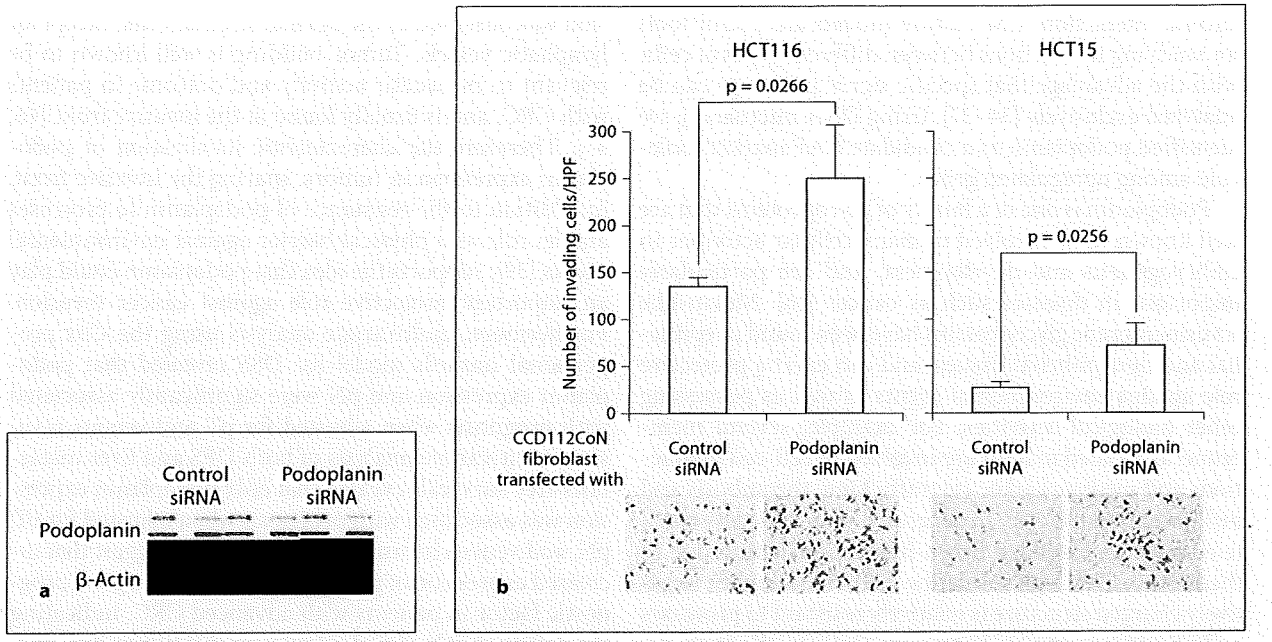

Fig. 3. Podoplanin expression in CCD112CoN fibroblast cells transfected with siRNAs and invasiveness of the cocultured CRC cell lines HCT116 and HCT15. **a** Western blotting using anti-D2–40 antibody (1:100; Dako) in the CCD112CoN fibroblast cells transfected with siRNAs. Podoplanin siRNA reduced podoplanin expression at the protein level almost completely. β-Actin was used as a loading control. **b** Invasiveness of HCT116 and HCT15 cells cocultured with fibroblasts transfected with podoplanin siRNA and control siRNA and in the Matrigel invasion system. After 24 h of coculture, CRC cell lines cocultured with fibroblasts transfected with podoplanin siRNA exhibited a 1.8- to 2.6-fold increase in the number of cells invading the Matrigel-coated insert. HPF = High-power field.

Table 3. Multivariate Cox proportional hazards analysis in patients with advanced CRC (stages II and III)

Prognostic factors	Disease-specific survival			Recurrence-free survival			Liver metastasis-free survival		
	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value
Expression of podoplanin (group A/group B)	0.161	0.037–0.708	0.0157	0.153	0.046–0.510	0.0023	0.089	0.012–0.682	0.0198
Depth of invasion (T2, T3/T4)	0.339	0.075–1.523	0.1582	0.308	0.089–1.066	0.0630	0.396	0.089–1.775	0.2264
Lymph node metastasis (absence/presence)	0.237	0.066–0.849	0.0270	0.488	0.201–1.184	0.1125	0.864	0.297–2.515	0.7888
Venous invasion (presence/absence)	0.858	0.269–2.739	0.7956	1.517	0.551–4.175	0.4194	5.745	0.727–45.380	0.0973

HR = Hazard ratio; CI = confidence interval.

planin, the number of invading cells was significantly increased ($p = 0.027$ and $p = 0.026$, Student's t test for coculture with HCT116 and HCT15, respectively; fig. 3b). As a negative control, when fibroblasts transfected with control siRNA and podoplanin siRNA were cultured only in CM-HT29 without CRC cell lines, almost no invading cells were evident (data not shown).

Discussion

To help understand the difference between CAFs and uninvolved fibroblasts, and to further evaluate the role of CAFs, we compared their genome-wide expression profiles using in vitro CM culture models in which soluble factors originating from cancer cells exerted a paracrine action on the surrounding fibroblasts involved in tumor-

stroma interaction. CM culture models are useful tools for studying interactions between different types of cells, with the advantage that specific signal pathways can be analyzed exclusively [34–37]. Using DNA microarray, we identified podoplanin as a candidate CAF marker molecule among upregulated genes.

Podoplanin is one of a family of glycoproteins that are well known to be involved in many cellular activities in embryogenesis and development, and are particularly important in diseases such as cancer [16]. Mucin-like transmembrane glycoproteins have been found in epithelial and nonepithelial tissues, and can exert a protective role against environmental agents as well as possessing other biological activities. For example, several membrane-associated mucins are involved in cell-cell interactions and mediate leukocyte trafficking, thrombosis and inflammation [38–40]. In general, mucin-type glycoproteins have an extended brush-like conformation due to their extensive O-glycosylation [39]. This highly negatively charged structure is relatively resistant to proteases and provides a physical barrier protecting cells from environmental agents.

In the present study, immunohistochemical localization of podoplanin was confined exclusively to CAFs in the cancer stroma. Normal stroma, epithelial cells and tumor cells were completely negative for podoplanin in all cases tested, and only lymphatic vessels were positive. Podoplanin expression in CAFs of cancer stroma was significantly correlated with more distal tumor localization and a shallower depth of tumor invasion. Invasion of CRC cell lines was augmented upon coculture with fibroblasts in which podoplanin expression was reduced by siRNA. These results indicate that podoplanin could play an important protective role against cancer invasion.

Expression of podoplanin by cancer cells of oral and uterine cervix squamous cell carcinoma has been reported to be associated with prognosis [41, 42]. However, previous studies have found that adenocarcinoma cells rarely express podoplanin [43, 44]. Podoplanin-positive CAFs are reportedly present in invasive adenocarcinoma of the lung, but not in noninvasive adenocarcinoma [45]. Podoplanin expression by CAFs is reported to be significantly associated with a poor outcome in patients with lung adenocarcinoma. However, multivariate analysis failed to show that podoplanin expression was an independent prognostic factor [45]. In the present study, the localization of podoplanin expression was intriguing because it was seen in CAFs located mainly in the superficial to deep area of the tumor, sparing the invasive front where tumor budding is often observed. No podoplanin expression was observed in the normal stromal cells, except for lymphatic vessels. Tumor budding is well known to be relevant to metastatic acitivity and outcome in patients with CRC, and is usually found at the invasive front [46, 47]. Therefore the characteristic localization of podoplanin expression in tumors, sparing the invasive front, in addition to the resistance of podoplanin to proteases and its role as a physical barrier against environmental agents [39], supports the idea that podoplanin could play an important protective role against cancer invasion. Furthermore, multivariate analysis using the Cox proportional hazards model for DSS revealed that podoplanin expression and pN were significantly associated with prognosis when adjusted for pT and venous invasion. Multivariate analysis of both DFS and liver metastasis-free survival revealed that only podoplanin expression was associated with prognosis when adjusted for pT, pN, and venous invasion. These findings suggest that increased expression of podoplanin in CAFs is a good prognostic factor in patients with advanced CRC, indicating the defensive role of podoplanin against tumor invasion. In terms of clinical use, podoplanin expression in CRC might be helpful for selecting patients who should undergo adjuvant chemotherapy, or those for whom it is unnecessary. However, in order for podoplanin expression to be applied for practical clinical care, it must be validated in a large-scale prospective clinical trial.

Furthermore, our coculture invasion assay indicated that podoplanin expressed in CAFs could have a suppressive effect on the invasion of tumor cells, although it is not yet clear whether CAFs have both an inductive and a suppressive effect on tumor progression and regulate tumorigenesis. Other constituents of the desmoplastic extracellular matrix have also been shown to inhibit tumor progression. For example, injection of L-3, 4-dehydroproline, which inhibits the formation of collagen fibrils, increases tumor cell invasion in mice with B16F10 melanoma subcutaneous tumors [48]. In addition, extracellular matrix accumulation in tumors contributes to increased interstitial fluid pressure and hinders the diffusion of macromolecules and oxygen, leading to tumor cell necrosis [49, 50]. The overall effect of altered extracellular matrix in tumors and the effect of CAFs during tumor progression are still poorly understood. Further studies directed at disrupting the complex interaction between tumor cells and stromal composition may define new strategies for diagnosis of tumors and suitable therapeutic interventions.

In conclusion, podoplanin, a mucin-type transmembrane glycoprotein, was found to be upregulated in CAFs

in vitro and to be overexpressed in CAFs surrounding CRC cells in vivo. Multivariate analysis of both DFS and liver metastasis-free survival revealed that only podoplanin expression was associated with prognosis when adjusted for pT, pN, and venous invasion. In addition, invasiveness of CRC cells was increased significantly by coculture with podoplanin-suppressed CAFs. These findings suggest that increased podoplanin expression in stromal fibroblasts is a significant indicator of good prognosis in patients with advanced CRC, reflecting its defensive role against cancer invasion.

Acknowledgement

This study was supported by a grant-in-aid for the Third Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan. T. Y. is a recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research in Japan.

References

1 Bhowmick NA, Neilson EG, Moses HL: Stromal fibroblasts in cancer initiation and progression. Nature 2004;432:332–337.
2 De Wever O, Mareel M: Role of tissue stroma in cancer cell invasion. J Pathol 2003;200:429–447.
3 Kalluri R, Zeisberg M: Fibroblasts in cancer. Nat Rev Cancer 2006;6:392–401.
4 Micke P, Ostman A: Exploring the tumour environment: cancer-associated fibroblasts as targets in cancer therapy. Expert Opin Ther Targets 2005;9:1217–1233.
5 Bhowmick NA, Chytil A, Plieth D, et al: TGF-β signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science 2004;303:848–851.
6 Orimo A, Gupta PB, Sgroi DC, et al: Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 2005;121:335–348.
7 Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR: Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res 1999;59:5002–5011.
8 Dimanche-Boitrel MT, Vakaet L Jr, Pujuguet P, et al: In vivo and in vitro invasiveness of a rat colon-cancer cell line maintaining E-cadherin expression: an enhancing role of tumor-associated myofibroblasts. Int J Cancer 1994;56:512–521.
9 Sternlicht MD, Lochter A, Sympson CJ, et al: The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. Cell 1999;98:137–146.
10 Lochter A, Galosy S, Muschler J, Freedman N, Werb Z, Bissell MJ: Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. J Cell Biol 1997;139:1861–1872.
11 Cornil I, Theodorescu D, Man S, Herlyn M, Jambrosic J, Kerbel RS: Fibroblast cell interactions with human melanoma cells affect tumor cell growth as a function of tumor progression. Proc Natl Acad Sci USA 1991;88:6028–6032.
12 Mishima K, Kato Y, Kaneko MK, Nishikawa R, Hirose T, Matsutani M: Increased expression of podoplanin in malignant astrocytic tumors as a novel molecular marker of malignant progression. Acta Neuropathol (Berl) 2006;111:483–488.
13 Yuan P, Temam S, El-Naggar A, et al: Overexpression of podoplanin in oral cancer and its association with poor clinical outcome. Cancer 2006;107:563–569.
14 Schacht V, Dadras SS, Johnson LA, Jackson DG, Hong YK, Detmar M: Up-regulation of the lymphatic marker podoplanin, a mucin-type transmembrane glycoprotein, in human squamous cell carcinomas and germ cell tumors. Am J Pathol 2005;166:913–921.
15 Zimmer G, Oeffner F, Von Messling V, et al: Cloning and characterization of gp36, a human mucin-type glycoprotein preferentially expressed in vascular endothelium. Biochem J 1999;341:277–284.
16 Brockhausen I, Schutzbach J, Kuhns W: Glycoproteins and their relationship to human disease. Acta Anat (Basel) 1998;161:36–78.
17 Martin-Villar E, Scholl FG, Gamallo C, et al: Characterization of human PA2.26 antigen (T1α-2, podoplanin), a small membrane mucin induced in oral squamous cell carcinomas. Int J Cancer 2005;113:899–910.
18 Ordonez NG: Podoplanin: a novel diagnostic immunohistochemical marker. Adv Anat Pathol 2006;13:83–88.
19 Breiteneder-Geleff S, Soleiman A, Kowalski H, et al: Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. Am J Pathol 1999;154:385–394.
20 Schacht V, Ramirez MI, Hong YK, et al: T1α/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. Embo J 2003;22:3546–3556.
21 Wicki A, Lehembre F, Wick N, Hantusch B, Kerjaschki D, Christofori G: Tumor invasion in the absence of epithelial-mesenchymal transition: podoplanin-mediated remodeling of the actin cytoskeleton. Cancer Cell 2006;9:261–272.
22 Sugimoto Y, Watanabe M, Oh-hara T, Sato S, Isoe T, Tsuruo T: Suppression of experimental lung colonization of a metastatic variant of murine colon adenocarcinoma 26 by a monoclonal antibody 8F11 inhibiting tumor cell-induced platelet aggregation. Cancer Res 1991;51:921–925.
23 Watanabe M, Okochi E, Sugimoto Y, Tsuruo T: Identification of a platelet-aggregating factor of murine colon adenocarcinoma as determined by monoclonal antibodies. Cancer Res 1988;48:6411–6416.
24 Kato Y, Kaneko M, Sata M, Fujita N, Tsuruo T, Osawa M: Enhanced expression of Aggrus (T1α/podoplanin), a platelet-aggregation-inducing factor in lung squamous cell carcinoma. Tumour Biol 2005;26:195–200.
25 Kimura N, Kimura I: Podoplanin as a marker for mesothelioma. Pathol Int 2005;55:83–86.
26 Ordonez NG: D2–40 and podoplanin are highly specific and sensitive immunohistochemical markers of epithelioid malignant mesothelioma. Hum Pathol 2005;36:372–380.
27 Roy S, Chu A, Trojanowski JQ, Zhang PJ: D2–40, a novel monoclonal antibody against the M2A antigen as a marker to distinguish hemangioblastomas from renal cell carcinomas. Acta Neuropathol (Berl) 2005;109:497–502.

28 Kato Y, Sasagawa I, Kaneko M, Osawa M, Fujita N, Tsuruo T: Aggrus: a diagnostic marker that distinguishes seminoma from embryonal carcinoma in testicular germ cell tumors. Oncogene 2004;23:8552–8556.
29 Shibahara J, Kashima T, Kikuchi Y, Kunita A, Fukayama M: Podoplanin is expressed in subsets of tumors of the central nervous system. Virchows Arch 2006;448:493–499.
30 Mishima K, Kato Y, Kaneko MK, et al: Podoplanin expression in primary central nervous system germ cell tumors: a useful histological marker for the diagnosis of germinoma. Acta Neuropathol (Berl) 2006;111:563–568.
31 Gandarillas A, Scholl FG, Benito N, Gamallo C, Quintanilla M: Induction of PA2.26, a cell-surface antigen expressed by active fibroblasts, in mouse epidermal keratinocytes during carcinogenesis. Mol Carcinog 1997;20:10–18.
32 Sobin LH, Fleming ID: TNM Classification of Malignant Tumors, ed 5 (1997). Union Internationale contre le Cancer and the American Joint Committee on Cancer. Cancer 1997;80:1803–1804.
33 World Health Organization: Classification of Tumours. Lyon, IARC Press, 2000.
34 DeCosse JJ, Gossens C, Kuzma JF, Unsworth BR: Embryonic inductive tissues that cause histologic differentiation of murine mammary carcinoma in vitro. J Natl Cancer Inst 1975;54:913–922.
35 DeCosse JJ, Gossens CL, Kuzma JF, Unsworth BR: Breast cancer: induction of differentiation by embryonic tissue. Science 1973;181:1057–1058.
36 Cooper M, Pinkus H: Intrauterine transplantation of rat basal cell carcinoma as a model for reconversion of malignant to benign growth. Cancer Res 1977;37:2544–2552.
37 Maehara N, Matsumoto K, Kuba K, Mizumoto K, Tanaka M, Nakamura T: NK4, a four-kringle antagonist of HGF, inhibits spreading and invasion of human pancreatic cancer cells. Br J Cancer 2001;84:864–873.
38 Gendler SJ, Spicer AP: Epithelial mucin genes. Annu Rev Physiol 1995;57:607–634.
39 Hilkens J, Ligtenberg MJ, Vos HL, Litvinov SV: Cell membrane-associated mucins and their adhesion-modulating property. Trends Biochem Sci 1992;17:359–363.
40 Varki A: Selectin ligands. Proc Natl Acad Sci USA 1994;91:7390–7397.
41 Yuan P, Temam S, El-Naggar A, Zhou X, Liu DD, Lee JJ, Mao L: Overexpression of podoplanin in oral cancer and its association with poor clinical outcome. Cancer 2006;107:563–569.
42 Dumoff KL, Chu CS, Harris EE, Holtz D, Xu X, Zhang PJ, ACS G: Low podoplanin expression in pretreatment biopsy material predicts poor prognosis in advanced-stage squamous cell carcinoma of the uterine cervix treated by primary radiation. Mod Pathol 2006;19:708–716.
43 Ordonez NG: D2–40 and podoplanin are highly specific and sensitive immunohistochemical markers of epithelioid malignant mesothelioma. Hum Pathol 2005;36:372–380.
44 Chu AY, Litzky LA, Pasha TL, Acs G, Zhang PJ: Utility of D2–40, a novel mesothelial marker, in the diagnosis of malignant mesothelioma. Mod Pathol 2005;18:105–110.
45 Kawase A, Ishii G, Nagai K, Ito T, Nagano T, Murata Y, Hishida T, Nishimura M, Yoshida J, Suzuki K, Ochiai A: Podoplanin expression by cancer associated fibroblasts predicts poor prognosis of lung adenocarcinoma. Int J Cancer 2008;123:1053–1059.
46 Okuyama T, Oya M, Ishikawa H: Budding as a risk factor for lymph node metastasis in pT1 or pT2 well-differentiated colorectal adenocarcinoma. Dis Colon Rectum 2002;45:628–634.
47 Ueno H, Price AB, Wilkinson KH, Jass JR, Mochizuki H, Talbot IC: A new prognostic staging system for rectal cancer. Ann Surg 2004;240:832–839.
48 Barsky SH, Gopalakrishna R: Increased invasion and spontaneous metastasis of BL6 melanoma with inhibition of the desmoplastic response in C57 BL/6 mice. Cancer Res 1987;47:1663–1667.
49 Netti PA, Berk DA, Swartz MA, Grodzinsky AJ, Jain RK: Role of extracellular matrix assembly in interstitial transport in solid tumors. Cancer Res 2000;60:2497–2503.
50 Brown EB, Boucher Y, Nasser S, Jain RK: Measurement of macromolecular diffusion coefficients in human tumors. Microvasc Res 2004;67:231–236.

Intraductal carcinoma component as a favorable prognostic factor in biliary tract carcinoma

Hidenori Ojima,[1] Yae Kanai,[1,5] Motoki Iwasaki,[2] Nobuyoshi Hiraoka,[1] Kazuaki Shimada,[3] Tsuyoshi Sano,[3] Yoshihiro Sakamoto,[3] Minoru Esaki,[3] Tomoo Kosuge,[3] Michiie Sakamoto[4] and Setsuo Hirohashi[1]

[1]Pathology Division, National Cancer Center Research Institute, [2]Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, [3]Hepatobiliary and Pancreatic Surgery Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045; [4]Department of Pathology, Keio University School of Medicine, 35 Shinanomachi, Shinjyuku-ku, Tokyo 160-0016, Japan

(Received July 5, 2008/Revised September 9, 2008/Accepted September 16, 2008/Online publication November 25, 2008)

The aim of this study is to evaluate the prognostic impact of an intraductal carcinoma component and bile duct resection margin status in patients with biliary tract carcinoma. An intraductal carcinoma component was defined as carcinoma within the bile duct outside the main tumor nodule consisting of a subepithelial invasive component. Surgically resected materials from 214 patients were evaluated by histological observations. Seventy-nine patients (36.9%) with an intraductal carcinoma component infrequently developed large tumors and infrequently showed deep invasion and venous, lymphatic and perineural involvement in the main tumor nodule. An intraductal carcinoma component was inversely correlated with advanced clinical stage, and was shown to be a significantly favorable prognostic factor by both univariate and multivariate analyses. Proximal (hepatic) side bile duct resection margin status was categorized into negative for tumor cells, positive with only an intraductal carcinoma component [R1 (is)], and positive with a subepithelial invasive component (R1). Forty-five patients (21.0%) with an R1 resection margin had a poorer prognosis than 148 patients (69.2%) with a negative resection margin, whereas 21 patients (9.8%) with an R1 (is) resection margin did not. In patients with an R1 resection margin, the risk of anastomotic recurrence was higher, and the period until anastomotic recurrence was shorter, than in patients with an R1 (is) resection margin. Surgeons should not be persistent in trying to achieve a negative surgical margin when the intraoperative frozen section diagnosis is R1 (is), and can choose a safe surgical procedure to avoid postoperative complications. (*Cancer Sci* 2009; 100: 62–70)

Biliary tract carcinoma still has a poor prognosis, and most cases are at an advanced stage when patients present with symptoms. Previous studies of extrahepatic bile duct carcinoma and hilar cholangiocarcinoma have indicated that surgical resection is the only curative treatment for affected patients.[1–10] Biliary tract carcinoma is remarkable because of its tendency for superficial extension by wide intraductal carcinoma.[11–14] However, it is difficult to accurately estimate the extent of the intraductal carcinoma component in the biliary tract on the basis of preoperative imaging studies.[13,15–18] It is feasible that intraoperative histological diagnosis using frozen sections may detect tumor involvement at the bile duct resection margin. Surgeons are required to make an immediate decision about the resection area based on intraoperative frozen section diagnosis. However, to our knowledge, no previous study has examined the clinicopathological significance and prognostic impact of an intraductal carcinoma component with reference to bile duct resection margin status in patients with biliary tract carcinoma.

In this retrospective study, the presence or absence of an intraductal carcinoma component and bile duct resection margin status were evaluated by histological observations of all surgically resected materials from 214 patients with biliary tract carcinoma who underwent radical surgery with curative intent. In order to provide a yardstick for surgeons who depend on the results of frozen section diagnosis during surgery, we examined the correlation between an intraductal carcinoma component and bile duct resection margin status on the one hand, and clinicopathological parameters on the other, and also the prognostic impact of an intraductal carcinoma component and bile duct resection margin status.

Materials and Methods

Patients and specimens. The study included 214 patients with biliary tract carcinoma who underwent radical surgery with curative intent at the National Cancer Center Hospital, Tokyo, Japan, between May 1965 and December 2003. Patients who died in hospital or within 100 days after surgery, and patients who underwent biopsy or palliative surgery, were not included. The included patients comprised 150 men and 64 women, ranging in age from 33 to 83 (mean 63.4) years.

The main tumor nodule was located in the lower, middle and upper thirds of the extrahepatic bile duct, the entire extrahepatic bile duct, the hilar bile duct, and intrahepatic bile duct adjacent to the hilar area in 27, 38, 14, 5, 77, and 53 patients, respectively. Patients with carcinoma of the peripheral intrahepatic bile duct were excluded. Pancreatoduodectomy (PD), extrahepatic bile duct resection (EHBD), hepatic resection with extrahepatic bile duct resection (HR+EHBD), hepatic resection (HR) and combined hepatectomy and pancreatoduodectomy (HPD) were performed in 47, 19, 124, 16 and 8 patients, respectively. The formalin-fixed surgically resected specimens were cut into slices at intervals of 0.5–0.7 cm, and all the sections were embedded in paraffin and routinely processed for microscopical examination. All tumors were classified according to the pathological tumor-node-metastasis (TNM) classification.[19] Intrahepatic bile duct carcinomas adjacent to the hilar area, for which TNM criteria have never been established, were classified according to the TNM classification for extrahepatic bile duct carcinoma. This study was approved by the Ethical Committee of the National Cancer Center, Tokyo.

Evaluation of an intraductal carcinoma component and bile duct resection margin status. The intraductal carcinoma component was defined as carcinoma within the bile duct and its small branch outside the main tumor nodule consisting of a subepithelial invasive component (Fig. 1). For cases in which intraoperative frozen section diagnosis of the ductal stump had been performed, the proximal (hepatic) side bile duct resection margin status was histologically assessed by review of the frozen section and its re-fixed permanent section with reference

[5]To whom correspondence should be addressed. E-mail: ykanai@ncc.go.jp

doi: 10.1111/j.1349-7006.2008.01009.x

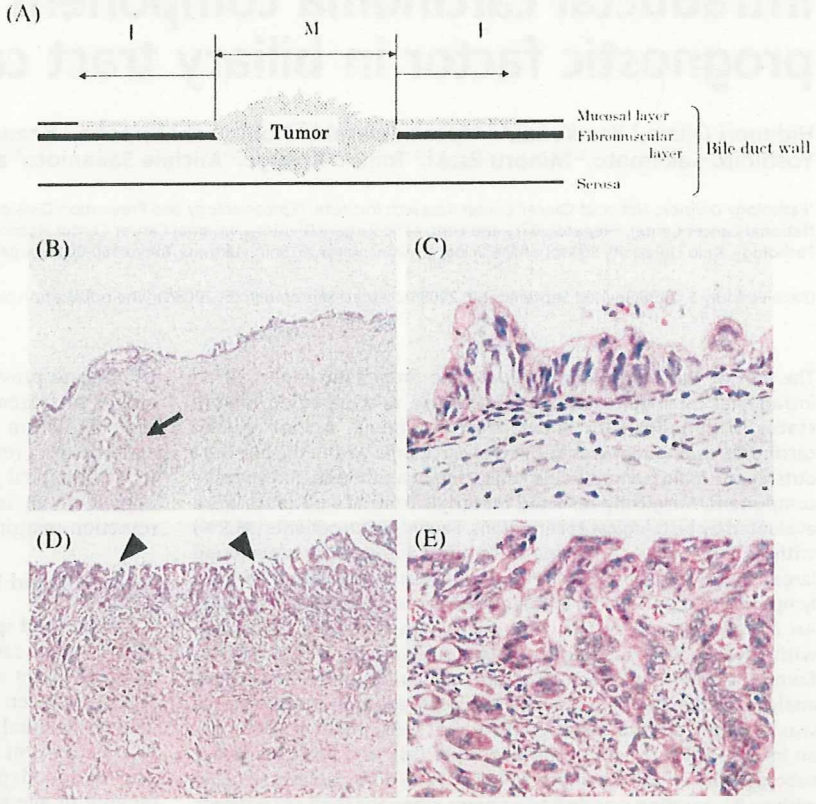

Fig. 1. Definition of an intraductal carcinoma component (I). (A) I is defined as intraductal carcinoma in the bile duct and its small branch outside the main tumor nodule (M) consisting of a submucosal invasive component. (B and C) Microscopic view of an example of I in the bile duct and its small branch (arrow). Hematoxylin and eosin (H&E) stain, original magnification ×40 (B) and ×400 (C). (D and E) Microscopic view of an example of M. Carcinoma *in situ* inside M (arrow heads) is not considered as I in this study. H&E stain, original magnification ×40 (D) and ×200 (E).

to the extent of the tumor in formalin-fixed surgically resected specimens. For cases in which intraoperative frozen section diagnosis of the ductal stump had not been performed, proximal side bile ductal resection margin status was histologically assessed by review of the formalin-fixed surgically resected specimens.

Follow-up and assessment of anastomotic recurrence at the bile duct resection margin. All 214 patients were followed for more than 100 days, and the mean duration of follow-up was 1215 days. Follow-up examination was performed using computed tomography, abdominal ultrasonography, and measurement of the serum carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) levels every 3–6 months by surgeons. Anastomotic recurrence at the proximal side of the bile duct resection margin was diagnosed only in patients with a positive resection margin. In such patients, when a mass lesion was detected after dilatation of the bile duct in the residual liver because of obstruction of the anastomosis site, using radiological evaluation including computed tomography and ultrasonography, surgeons considered that the patient had anastomotic recurrence (not local recurrence in which perineural invasion around the hepatic artery and/or involved regional nodes first formed a mass lesion). Causes of death were determined from the medical records.

Statistical analyses. Correlations between presence or absence of an intraductal carcinoma component and bile duct resection margin status on the one hand and clinicopathological parameters on the other were analyzed using chi-squared test.

Person-days of follow-up were calculated from the date of surgical resection until date of death or end of the study period (March 8, 2005), whichever occurred first. The crude rate of all-cause deaths was calculated by dividing the number of deaths by the number of person-days. Similarly, person-days of follow-up were calculated from the date of surgical resection until date of death, date of diagnosis of anastomotic recurrence, or end of the study period (March 8, 2005), whichever occurred first. The crude rate of recurrence at the proximal side bile duct resection margin was calculated by dividing the number of cases with recurrence by the number of person-days. Survival curves were constructed using the Kaplan–Meier method, and differences in survival were evaluated using the log-rank test. The Cox proportional hazards model was used to estimate hazard ratio (HR) and 95% confidence interval (CI) of death or anastomotic recurrence by clinicopathologic factors using the SAS program (PROC PHREG) (SAS Institute Inc., Cary NC, US). All tests were two-sided and differences at $P < 0.05$ were considered statistically significant.

Results

Univariate analysis of correlation between an intraductal carcinoma component and clinicopathological parameters. An intraductal carcinoma component was positive in 79 (36.9%) of the 214 examined patients. Correlations between an intraductal carcinoma component and clinicopathological parameters were examined using univariate analysis (Table 1). Location of the main tumor nodule ($P = 0.007$), and histologic type ($P < 0.0001$) were significantly correlated with an intraductal carcinoma component (Table 1). Tumor size ($P = 0.01$), depth of invasion ($P < 0.0001$), venous involvement ($P < 0.0001$), lymphatic involvement ($P = 0.0006$), perineural involvement ($P < 0.0001$), the pathological assessment of the primary tumor (pT) ($P < 0.0001$), and pathological TNM stage ($P < 0.0001$) were each inversely correlated with presence of an intraductal carcinoma component: patients with an intraductal carcinoma component infrequently developed large tumors, infrequently showed deep invasion into the bile duct wall and venous, lymphatic and perineural involvement in the main tumor nodule, and were infrequently at an advanced stage when diagnosed (Table 1).

Table 1. Correlation between an intraductal carcinoma component and clinicopathological parameters in patients with biliary tract carcinoma

	No. of cases		*P* for difference*
	Intraductal carcinoma component		
	Negative (*n* = 135)	Positive (*n* = 79)	
Age (years)			0.01
<65	73	29	
≥65	62	50	
Sex			0.85
Male	94	56	
Female	41	23	
Location of the main tumor nodule			0.007
Lower third of extrahepatic bile duct	15	12	
Middle third of extrahepatic bile duct	17	21	
Upper third of extrahepatic bile duct	6	8	
Entire extrahepatic bile duct	2	3	
Hilar bile duct	54	23	
Intrahepatic bile duct	41	12	
Histologic type			<0.0001
Adenocarcinoma	129	55	
Papillary adenocarcinoma	1	21	
Others	5	3	
Tumor size (cm)			0.13
<3	54	40	
≥3	81	39	
Differentiation of adenocarcinoma			0.50
Well	34	18	
Moderate	80	29	
Poor	15	8	
Depth of invasion			<0.0001
Carcinoma *in situ* or invasion to fibromuscular layer	1	16	
Invasion into subserosa or beyond bile duct wall	134	63	
Venous involvement			<0.0001
Absent	6	19	
Present	129	60	
Lymphatic involvement			0.0006
Absent	9	18	
Present	126	61	
Perineural involvement			<0.0001
Absent	10	24	
Present	125	55	
pT classification			<0.0001
pT1-2	11	40	
pT3-4	124	39	
pN classification			0.06
pN0	64	48	
pN1	71	31	
TNM stage			<0.0001
0, IA, IB	8	32	
IIA	53	14	
IIB	62	28	
III	12	5	

*Chi-squared test.

Univariate analysis of correlation between an intraductal carcinoma component or clinicopathological parameters on the one hand and prognosis of patients on the other. Overall survival rates after resection were 33.2% at 5 years and 22.9% at 10 years. Hazard ratio (HR) and 95% confidence interval (CI) of all-cause deaths by an intraductal carcinoma component and other clinicopathological parameters were examined using univariate analysis (Table 2). Patients with an intraductal carcinoma component showed a significantly more favorable prognosis than patients without such a component (Table 2).

Multivariate analysis of prognostic impact of an intraductal carcinoma component. When all 214 patients were examined by multivariate analysis adjusted for age, operation day, type of surgical resection, tumor size, histologic type and tumor differentiation, depth of invasion, venous involvement, lymphatic involvement, perineural involvement and TNM stage, patients with an intraductal carcinoma component showed a significantly more favorable prognosis than patients without such a component (Table 3). When only the 117 patients who underwent complete resection (proximal side bile duct resection margin for all

doi: 10.1111/j.1349-7006.2008.01009.x

Table 2. Crude hazard ratio (HR) and 95% confidence interval (CI) of all-cause deaths by an intraductal carcinoma component and clinicopathological parameters

	No. of deaths	Person-days	Crude death rate†	Crude HR	95% CI	*P* for trend
Intraductal carcinoma component						
Negative	96	136 804	70.2	1.00		
Positive	35	123 209	28.4	0.39	0.27, 0.58	
Age (years)						
<65	58	137 562	42.2	1.00		
≥65	73	122 451	59.6	1.33	0.94, 1.87	
Sex						
Male	94	179745	52.3	1.00		
Female	37	80 268	46.1	0.84	0.57, 1.23	
Location of the main tumor nodule						
Lower third of extrahepatic bile duct	17	43 899	38.7	1.00		
Middle third of extrahepatic bile duct	23	43 696	52.6	1.04	0.56, 1.96	
Upper third of extrahepatic bile duct	10	18 228	54.9	1.17	0.54, 2.56	
Entire of extrahepatic bile duct	3	4837	62.0	1.07	0.31, 3.67	
Hilar bile duct	46	87 502	52.6	1.09	0.63, 1.91	
Intrahepatic bile duct	32	61 851	51.7	1.14	0.63, 2.06	
Histologic type						
Adenocarcinoma	123	211 330	58.2	1.00		
Papillary adenocarcinoma	5	37 000	13.5	0.25	0.10, 0.62	
Others	3	11 683	25.7	0.51	0.16, 1.61	
Tumor size (cm)						
<3	46	134 392	34.2	1.00		
≥3	85	125 621	67.7	1.82	1.27, 2.61	
Differentiation of adenocarcinoma						
Well	36	70 278	51.2	1.00		
Moderately	67	126 210	53.1	1.13	0.75, 1.69	
Poorly	20	14 842	134.8	2.56	1.47, 4.44	
Depth of invasion						
Carcinoma *in situ* or invasion to fibromuscular layer	3	37 435	8.0	1.00		
Invasion into subserosa or beyond bile duct wall	128	222 578	57.5	6.44	2.04, 20.3	
Venous involvement						
Absent	6	63 305	9.5	1.00		
Present	125	196 708	63.5	5.80	2.54, 13.3	
Lymphatic involvement						
Absent	9	76 294	11.8	1.00		
Present	122	183 719	66.4	4.67	2.25, 9.67	
Perineural involvement						
Absent	11	72 565	15.2	1.00		
Present	120	187 448	64.0	3.67	1.95, 6.89	
pT classification						
pT1-2	23	89 367	25.7	1.00		
pT3-4	108	170 646	63.3	2.32	1.47, 3.66	
pN classification						
pN0	57	176 738	32.3	1.00		
pN1	74	83 275	88.9	2.56	1.80, 3.65	
TNM stage						
0,IA,IB	15	77 359	19.4	1.00		<0.01
IIA	39	91 428	42.7	2.26	1.24, 4.12	
IIB	65	75 858	85.7	4.21	2.37, 7.46	
III	12	15 368	78.1	3.80	1.77, 8.15	

†per 100 000 person-days.

patients, distal [duodenal] side bile duct resection margin for patients who underwent HR + EHBR, resected margin of the pancreas for patients who underwent PD were all negative) were examined in order to eliminate the effect of surgical curability, an intraductal carcinoma component was still a favorable prognostic factor (Table 3).

Correlation between an intraductal carcinoma component and bile duct resection margin status. Although an intraductal carcinoma component has been proven to be a favorable prognostic factor, it is feasible that patients with such components frequently have tumor involvement at the bile duct resection margin. Therefore, the correlation between an intraductal carcinoma component and proximal side bile duct resection margin status (negative or positive) was examined statistically (Table 4). An intraductal carcinoma component was found to be correlated with bile duct resection margin status: patients with an intraductal carcinoma component more frequently had a positive resection margin than patients without such a component ($P = 0.0192$, Table 4). In

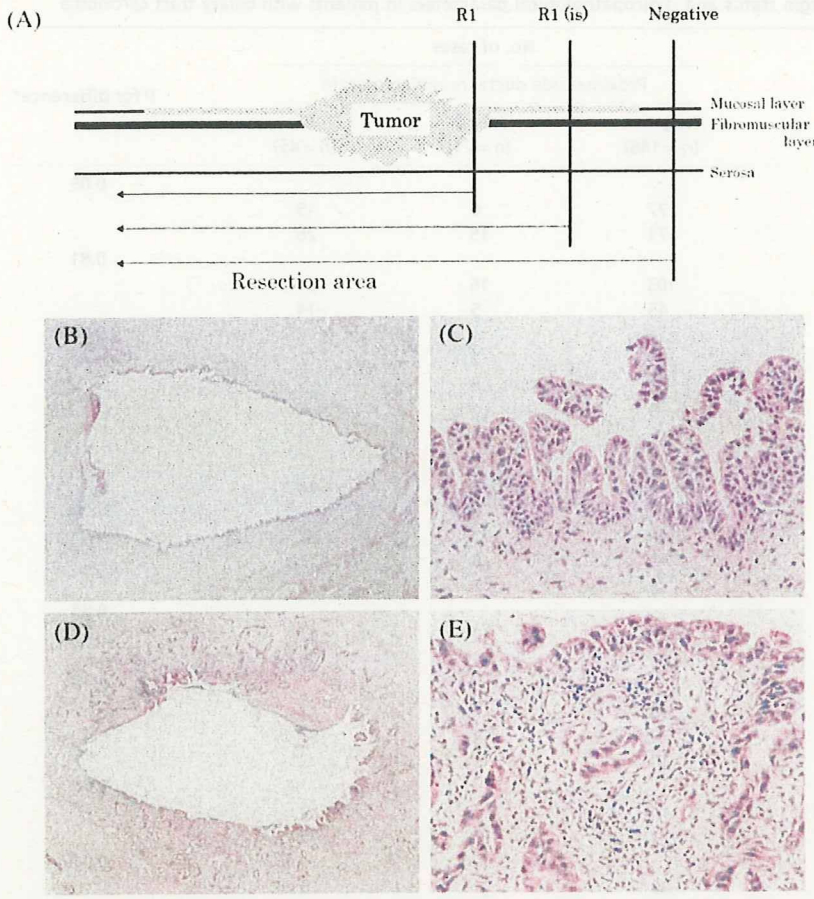

Fig. 2. Definition of bile duct resection margin status. (A) Bile duct resection margin status was categorized as negative for tumor cells (negative), positive with only an intraductal carcinoma component [R1 (is)], and positive with a subepithelial invasive component (R1). (B and C) Microscopic view of an example of an R1 (is) bile duct resection margin. Hematoxylin and eosin (H&E) stain, original magnification ×12.5 (B) and ×200 (C). (D and E) Microscopic view of an example of an R1 bile duct resection margin. H&E stain, original magnification ×12.5 (D) and × 200 (E).

Table 3. Adjusted hazard ratio (HR) and 95% confidence interval (CI) of all-cause death by an intraductal carcinoma component

	Adjusted HR	95% CI
Total (214 cases)		
Intraductal carcinoma component†		
Negative	1.00	
Positive	0.50	0.31, 0.79
Complete resection cases (117 cases)		
Intraductal carcinoma component†		
Negative	1.00	
Positive	0.31	0.13, 0.73

†Adjusted for age, operation day, type of surgical resection, tumor size, histologic type and tumor differentiation, depth of invasion, venous involvement, lymphatic involvement, perineural involvement, and TNM stage.

Table 4. Correlation between an intraductal carcinoma component and proximal side bile duct resection margin status

	No. of cases		*P* for difference*
	Bile duct resection margin status		
	Negative ($n = 148$)	Positive ($n = 66$)	
Intraductal carcinoma component			0.0192
Negative	101	34	
Positive	47	32	

*Chi-squared test.

order to further examine the clinicopathological significance and prognostic impact of bile duct resection margin status with reference to an intraductal carcinoma component, proximal side bile duct resection margin status was categorized as negative for tumor cells (negative), positive with only an intraductal carcinoma component [R1 (is)], or positive with a subepithelial invasive component (R1) (Fig. 2). According to the International Union Against Cancer, when invasive carcinoma is completely resected but histology shows an *in situ* component at the resection margin, the residual tumor is defined as R1 (is).[20] When the surgeon considers that resection has been complete but histology shows invasive carcinoma at the resection margin, the residual tumor is defined as R1.[20]

Univariate analysis of correlations between bile duct resection margin status and clinicopathological parameters. Bile duct resection margin status was negative, R1 (is) and R1 in 148 (69.2%), 21 (9.8%) and 45 (21.0%) of the 214 examined patients, respectively. Correlations between bile duct resection margin status and clinicopathological parameters were examined by univariate analysis (Table 5). Location of the main tumor nodule ($P = 0.0004$), histological type ($P = 0.008$) and venous involvement ($P = 0.009$) were each significantly correlated with bile duct resection margin status (Table 5).

doi: 10.1111/j.1349-7006.2008.01009.x

Table 5. Correlation between bile duct resection margin status and clinicopathological parameters in patients with biliary tract carcinoma

	No. of cases			*P* for difference*
	Proximal side ductal resection margin			
	Negative ($n = 148$)	R1 (is) ($n = 21$)	R1 ($n = 45$)	
Age (years)				0.09
< 65	77	6	19	
≥ 65	71	15	26	
Sex				0.81
Male	103	16	31	
Female	45	5	14	
Location of the main tumor nodule				0.004
Lower third of extrahepatic bile duct	23	2	2	
Middle third of extrahepatic bile duct	25	8	5	
Upper third of extrahepatic bile duct	8	1	5	
Entire of extrahepatic bile duct	0	1	4	
Hilar bile duct	53	7	17	
Intrahepatic bile duct	39	2	12	
Histologic type				0.008
Adenocarcinoma	129	13	42	
Papillary adenocarcinoma	15	6	1	
Others	4	2	2	
Tumor size (cm)				0.34
< 3	65	12	17	
≥ 3	83	9	28	
Differentiation of adenocarcinoma				0.16
Well	33	7	12	
Moderately	78	4	27	
Poorly	18	2	3	
Depth of invasion				0.06
Carcinoma *in situ* or invasion to fibromuscular layer	14	3	0	
Invasion into subserosa or beyond bile duct wall	134	18	45	
Venous involvement				0.009
Absent	20	5	0	
Present	128	16	45	
Lymphatic involvement				0.18
Absent	22	3	2	
Present	126	18	43	
Perineural involvement				0.57
Absent	26	3	5	
Present	122	18	40	
pT classification				0.08
pT1-2	37	8	6	
pT3-4	111	13	39	
pN classification				0.48
pN0	80	12	20	
pN1	68	9	25	
TNM stage				0.35
0, IA, IB	28	7	5	
IIA	48	5	14	
IIB	59	9	22	
III	13	0	4	

*Chi-squared test.

Univariate and multivariate analysis of prognostic impact of bile duct resection margin status. Univariate analysis revealed that although an R1 (is) bile duct resection margin had no prognostic impact in comparison with a negative bile duct resection margin, patients with an R1 bile duct resection margin showed a poorer prognosis than patients with a negative bile duct resection margin (Table 6). Surgical resection procedure, which was not examined in Table 3, is addressed in Table 6. None of the 66 patients with a positive resection margin [both R1 (is) and R1] had received any adjuvant therapy until recurrence was diagnosed.

When adjusted for age, operation day, surgical resection procedure, tumor size, histologic type, tumor differentiation, depth of invasion and venous involvement, although an R1 (is) bile duct resection margin had no prognostic impact in comparison with a negative bile duct resection margin, patients with an R1 bile duct resection margin showed a poorer prognosis than patients with a negative bile duct resection margin (Table 7).