TABLE IV. Relationship Between the Degree of BDTT Extension and Other Clinicopathological Factors

Factor	Degree of extension of bile duct invasion		P^d
	Microscopic (n = 19)	Macroscopic (n = 19)	
Sex			
M	13	19	0.008
F	6	0	
Symptoms[a]			
Yes	14	4	0.001
No	5	15	
ICGR15(%)[b]	18 ± 10	12 ± 8	0.044
Surgical procedure			
Limited resection	11	1	0.000
Segmentectomy, hepatectomy	8	18	
Bile duct resection or direct removal of thrombosis			
Yes	0	8	0.000
No	19	11	
Size (cm)	6.2 ± 5.7	6.2 ± 2.9	n.s.
Number			
Solitary	9	10	n.s.
Multiple	10	9	
Portal vein involvement			
Yes	16	11	0.074
No	3	8	
Intrahepatic metastases			
Yes	9	10	n.s.
No	10	9	
Noncancerous liver			
Cirrhosis	7	3	n.s.
No cirrhosis	12	16	
Histologic differentiation			
Well/mod	9	11	n.s.
Poor	6	5	
Combined[c]	3	3	
Surgical curability			
Yes	15	15	n.s.
No	4	4	

n.s., not significant.
[a]Symptoms included jaundice, fever, or abdominal pain.
[b]ICG15R, the indocyanine green retention rate at 15 min.
[c]Combined means mixed hepatocellular carcinoma and cholangiocarcinoma.
[d]The unpaired *t*-test or Chi-square test.

can improve surgical outcome in patient with macroscopic bile duct invasion without direct bile duct wall invasion. The clinical significance of combined resection of bile duct should be further evaluated in a large series study.

In conclusion, we found favorable long-term results for HCC patients especially with macroscopic invasion. Even if HCC tumor thrombosis is recognized in the major branches of bile duct, extensive surgical procedure should be strongly recommended when curative and safe surgery can be expected in patients without intrahepatic metastases.

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Prognostic Significance of Tissue Factor in Pancreatic Ductal Adenocarcinoma

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Abstract Tissue factor (TF) is a transmembrane glycoprotein that plays roles in the blood coagulation and intracellular signaling pathways, and has also been suggested to modulate the biological behavior of cancer cells. In order to examine the clinicopathologic significance of TF expression in pancreatic ductal adenocarcinoma, TF expression was determined by immunohistochemistry using a newly raised anti-TF monoclonal antibody in 113 patients who had undergone surgical resection of pancreatic ductal adenocarcinoma. According to the incidence of tumor cell immunopositivity, patients were divided into "negative TF" (0%), "weak TF" (<25%), or "high TF" (25% or more) groups, which accounted for 11.6% (n = 13), 44.2% (n = 50), and 44.2% (n = 50) of the total, respectively. Increased TF expression was correlated with the extent of the primary tumor (P = 0.0043), lymph node metastasis (P = 0.0043), lymphatic distant metastasis (P = 0.0039), advanced tumor-node-metastasis stage (P = 0.0002), and high tumor grade (P = 0.0164). Multivariate analysis using the Cox proportional hazards model showed that high TF expression was an independent negative predictor for survival (hazard ratio, 2.014; P = 0.0076). Moreover, patients with TF-negative tumors had a significantly better prognosis even if lymph node metastasis was present (P < 0.0001). We also showed that TF knockdown by RNA interference suppressed the invasiveness of a pancreatic adenocarcinoma cell line *in vitro*. These results indicate that TF expression may contribute to the aggressiveness of pancreatic ductal adenocarcinoma by stimulating tumor invasiveness, and that evaluation of the primary tumor for TF expression may identify patients with a poor prognosis.

Tissue factor (TF) is a transmembrane glycoprotein that functions as a cellular receptor for coagulation factor VII (FVII) and modulates it to produce the activated form, FVIIa. The TF/FVIIa complex is regarded as the initiator of the extrinsic blood coagulation cascade, which ultimately leads to the generation of thrombin (1). In normal human tissues, TF is expressed only in extravascular cells, including the vascular adventitia and organ capsules (2). Based on this cellular distribution, under physiologic conditions, TF is thought to act mainly as a hemostatic barrier to prevent blood loss. In addition to its role as a hemostatic initiator, the binding of FVIIa with TF has been suggested to be involved in intracellular signaling mechanisms (3), such as the mitogen-activated protein kinase pathway (4) and the Src family member/PI3K/Rac-dependent signaling pathway (5), at least in some cell types.

TF is also involved in many pathophysiologic conditions, such as inflammation, atherosclerosis, and malignancies. With regard to malignancies, it has been well recognized that patients with malignant diseases are predisposed to hypercoagulation since Trousseau (6) first reported the increased frequency of thrombosis in patients with gastrointestinal cancers, and this hypercoagulable state is associated with TF (7). Immunohistochemical analysis has revealed that TF is expressed in a wide variety of malignancies (8). Metastatic melanoma cells express higher levels of TF than nonmetastatic cells (9), and a metastatic rectal carcinoma subline showed enhanced TF expression in comparison to its parental line (10). Transfection of TF promoted the metastasis of melanoma in a mouse model (11), and enhanced primary tumor growth in a pancreatic adenocarcinoma cell line (12). Therefore, TF not only contributes to the development of a hypercoagulable state in cancer patients but also modulates the biological behavior of cancer cells.

Pancreatic adenocarcinoma is one of the most clinically aggressive malignancies; indeed, the 3-year survival rate after surgical resection of the primary tumor has been reported as only 17% (13). Therefore, identification of molecules that

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might predict a poor prognosis is important in selecting patients who would benefit from radical treatment or molecular targeting therapy. Although a few immunohistochemical studies on TF expression in pancreatic ductal carcinoma have been done (8, 14, 15), no detailed clinicopathologic study using multivariate-type analysis has been carried out to date. In the present immunohistochemical study, we used a newly raised anti-TF antibody named NCC-7C11 to examine TF expression in a large series of surgically resected pancreatic ductal adenocarcinomas, and investigated the correlations between TF expression and various clinicopathologic parameters, including the clinical outcome. Furthermore, we investigated the effect of TF knockdown on the invasiveness of a pancreatic cancer cell line using RNA interference, a new gene-silencing technique.

Materials and Methods

Production of the monoclonal antibody. Female BALB/c (*nu/nu*) mice were immunized with the scirrhous gastric carcinoma cell line HSC-44PE by means of a rejection method, and hybridomas were produced as described previously (16). The hybridomas were then selected on the basis of their immunohistochemical reactivity with various cancerous tissues, and a hybridoma that produced the monoclonal antibody (mAb) NCC-7C11 (IgG_1, k), which reacted with the invasive front of pancreatic ductal adenocarcinoma, was obtained.

Cell lines and reagents. All pancreatic cancer cell lines (BxPC-3, SU 86.86., AsPC-1, Capan-1, Capan-2, PK-59, HPAC, MPanc-96, CFPAC-1, PANC-1, and MIAPaCa-2) were obtained from the American Type Culture Collection (Rockville, MD). The scirrhous gastric carcinoma cell line HSC-44PE was established by Yanagihara (17). The cells were maintained in RPMI 1640 (BxPC-3, SU86.86., AsPC-1, Capan-1, PK-59, HPAC, CFPAC-1 and HSC-44PE) or DMEM (Capan-2, MPanc-96, PANC-1, and MIAPaCa-2), supplemented with either 20% (Capan-1) or 10% (others) heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen Corp., Carlsbad, CA) at 37°C in a humidified atmosphere containing 5% carbon dioxide. Another murine anti-human TF mAb (TFE), recombinant human TF apoprotein, and normal murine IgG_1k were purchased from Enzyme Research Laboratories, Inc. (South Bend, IN), Angiopharm (O'Fallon, MO), and Becton Dickinson and Company (Franklin Lakes, NJ), respectively.

Immunoprecipitation. The BxPC-3 pancreatic carcinoma cell line was used for immunoprecipitation. The cells were washed with ice-cold Ca^{2+}/Mg^{2+}-free PBS and treated with radioimmunoprecipitation assay buffer containing a proteinase inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) on ice for 30 minutes. After centrifugation (15,000 rpm for 30 minutes), the supernatant was collected and precleared with protein G sepharose (50% slurry) at 4°C overnight. To conjugate the primary antibodies, 1 μg primary antibody and 25 μL protein G sepharose beads suspended in RIPA buffer were incubated with mixing at 4°C overnight. After centrifugation, ~500 μg of total cellular protein from the precleared supernatant and the antibody-sepharose conjugate were incubated with mixing at 4°C for 3 hours. The immunoprecipitates were collected by centrifugation at 2,500 rpm for 5 minutes at 4°C. After washing four times with RIPA buffer, the supernatant was carefully removed and the pellets were resuspended in 40 μL of 2× electrophoresis sample buffer.

Protein identification by mass spectrometry. The protein immunoprecipitated by mAb NCC-7C11 from the BxPC-3 lysate was subjected to SDS-PAGE. The protein was visualized using a negative gel stain kit (Wako Pure Chemical Industries, Ltd., Japan) and its band was excised from the gel. In-gel digestion was carried out with trypsin (Promega, Madison, WI), as described in the literature (18). Mass spectrometric analyses of the trypsin digests were done using Voyager (Applied Biosystems, Framingham, MA), and peptide mass mapping was carried out with reference to the MASCOT database.

Western blot analysis. Samples were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking, the filters were incubated with the primary antibodies, then with peroxidase-conjugated secondary antibodies (Amersham Biosciences Corp., Piscataway, NJ). The peroxidase-labeled bands were visualized using an electrochemiluminescence kit (Amersham Biosciences). As a loading control, the same membrane was reprobed with an anti--actin mAb (Sigma-Aldrich), as described in the literature (19).

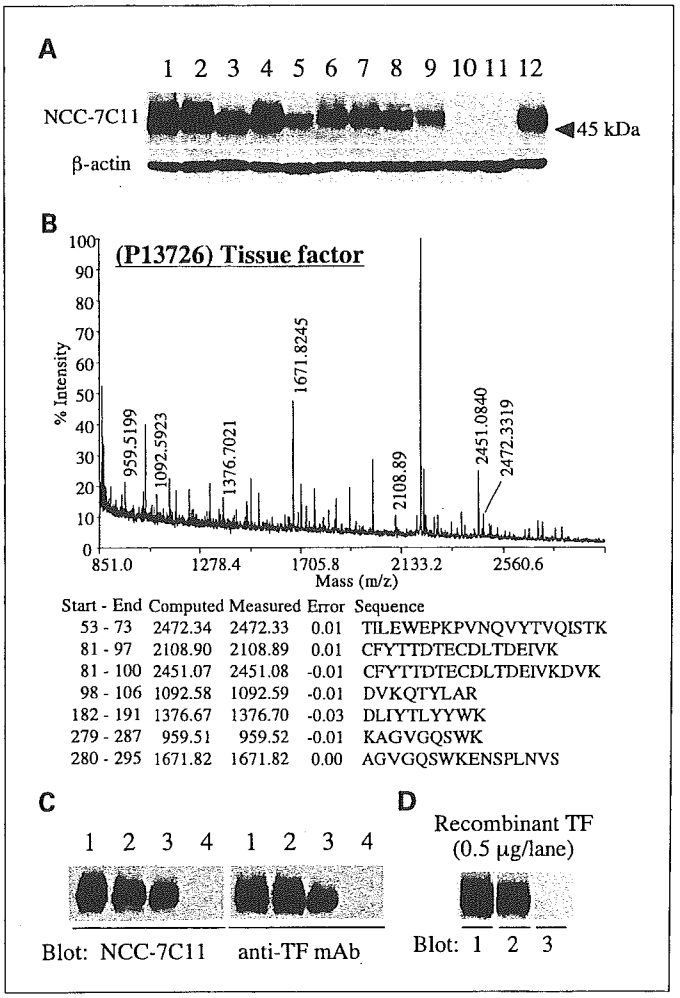

Fig. 1. Identification of the antigen recognized by mAb NCC-7C11. *A*, Western blot analysis of the NCC-7C11 antigen in various pancreatic cancer cell lines. *Lane 1*, BxPC-3; *lane 2*, SU 86.86.; *lane 3*, AsPC-1; *lane 4*, Capan-1; *lane 5*, Capan-2; *lane 6*, PK-59; *lane 7*, HPAC; *lane 8*, MPanc-96; *lane 9*, CFPAC-1; *lane 10*, PANC-1; *lane 11*, MIAPaCa-2; *lane 12*, HSC-44 (scirrhous gastric carcinoma cell line; Immunogen). Forty micrograms of whole cell lysate were applied to each lane and separated by SDS-PAGE under reducing condition. Position of the 45 kDa molecular size marker (*right*); *B*, identification of the protein immunoprecipitated by NCC-7C11 using mass spectrometry. After trypsin digestion, the ion peak spectra matched the seven peptide sequences of TF (P13726); *C*, reciprocal coimmunoprecipitations from BxPC-3 cells. Immunoprecipitates with NCC-7C11 (*lane 1*), a commercially available anti-TF mAb (TFE; *lane 2*) and mouse immunoglobulin (*lane 4*; negative control) were subjected to SDS-PAGE under nonreducing conditions. Whole cell lysates served as a positive control (*lane 3*); *D*, reactivity of antibodies with recombinant human TF apoprotein (0.5 μg/lane) by Western blot analysis under reducing condition. *Lane 1*, NCC-7C11; *lane 2*, the anti-TF mAb TFE; *lane 3*, mouse immunoglobulin.

Patients and tissue specimens. Formalin-fixed, paraffin-embedded tumor specimens were obtained from a series of 113 consecutive patients with pancreatic ductal adenocarcinoma who had undergone surgical resection at the National Cancer Center Hospital in Tokyo, Japan between 1990 and 1999. Patients with pancreatic tumors of a special type, such as mucinous cystadenocarcinoma, intraductal papillary-mucinous adenocarcinoma, or adenosquamous carcinoma, were excluded. Three patients who died in the immediate postoperative period were also excluded. The patients consisted of 72 men (63.7%) and 41 women (36.3%), who ranged in age from 45 to 82 years, with a mean age of 63.1 years. The median duration of follow-up was 16 months (range 2.9-72 months). The surgical procedures were total pancreatectomy in 6 patients, distal pancreatectomy in 35 patients, pylorus-preserving pancreaticoduodenectomy in 20 patients, and pancreaticoduodenectomy in 52 patients. Intraoperative radiation was done in 77 patients and postoperative chemotherapy was given to 44 patients. The resected specimens were staged according to the International Union against Cancer tumor-node-metastasis (TNM) classification (20). Histologic grading of the tumors was done according to the WHO classification system (21). Other pathologic variables (lymphatic invasion, vascular invasion, perineural invasion, and growth pattern) were based on the Japan Pancreas Society's classification system for pancreatic carcinoma (22).

Immunohistochemistry. The avidin-biotin-peroxidase complex method was used for immunostaining, as described in the literature (23). Briefly, formalin-fixed, paraffin-embedded sections (4 μm thick) containing the maximum diameter of the tumor were deparaffinized using a graded ethanol and xylene series, treated with 0.3% hydrogen peroxide in methanol and immersed in 10 mmol/L citrate buffer (pH 6.0). After autoclaving, the sections were incubated with normal swine serum for 10 minutes to block nonspecific antibody reactions, exposed to the primary antibody (final concentration, 1 μg/mL) overnight at 4°C, then incubated sequentially with biotinylated goat anti-mouse IgG and avidin-biotinylated-peroxidase complex as supplied in the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The color reaction was developed over 5 minutes using diaminobenzidine tetrahydrochloride and 0.02% hydrogen peroxide, and nuclear counterstaining with hematoxylin was done. The positive control included in every assay was a section composed of formalin-fixed, paraffin-embedded cell pellets of the human pancreatic carcinoma cell line BxPC-3, which was confirmed to express the NCC-7C11 antigen by Western blot analysis. Negative control staining, which was done using the same class of mouse immunoglobulin as the primary antibody, yielded negative results in every specimen.

RNA interference, immunocytochemistry, and invasion assays. The sequences used to design the small interfering RNAs (siRNA) were selected according to a previously described strategy (24-26). The siRNA sequences chosen to target TF (Genbank accession number NM 001993) were positions 489 to 509 ($siRNA_{TF}489$) and 653 to 673 ($siRNA_{TF}653$), numbered from the start codon, and the siRNAs were purchased from Dharmacon, Inc. (Lafayette, CO). Control experiments were done using two unrelated siRNAs. $siRNA_{Luc}$ was Cy3 labeled siRNA directed against Luciferase mRNA (Dharmacon) and $siRNA_{NC}$ (mock) was Nonspecific Control Duplex X (Dharmacon). The sequence of the latter (5′-NNATTCTATCACTAGCGTGAC-3′) was confirmed to have no homology with any known mRNA by a BLAST search; however, it had the same GC content as $siRNA_{TF}$.

At first, we examined transfection efficiencies among the TF-positive cell lines BxPC-3, SU 86.86., and AsPC-1 by using Cy3-labeled siRNA against luciferase. In >60% of BxPC-3 cells, Cy3 was observed by fluorescence microscopy, and therefore the BxPC-3 cell line was selected. This Cy3-labeled siRNA against luciferase was used as a negative control in each experiment, so we confirmed the transfection efficiency every time we did the siRNA knockdown and invasion assay. Reduction of TF expression on the surface of cells was confirmed by immunocytochemistry using anti-TF antibody NCC-7C11, biotinylated goat anti-mouse IgG, and avidin-FITC (Vector Laboratories) under fluorescence microscopy.

RNA interference and invasion assays were done as described in the literature (27). BxPC-3 cells were exposed to 40 nmol/L siRNA, in the presence of Lipofectamine 2000 (Invitrogen), for 6 hours. The transfected cells were subjected to either immunoblot assays or invasion assays 24 hours after the removal of the transfection reagent.

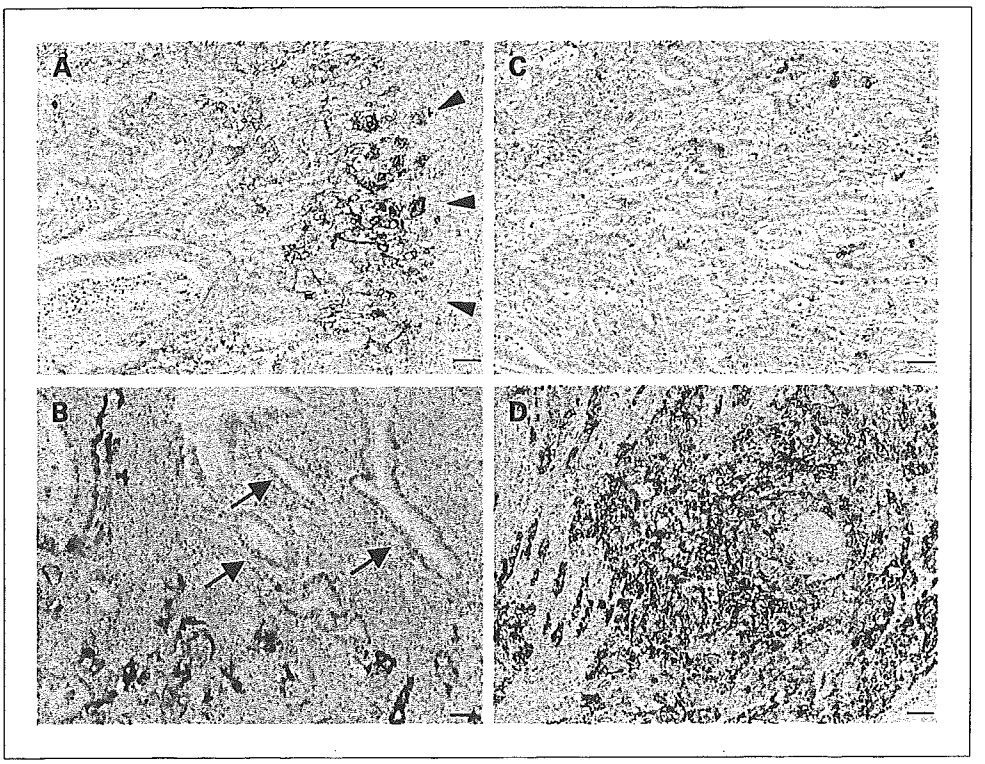

Fig. 2. Immunohistochemical staining pattern of the anti-TF mAb NCC-7C11. *A*, NCC-7C11 reacted preferentially with the invasive tumor front, as shown in this moderately differentiated pancreatic ductal adenocarcinoma (*arrowheads*); *B*, pancreatic ductal adenocarcinoma cells were stained by NCC-7C11, whereas the adjacent normal pancreatic ducts showed no immunoreactivity (*arrows*). Representative staining patterns (*C* and *D*); *C*, this moderately differentiated adenocarcinoma was classified as showing low TF expression; *D*, this poorly differentiated adenocarcinoma was markedly stained by NCC-7C11 and was classified as showing high TF expression. *Bar*, 100 μm.

Table 1. Association between TF expression and clinicopathologic variables

Variables	TF expression			
	Low TF (<25%, n = 63)			
	Negative TF (0%, n = 13)	Weak TF (0%<, n = 50)	High TF (≥ 25%, n = 50)	P value (Low vs. High)
Age (y)				
<65	8	26	24	
≥65	5	24	26	0.5284
Gender				
Male	7	31	34	
Female	6	19	16	0.3989
Extent of the primary tumor spread				
pT_1	3	3	1	
pT_2	3	9	5	
pT_3	4	20	15	
pT_4	3	18	29	0.0043*
Lymph node metastasis				
pN_0	2	7	5	
pN_{1a}	10	20	11	
pN_{1b}	1	23	34	0.0043*
Distant metastasis				
pM_0	9	42	28	
pM_1	4	8	22	0.0041*
M_1 (LYM)†	2	7	19	0.0039*
M_1 (HEP)†	1	1	1	0.9999
M_1 (PER)†	1	0	2	0.5508
Stage				
I	1	4	1	
II	0	2	2	
III	5	23	7	
IVA	3	13	18	
IVB	4	8	22	0.0002*
Histopathologic tumor grade				
G_1	7	24	16	
G_2	5	23	23	
G_3	1	3	11	0.0164*
Lymphatic invasion‡				
Negative	4	5	8	
Positive	9	45	42	0.8001
Vascular invasion‡				
Negative	8	14	12	
Positive	5	36	38	0.2087
Perineural invasion‡				
Negative	2	6	4	
Positive	11	44	46	0.5443
Growth pattern‡				
Expansive + intermediate	9	30	21	
Infiltrative	4	20	29	0.0392*
Surgical margin				
Negative	10	34	30	
Positive	3	16	20	0.2744

*Significant.
†LYM, lymphatic metastasis; HEP, hepatic metastasis; PER, peritoneal metastasis.
‡Classified according to the classification of Pancreatic Carcinoma of Japan Pancreas Society.

The relative density of the chemiluminescence signal was determined using Image Gauge Software (Fuji Photo Film Co., Ltd., Japan) and standardized by using the relative density of the β-actin signal. For the invasion assays, Biocoat Matrigel Invasion Chambers (Becton Dickinson Labware) were utilized according to the manufacturer's instructions. We used Accutase (Innovative Cell Technologies, Inc., San Diego, CA) to harvest cells for use in the invasion assay, and the harvested cells were washed with ice-cold PBS containing 0.1% bovine serum albumin before seeding. Transfected cells (4×10^5) in 500 μL RPMI 1640 containing 0.1% bovine serum albumin were seeded into each insert chamber. Then, 750 μL RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum was added to each lower chamber, and the plates were incubated at 37°C in a 5% CO_2/95% air incubator for 18 hours. After incubation, the noninvading cells were carefully removed from the top of each insert chamber with a cotton swab. The invading cells were then fixed and stained using a Diff-Quik kit (Sysmex Corp., Japan), and the total number of invading cells was counted under a microscope. Each run was done in triplicate, and the experiment was repeated independently thrice.

Statistical analysis. Correlations between TF immunoreactivity and patients' clinicopathologic variables were analyzed using the Mann-Whitney *U* test for the extent of the primary tumor spread (pT), lymph node metastasis, histologic tumor grade, and pTNM stage, and either the χ^2 test or Fisher's exact test for the remaining variables. The Kaplan-Meier method was used to generate survival curves, and differences in survival were analyzed using the log-rank test, based on the TF expression status. Univariate and multivariate analyses were done using the Cox proportional hazards model. Matrigel invasion assays and densitometric analyses were compared using the Mann-Whitney *U* test. Probability values <0.05 were considered statistically significant. All analyses were done using statistical analysis software (Statview, version 5.0; SAS Institute, Inc., Cary, NC).

Results

Monoclonal antibody characterization. Western blotting under reducing condition showed that about half of the pancreatic cancer cell lines expressed moderate to high levels of the NCC-7C11 antigen (Fig. 1A). A peptide mass fingerprint of tryptic digests of the antigen immunoprecipitated from the BxPC-3 cell lysates was obtained by mass spectrometry and a search of the MASCOT database identified this antigen as TF (Fig. 1B). To confirm the identity of TF, we did reciprocal coimmunoprecipitation assays using a commercially available anti-TF mAb TFE under nonreducing conditions (Fig. 1C). We also showed the reactivity of NCC-7C11 and TFE mAbs to recombinant TF apoprotein by immunoblotting (Fig. 1D). Together, these data confirmed that NCC-7C11 was an anti-TF mAb. We examined the TF expression pattern of the cell lines by Western blotting with a commercially available polyclonal antibody against TF (clonal, American Diagnostic, Inc, Greenwich, CT), and thus confirmed the results of our Western blot analysis (data not shown).

Immunohistochemical analysis of tissue factor expression in pancreatic ductal adenocarcinoma. The immunostaining pattern of NCC-7C11 is shown in Fig. 2. TF expression occurred preferentially at the invasive front of the tumor (Fig. 2A), whereas no TF was expressed in adjacent normal ductal cells (Fig. 2B), as previously described in the literature (14). According to the proportion of TF-positive cancer cells, TF expression was classified as "low TF" (0-25% of cells showing immunopositivity, Fig. 2C) or "high TF" (25% or more of cells showing immunopositivity, Fig. 2D). Low TF included patients with completely TF-negative tumors ("negative TF", 0% of cells showing immunopositivity), and those with weakly TF-positive tumors ("weak TF", >0% and <25% of cells showing immunopositivity). The cutoff point for weak/high TF was set at the median value for the entire sample without the TF-negative sample. When comparing the high TF group with the low TF group, increased TF expression was positively correlated with the extent of primary tumor spread, lymph node metastasis, the presence of lymphatic distant metastasis, high tumor grade, advanced TNM stage, and an infiltrative growth pattern (Table 1).

Prognostic significance of tissue factor expression. The survival curves of the patients, grouped according to the level of TF staining in their tumors, are shown in Fig. 3A. The high TF expression group had a significantly poorer prognosis than the

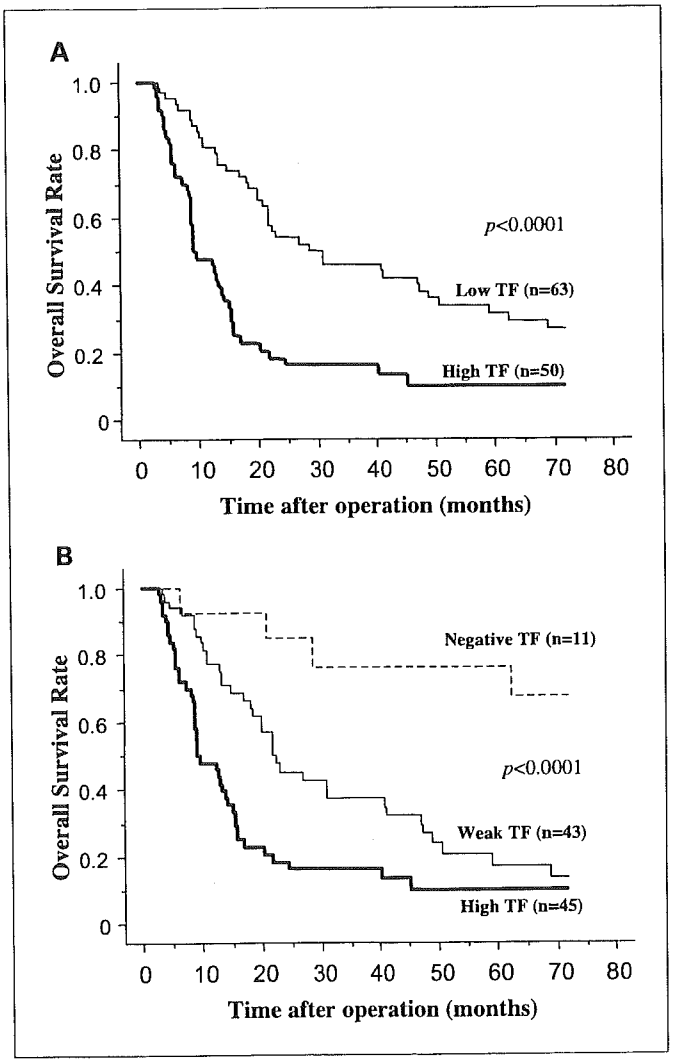

Fig. 3. Kaplan-Meier survival curves for patients who had undergone surgical resection of pancreatic ductal adenocarcinoma, stratified according to the level of expression of TF in their tumors. *A*, overall survival of patients with pancreatic ductal adenocarcinoma (lowTF, 0-25%; highTF, 25% or more of the cells showing immunopositivity; log-rank test, $P < 0.0001$); *B*, overall survival of patients who had tumors with lymph node metastasis (negativeTF, 0%; weakTF, >0% and <25%; highTF, 25% or more of the cells showing immunopositivity; log-rank test, $P < 0.0001$).

low TF expression group (log-rank test, $P < 0.0001$). Upon univariate analysis with the Cox proportional hazards model, the extent of the primary tumor ($P = 0.0497$), lymph node metastasis ($P = 0.0102$), distant metastasis ($P = 0.0027$), histologic tumor grade ($P = 0.0070$), growth pattern ($P = 0.0173$), and TF immunopositivity ($P < 0.0001$) were all positively correlated with a poor prognosis. Multivariate analyses indicated that TF expression was an independent predictor of an unfavorable prognosis ($P = 0.0076$; risk ratio, 2.014; 95% confidence interval, 1.205-3.366), as were the presence of lymph node metastasis ($P = 0.0103$) and histologic tumor grade ($P = 0.0154$; Table 2). The survival of the patients with lymph node metastasis was further analyzed, grouped according to three TF staining levels, i.e., negative TF, weak TF, and high TF (Fig. 3B). The survival of the TF-negative group was markedly better and increased TF expression was significantly correlated with a poor prognosis (log-rank test, $P < 0.0001$).

The effects of small interfering RNAs targeted against tissue factor on tumor invasion. TF overexpression proved to be linked with the aggressiveness of pancreatic cancer in our immunohistochemical analysis. In order to determine whether down-regulation of endogenous TF would suppress the invasive behavior of pancreatic cancer, we synthesized siRNAs that, when transfected into cells, target TF mRNA for degradation, thus reducing the expression of TF protein. High transfection efficiency of siRNAs into BxPC-3 cells has been achieved with Lipofectamine 2000 (Fig. 4A, *top*) and reduction of TF expression by $siRNA_{TF}653$ against TF, compared with control $siRNA_{NC}$, has been ascertained under fluorescence microscopy by immunocytochemistry (Fig. 4A, *middle* and *bottom*). Densitometric analyses (Fig. 4B) and invasion assays (Fig. 4C) showed that transfection with either $siRNA_{TF}489$ or $siRNA_{TF}653$ significantly reduced TF expression by, and the invasiveness of, BxPC-3 cells compared with mock-transfected cells ($siRNA_{NC}$), whereas transfection with a siRNA targeted to an unrelated mRNA ($siRNA_{Luc}$) had no effect on TF expression or invasiveness.

Discussion

In the present study, we showed the clinicopathologic significance of TF expression in pancreatic ductal adenocarcinoma in an immunohistochemical analysis using a newly raised

Table 2. Prognostic factors in Cox's proportional hazards model

	Univariate			Multivariate		
Variables	Hazard ratio	95% Confidence interval	*P*	Hazard ratio	95% Confidence interval	*P*
Age (y)						
≥65/<65	1.202	0.782-1.846	0.4014			
Gender						
Female/male	0.909	0.582-1.420	0.6762			
Extent of the primary tumor spread						
pT_4/pT_1-pT_3	1.545	1.001-2.385	0.0497*	1.280	0.793-2.066	0.3125
Lymph node metastasis						
pN_{1a}, pN_{1b}/pN_0	2.770	1.274-6.023	0.0102*	2.953	1.292-6.752	0.0103*
Distant metastasis						
pM_1/pM_0	2.301	1.279-3.223	0.0027*	1.501	0.912-2.471	0.1101
Histologic tumor grade						
G_2, G_3/G_1	1.845	1.182-2.879	0.0070*	1.882	1.128-3.318	0.0154*
Lymphatic invasion						
Positive/negative	1.429	0.757-2.700	0.2708			
Vascular invasion						
Positive/negative	1.412	0.877-2.273	0.1554			
Tumor diameter (cm)						
≥3.5/<3.5	1.366	0.884-2.111	0.1604			
Growth pattern						
Infiltrative/expansive, intermediate	1.638	1.096-2.584	0.0173*	1.211	0.742-1.976	0.4446
Surgical margin						
Positive/negative	1.168	0.747-1.824	0.4959			
Chemoradiotherapy						
Not received/received	0.957	0.562-1.630	0.8708			
TF expression						
HighTF/lowTF	2.723	1.748-4.243	<0.0001*	2.014	1.205-3.366	0.0076*

*Significant.

anti-TF antibody. Our findings indicate that TF has prognostic significance in patients with resectable tumors. Moreover, we confirmed that TF contributed to the invasiveness of a pancreatic cancer cell line by inhibiting TF expression using the RNA interference technique *in vitro*.

It is well recognized that cancer cells at the invasive front express invasion-related molecules such as matrix metalloproteinases (28) and the laminin γ2 chain (29, 30). We confirmed that TF is another of these invasion-related molecules, since TF immunopositivity was clearly observed at the invasive fronts of the pancreatic ductal adenocarcinomas. Our immunohistochemical study also showed that TF expression in the primary tumors was correlated significantly with many aggressiveness-related factors, including the extent of primary tumor spread, lymph node metastasis, lymphatic distant metastasis, TNM stage, tumor grade, and growth pattern. Among previous immunohistochemical studies of TF expression in pancreatic ductal adenocarcinoma, only that reported by Kakkar et al. (14) showed correlations between TF expression and clinicopathologic characteristics, showing that TF expression is correlated with histologic tumor grade and possibly with lymph node metastasis. In agreement with their results, the present study clarified that TF expression was indeed correlated with tumor grade and the extent of lymph node metastasis. Although there was a tendency for TF to be frequently expressed in G_3 cells, it was also expressed in some well or moderately differentiated tumors. Moreover, it is very disconcerting that the least differentiated cell lines examined, such as MIAPaCa-2 and Panc-1, proved TF-negative. However, in agreement with the present study, MIAPaCa-2 and Panc-1 have actually been reported to express hardly any TF mRNA (31). Therefore, we speculate that TF is not merely an indicator of grade. It is unclear what value this spectrum of cell lines adds to the current proposal and whether they are incapable of expressing TF. Further analysis will be needed to reconcile this discrepancy between *in vitro* and *in situ* conditions. On the other hand, TF expression in lymph node metastases is of great interest since our immunohistochemical analysis seemed to indicate that TF was involved in lymph node metastasis. Therefore, we have additionally examined 10 lymph node metastases to determine whether TF expression is enriched in comparison with the expression in the primary tumor. We found that TF expression in lymph node metastases reflected that in the primary tumor, although it was not necessarily enriched (data not shown). Immunohistochemical studies on other cancers have also revealed correlations between TF expression and clinicopathologic characteristics. In colorectal carcinoma, TF expression was positively correlated with lymph node metastasis, liver metastasis, and Dukes' stage (32). In non-small cell lung cancers, TF expression was also associated with hematogenous or lymphogenous metastasis (33). These observations are consistent with our findings, in that TF

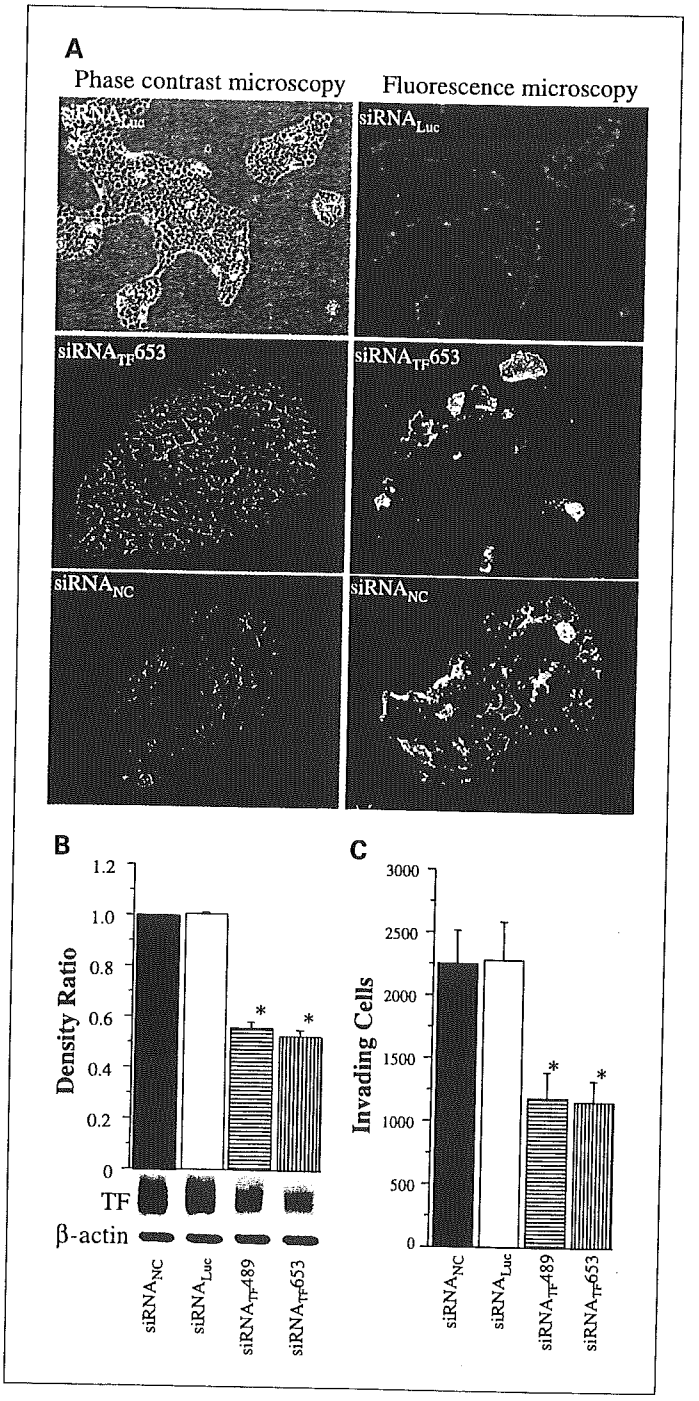

Fig. 4. Effect of TF knockdown by RNA interference on the invasiveness of human pancreatic cancer cells. BxPC-3 cells were transiently transfected with short interfering RNAs and subjected to either Western blot analysis or Matrigel invasion assays. siRNA$_{TF}$489 and siRNA$_{TF}$653 are directed against TF. Control experiments were done with a Cy3-labeled siRNA directed against an unrelated mRNA (Luciferase; siRNA$_{Luc}$) and an irrelevant siRNA (siRNA$_{NC}$; used as a mock-transfectant). Transfection efficiency was confirmed by using Cy3-labeled siRNA$_{Luc}$ in each assay, and representative pictures obtained by phase-contrast microscopy and fluorescence microscopy revealed a high efficiency of transfection of siRNA into BxPC-3 cells (*A, top*). Immunocytochemistry under fluorescence microscopy shows that many cells lack TF expression on their surface as a result of knockdown by siRNA$_{TF}$653 against TF (*A, middle*), whereas control siRNA$_{NC}$ has no effect on TF surface expression (*A, bottom*). Reduction of TF protein expression by siRNA against TF was determined by Western blot analysis and densitometric analysis. The relative density of the chemiluminescence signal was measured and standardized using the relative density of the β-actin signal. Transfection with either siRNA$_{TF}$489 or siRNA$_{TF}$653 significantly reduced TF compared with mock-transfected cells (siRNA$_{NC}$), whereas transfection with a siRNA targeted to an unrelated mRNA (siRNA$_{Luc}$) had no effect on TF expression (*B*). For the invasion assays, the transfectants were seeded onto Matrigel-coated invasion chambers and incubated for 18 hours, then the total number of cells on the underside of each filter was determined. Invading cells were significantly suppressed by siRNA against TF, as reflected in the observed reduction of protein expression (*C*). Columns, means; bars, SE ($n = 9$); *, $P < 0.01$ compared with both control groups.

expression was significantly correlated with lymphatic distant metastasis and TNM stage. In our series, TF expression did not correlate with either hepatic or peritoneal metastasis, but only with lymphatic distant metastasis, suggesting a potential specificity of this protein's role in invasion. However, it is rare for pancreatic tumors with distant metastasis, except lymphatic distant metastasis, to become operable. Therefore, it is difficult to conclude that there is no correlation between TF expression and distant metastasis besides lymphatic distant metastasis. The present study also revealed that high TF expression was associated with the extent of the primary tumor and an infiltrative growth pattern, suggesting that TF overexpression has a proinvasive effect.

The clinical significance of high-level TF expression was further substantiated by its correlation with a shorter overall survival time. Univariate analysis showed that TNM status, tumor grade, tumor size, growth pattern, and TF expression were all significantly correlated with patient survival. Moreover, multivariate analysis also showed that TF expression was an independent prognostic factor. Therefore, TF had significant predictive value for overall survival, suggesting that its expression could be a useful predictor of poor prognosis. Although the hazard ratio of lymph node status was higher than that of TF expression in multivariate analysis, lymph node status and TF expression were proven to be statistically significant and independent prognostic factors. Therefore, we believe that both factors are almost equally important in predicting prognosis in patients with pancreatic cancer. Indeed, among patients with lymph node metastasis, those with TF-negative tumors had a markedly better prognosis, and increased TF was also significantly correlated with a poorer prognosis. Thus, our findings suggest that TF contributes to the aggressiveness of pancreatic ductal adenocarcinoma. To our knowledge, this is the first study to have shown the clinicopathologic significance of TF expression in pancreatic ductal adenocarcinoma using multivariate-type analysis.

The present study revealed that knockdown of endogenous TF could suppress the invasiveness of a pancreatic adenocarcinoma cell line *in vitro*, suggesting that TF plays an important role in tumor invasion. The potential role of coregulation of TF and effector proteases such as matrix metalloproteinases has been reported previously for other cell types (34, 35). In a small cell lung cancer cell line, the transition of a small cell lung cancer from a suspension to adherent and aggressive growth was accompanied by expression of TF as well as matrix metalloproteinases-2 and -9 (35). Other mechanisms by which TF promotes tumor invasion have been suggested previously. Taniguchi et al. (31) showed that binding of FVIIa to TF induced overexpression of the urokinase plasminogen activator receptor gene, which is involved in proteolytic extracellular matrix degradation, resulting in increased migration of pancreatic cancer cells, whereas blockade of TF activity with neutralizing monoclonal antibodies inhibited FVIIa-dependent tumor invasion. Ott et al. (36) showed that the role of TF in cell migration and adhesion is mediated by an interaction with actin-binding protein. TF has also been shown to mediate intracellular signaling leading to the development of lamellipodia and filopodia (5). In our invasion assay, however, the number of invading control cells observed was higher than the levels reported previously (37). One reason for the high invasion may have been that the seeding density we used was more than 10 times higher than that reported previously. Another reason might be that we used Accutase to harvest the cells from culture, although Accutase has also been reportedly utilized for the invasion assay in a study of another cell type (38). Since Accutase is reported to maintain most cell surface antigens and some antibodies including anti-TF antibody and anti-urokinase plasminogen activator receptor antibody work well with Accutase according to the manufacturer (data not shown), cells treated with Accutase might retain their invasive ability. On the other hand, Accutase is a mixture of invasion-relevant proteases that are directly capable of degrading the reconstituted basement membrane used as a barrier in the invasion assay. So, although the cells were washed before being seeded, we cannot rule out the possibility that this assay might not represent an examination of the capability of BxPC-3 cells to invade *de novo*, but rather their ability to use extrinsic enzymes to effect invasion. Although the present study could not prove the mechanism by which TF promotes tumor invasion, our finding of a distinct association between TF and tumor invasiveness may have therapeutic as well as prognostic implications. Since retinoic acid (39), resveratol (40), vitamin D_3 (41), and pentoxifylline (42) have all been reported to down-regulate TF, the effects of these agents on TF expression in pancreatic cancer cells are worth evaluating. Recently, the relationship between TF expression and angiogenesis in various types of malignancies has also been emphasized (43–45); this may occur through regulation of the vascular endothelial growth factor (46). Therefore, down-regulation of TF expression might lead to the suppression of not only tumor invasiveness but also angiogenesis. However, although TF seems to be an attractive target for potential treatments of pancreatic ductal adenocarcinoma, we must always be concerned about the possible side effects of TF targeting therapy, including an increased bleeding tendency.

Finally, Kakkar et al. showed that the level of TF was higher in the plasma of cancer patients, including those with pancreatic cancer, than in healthy controls (47). Furthermore, the plasma concentration of TF was shown to reflect tumor TF, which was correlated with the prognosis of patients with breast cancer (48). Hence, measurement of the plasma TF concentration might be of predictive value for prognosis or selecting candidates for TF-targeting therapy, even in patients with inoperable pancreatic ductal carcinoma.

In conclusion, our present findings indicate that there is a significant association between TF expression and tumor aggressiveness in pancreatic ductal adenocarcinoma and suggest that TF expression is a useful prognostic marker in postoperative patients. In addition, TF expression may contribute to the aggressiveness of pancreatic ductal adenocarcinoma by stimulating tumor invasiveness.

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PROGNOSTIC FACTORS OF SURGICAL RESECTION IN MIDDLE AND DISTAL BILE DUCT CANCER: AN ANALYSIS OF 55 PATIENTS CONCERNING THE SIGNIFICANCE OF DUCTAL AND RADIAL MARGINS

by

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Prognostic factors of surgical resection in middle and distal bile duct cancer: An analysis of 55 patients concerning the significance of ductal and radial margins

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Background. *The surgical outcome of middle and/or distal bile duct cancer remains unsatisfactory. Although the resectional margin is known to be a predictive factor, the prognostic significance of a positive ductal margin and other radial margin has never been evaluated independently.*
Methods. *The clinicopathologic data of 55 patients who had undergone surgical resection for middle and/or distal bile duct cancer between 1987 and 2003 were reviewed retrospectively. The surgical procedures consisted of pancreatoduodenectomy in 42 patients (76%), extrahepatic bile duct resection in 8 patients (15%), major hemihepatectomy (Hx) in 3 patients (5%), and pancreatoduodenectomy plus Hx in 2 patients (4%). In all the patients, intraoperative diagnosis of the ductal margins was performed using frozen sections. Twenty-one clinicopathologic factors, including the status of the ductal margins and of other radial margins, were evaluated using univariate and multivariate analyses.*
Results. *The overall 5-year survival rate and the median survival time were 24% and 38 months, respectively. There were 4 (7%) postoperative deaths. Fifteen of the remaining 51 patients (29%) were determined to have positive hepatic-side ductal margins during operation, and 14 of them underwent additional resection of the bile duct (1.6[range, 1-3] times, on average). As a result, hepatic-side ductal margin (hm) and duodenal-side ductal margin were found to be positive in 6 and 0 patients on the final pathologic analysis, respectively. Two of the 6 patients (33%) with positive hm have developed ductal recurrence so far, but the status of hm was not found to be a significant predictor. The depth of neoplastic invasion into the bile duct wall, pancreatic invasion, radial margin, and blood transfusion were significant prognostic factors by the univariate analysis. Multivariate analysis revealed that the depth of neoplastic invasion and blood transfusion were the independent prognostic factors.*
Conclusions. *In the treatment of middle and distal bile duct cancer, it is of importance to secure a negative radial margin, although it may be less beneficial to obtain a negative hm. Surgeons should make efforts to obtain negative radial margins and to avoid blood transfusion. (Surgery 2005;137:396-402.)*

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proteins,[13] also may stimulate TLR4 and hence also cause some forms of SIRS and sepsis syndrome.

CONCLUSION

The discovery that both microbial toxins and endogenous agonists generated during infection and tissue injury activate TLRs may explain why anti-LPS therapy fails in some (or most) patients with sepsis and why SIRS mimics sepsis syndrome (Fig).[5] In patients with gram-negative infection, heparan sulfate and other endogenous agonists of TLR4 may precipitate sepsis syndrome, even if LPS is blocked. Our findings and the model emerging from these findings suggest that some attention might be usefully directed toward the breakdown products of tissues and the enzymes that generate them, in addition to microbial products, in efforts to better understand and treat sepsis syndrome and SIRS. Activation of the TLR4 may initiate a common downstream pathway for induction of sepsis and SIRS. Our findings also suggest that in some cases, "decay" of tissues causes sepsis.

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3

THE REPORTED 5-YEAR SURVIVAL RATES of surgical treatment for middle and distal bile duct cancer remain unsatisfactory (24-39%)[1-8] despite high resectability rates (up to 90%).[1-3] Numerous reports published until now[1,3-5,9-12] have suggested that a positive surgical margin may be associated with a poor prognosis. Bile duct cancer exhibits 2 major forms of tumor extension, superficial spread and submucosal infiltration,[13] which makes it difficult to obtain a cancer-free margin during resection. When a positive proximal ductal margin is detected during operation for middle and distal bile duct cancer, further exploration of the hilar bile duct is necessary to secure a negative surgical margin. However, this effort sometimes results in detection of a second positive ductal margin, necessitating subsequent reconstruction of multiple peripheral biliary ducts, which is technically demanding. Pancreatoduodenectomy combined with major hepatectomy may resolve the problem of ductal spread,[14] but this extensive operation is associated with a considerable risk of postoperative complications, such as liver failure or pancreatic fistula.[15,16] In addition, it is sometimes difficult to secure an adequate radial margin in the hepatoduodenal ligament during resection of middle bile duct cancer because of the anatomic location of the middle bile duct and the associated vascular structures. In this context, assessment of the prognostic significance of factors related to surgical resection for middle and distal bile duct cancer should take into consideration the significance of the status of the ductal margin and other radial margins independently.

We reviewed the medical records of 55 patients who had undergone surgical resection with a curative intent for middle and distal bile duct cancer during the last 17 years at our institution and analyzed the prognostic significance of the status of the surgical margins.

PATIENTS AND METHODS

From January 1987 to May 2003, 169 patients underwent resectional surgery for bile duct cancer with curative intent at the Hepato-Biliary and Pancreatic Surgery Division, Department of Surgery, National Cancer Center Hospital. One hundred and ten patients who were diagnosed to have hilar bile duct cancer and 2 patients who had apparent involvement of the entire extrahepatic biliary tree were excluded from the study. The remaining 57 patients (34%) were confirmed histologically to have bile duct cancer originating from the middle or distal bile duct. The middle bile duct was defined as the distal half of the extrahepatic bile duct, and the distal bile duct was referred to as the intrapancreatic bile duct.[17] Two of these 57 patients were excluded from the present analysis because curative resection proved impossible.

The remaining 55 patients consisted of 41 males and 14 females with a median age of 66 years (range, 43 to 81 years). The predominant sites of cancer were the distal bile duct in 26 patients (47%) and middle bile duct in 29 patients (53%). The surgical procedures consisted of standard pancreatoduodenectomy (PD) in 22 patients (40%), pylorus-preserving pancreatoduodenectomy (PPPD) in 20 patients (36%), extrahepatic bile duct resection (EHBR) in 8 patients (15%), extended right or left hemihepatectomy with extrahepatic bile duct resection (Hx) in 3 patients (5%), and extended right hemihepatectomy combined with PPPD (HPD) in 2 patients (4%). Before extended right hemihepatectomy, right portal vein embolization was performed to increase the remnant volume of the left hemiliver.[18] The surgical procedure of Hx is described in detail elsewhere.[9,11,12] Peripancreatic nodes and nodes around the hepatoduodenal ligament were dissected routinely. Intraoperative diagnosis of the ductal margins was performed using frozen sections. When a positive ductal margin was found, additional resection of the marginal bile duct was performed to the maximum extent possible.

Follow-up examinations were performed using abdominal ultrasonography, computed tomography, and measurement of the serum carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) levels every 3 to 6 months. No adjuvant chemotherapy or radiotherapy was administered in any of the patients.

Definition of the surgical margins. The hepatic-side ductal margin (*hm*), duodenal-side ductal margin (*dm*), and radial margins (*rm*) were defined in accordance with the General Rules for Surgical and Pathological Studies on Cancer of the Biliary Tract.[17] Briefly, *hm* was defined as the proximal (hepatic-side) ductal margin, and *dm* was defined as the distal (duodenal-side) ductal margin; *rm* was defined as the radial margin not including the hepatic- or duodenal-side ductal margin. When a cancer cell was detected on the cut surface of the resected margin, the margin was defined as positive.

Statistical analysis. A prognostic analysis was performed using the data from 51 patients, excluding the 4 patients who died within 1 month of the surgery. This criterion was based on the condition that the predictive value of the pathologic factors was the focus of the present study.

Table I. Univariate analysis of clinicopathologic factors in relation to the surgical resection of middle and distal bile duct cancer

Characteristic	*Number of patients (%)*	*Median survival time (months)*	*Five year survival rate (%)*	
			5 years	P *value*
Overall	51 (100)	38	26	
Predominant location of tumor				
Middle bile duct	26 (51)	39	24	.55
Distal bile duct	25 (49)	27	27	
Blood transfusion				
Not administered	30 (59)	51	31	.02
Administered	21 (41)	17	18	
Histopathological grading (G)				
pG1 or pG2	40 (78)	41	24	.34
pG3	11 (22)	17	28	
Depth of neoplastic invasion (T)				
pT1 or pT2	27 (53)	51	37	.02
pT3 or pT4	24 (47)	17	13	
Pancreatic invasion				
Absent	27 (53)	51	37	.02
Present	24 (47)	17	13	
Perineural invasion				
Absent	10 (20)	ND	58	.0503
Present	41 (80)	33	18	
Nodal involvement (N)				
Absent	24 (47)	38	42	.11
Present	27 (53)	27	16	
TNM staging				
Stage I	18 (35)	38	37	.15
Stage II, III	33 (65)	27	19	
Radial margin (*rm*)				
Negative	43 (84)	42	34	.03
Positive	7 (14)	17	0	
Proximal ductal margin (*hm*)				
Negative	45 (88)	38	27	.85
Positive	6 (12)	38	25	

Correlations with other clinicopathological factors did not reach statistical significance.

The following 21 clinicopathologic factors were analyzed by comparing subgroups divided according to each variable: age (< 60, ≥60 years), gender, serum levels of CEA (< 5, ≥5 ng/mL) and CA19-9 (< 38, ≥38 IU/L), preoperative biliary drainage, the operative procedure, blood loss during operation (<1000, ≥1000 mL), blood transfusion, clinically apparent pancreatic fistula, predominant site of the neoplasm, histopathologic grading (G category in the TNM classification of malignant neoplasms),[19] and depth of neoplastic invasion into the bile duct wall (T category), status of lymph node involvement (N category), TNM staging, status of the *hm*, status of the *dm*, status of the *rm* other than the *hm* or *dm*, status of pancreatic invasion, lymphatic invasion, venous invasion, and perineural invasion. Based on the results of the univariate analysis, a multivariate analysis was performed.

The cumulative survival rates were generated using the Kaplan-Meier method,[20] and survival analysis was performed using the log-rank test. A multivariate stepwise Cox regression analysis (backward elimination method) was performed to identify factors associated with death as a result of cancer recurrence.[21] The relationship between the location of the neoplasm and the various clinicopathologic factors was evaluated by Pearson's chi-square analysis. Statistical significance was defined as a *P* value of less than .05. The statistical analyses were performed using a statistical analysis software package (SPSS 9.0; SPSS Inc, Chicago, Ill).

RESULTS

The median survival of the 55 patients was 38 months (95% confidence interval [CI], 25-51; range, 3 months to 10.6 years). The overall survival

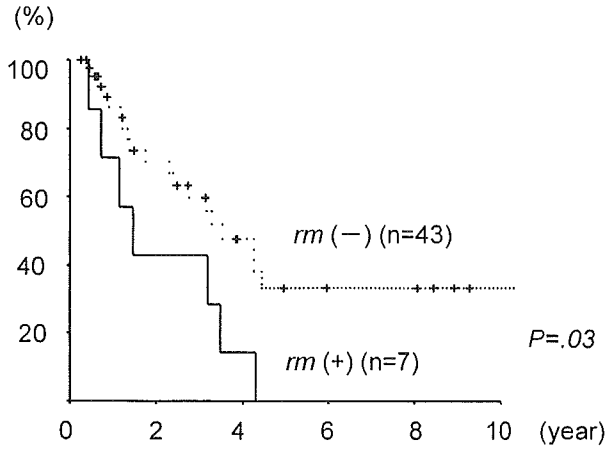

Fig 1. Kaplan-Meier survival curves after the resection of middle and distal bile duct cancer according to the status of the radial margin (*rm*; excluding the proximal ductal margin).

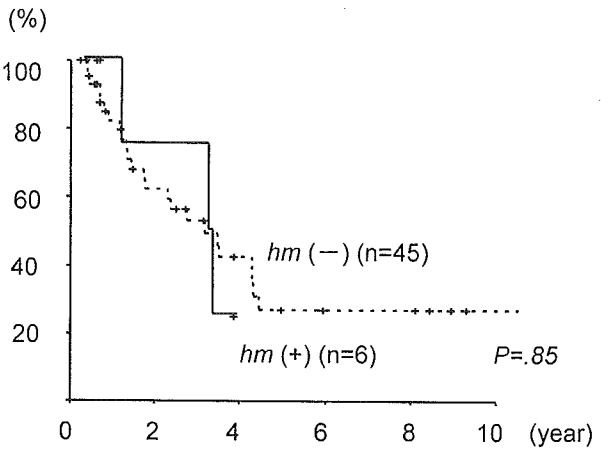

Fig 2. Kaplan-Meier survival curves after the resection of middle and distal bile duct cancer according to the status of the proximal (hepatic-side) ductal margin (*hm*).

rate was 78% at 1 year, 52% at 3 years, and 24% at 5 years. Four patients (7%) died within 1 month of the surgery because of acute heart failure (n = 2) or abdominal bleeding associated with a pancreatic fistula (n = 2). Seven patients (13%) survived for more than 5 years after the surgery. As of April 2004, the median survival period of the 51 patients who were included in the present prognostic analysis was 38 months (95% CI, 27-49; range, 3 months to 10.6 years). The overall 5-year survival rate was 26% (Table I).

Histopathologic analysis of neoplastic extension and the surgical margins. Intraoperative examination of the ductal margin was performed in all the 51 patients. Hepatic-side ductal margins were positive at the first dividing line in 15 patients. Additional resection of the peripheral hepatic-side bile duct was performed in 14 patients (mean, 1.6; range, 1-3). The remaining 1 patient did not undergo additional ductal resection because the duodenal margin was positive and additional extensive resection appeared dangerous. On the postoperative examination, *hm* proved to be positive in 4 out of 14 patients (29%) who underwent additional ductal resection. In one patient, intraoperative frozen section of the hepatic ductal status was negative; however, final diagnosis of *hm* was positive. As a result, 6 patients were found to have a positive *hm*, and the remaining 45 patients had a negative *hm*. In contrast, duodenal-side ductal margins were positive at the first dividing line in 3 patients. Additional resection of the intrapancreatic bile duct was performed 1 or 2 times, and *dm* proved to be negative in all of them. Seven patients had positive *rm*, the remaining 43 patients had negative *rm*, and one patient was excluded because the assessment of the *rm* status on the surgical specimen was very difficult (Table I). Hepatic-side ductal recurrence was found in 2 of the 6 patients with a positive *hm* (33%) and 4 of the 45 patients with a negative *hm* (9%), with a mean follow-up of 2.5 (0.6-3.2) years, but there was no significant difference in recurrence between the patients with positive *hm* and negative *hm* ($P = .08$).

Table II. Multivariate analysis for independent risk factors

Variable	*β*	*Risk ratio*	*95% CI*	P *value*
pT factor	0.774	2.168	0.958-4.905	.063
Blood transfusion	0.708	2.030	0.905-4.555	.086

Univariate and multivariate analyses. The depth of neoplastic invasion (T category), pancreatic invasion, status of the *rm* (Fig 1), and blood transfusion were found to be significant prognostic factors by the univariate analysis (Table I).

Other clinical factors, such as the predominant location of the neoplasm (middle or distal bile duct), status of the *hm* (Fig 2), and the TNM stage were not found to be significant predictors on prognosis. Patients with positive nodal metastasis or perineural invasion had worse survival than patients with a negative nodal status or perineural invasion, but these differences did not reach statistical significance (Table I). A multivariate analysis was then performed using the 6 clinicopathologic factors, which proved to be significant in the univariate analysis or other marginal predictors,

Table III. A chi-square analysis of location of the neoplasm and clinicopathologic characteristics

	Distal bile duct (n = 25)	*Middle bile duct (n = 26)*	P *value*
Surgical procedures			
PD or PPPD or HPD	25	15	.003
EHBR	0	7	
Hx	0	3	
Blood transfusion			
Not administered	13	17	.33
Administered	12	9	
Depth of neoplastic invasion			
pT1 or pT2	7	20	< .0001
pT3 or pT4	18	6	
Pancreatic invasion			
Absent	7	20	< .0001
Lymph node metastasis			
Absent	12	12	.89
Present	13	14	
TNM stage			
I	5	13	.077
II	19	12	
III	1	1	
Radial margin (*rm*)			
Negative	22	21	.68
Hepatic-side ductal margin (*hm*)			
Negative	24	21	.09

PD, Pancreatoduodenectomy; *PPPD*, pylorus-preserving pancreatoduodenectomy; *HPD*, extended hemihepatectomy plus PD; *EHBR*, extrahepatic bile duct resection; *Hx*, extended right or left hemihepatectomy combined with extrahepatic duct resection.

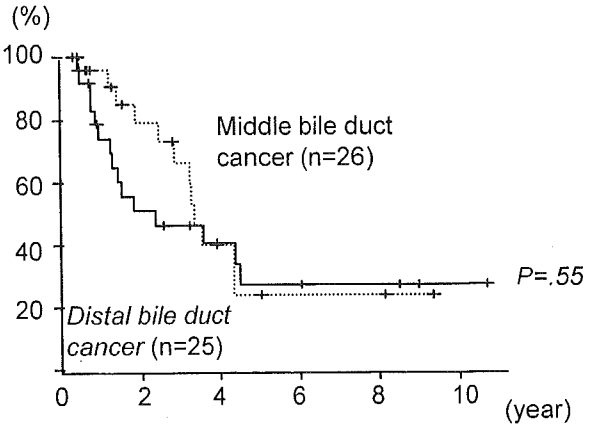

Fig 3. Kaplan-Meier survival curves after the resection of middle and distal bile duct cancer.

that is, the presence of lymph node metastasis and perineural invasion. The results of the multivariate analysis suggested that the depth of neoplastic invasion and blood transfusion were independent predictors of prognosis (Table II).

Location of neoplasm and other clinicopathologic factors. The depth of neoplastic invasion and pancreatic invasion was deeper in patients with distal bile duct cancer than in patients with middle bile duct cancer (Table III); however, no survival difference was observed between the two groups (Fig 3).

DISCUSSION

We found that the depth of neoplastic invasion including pancreatic invasion, blood transfusion, and status of the *rm*, but not that of the *hm*, were significant predictors in the surgical treatment of middle and/or distal bile duct cancer. Univariate analysis revealed that factors reflecting extension of the primary neoplasm, such as the depth of neoplastic invasion, pancreatic invasion, lymph node metastasis, and perineural invasion, were of prognostic importance. These results indicated that local or lymphatic invasion of the bile duct cancer largely controls the prognosis of the patients. Thus, factors related to the operative interventions, except for the status of the *rm* and blood transfusion, may have less influence on the survival than the biologic behavior of the primary tumor itself.

Ductal recurrence was found in 33% (n = 2 of 6) of the patients with a cancer-positive ductal margin. In a study examining 62 patients who underwent surgical treatment for hilar bile duct cancer, Sakamoto et al[9] observed no anastomotic recurrence in patients who had a negative *hm*, while 16% of patients with a positive *hm* developed ductal recurrence. Although we concede that the status of the hepatic ductal margin may have some clinical significance, we consider that it is of great importance to obtain a negative *rm* against vertical (radial) tumor infiltration; it appears to be relatively less beneficial to obtain a negative *hm* against longitudinal extension. In other words, practically, when *rm* is apparently positive for cancer, for example, in the hepatoduodenal ligament, additional resection of the peripheral bile would not be expected to improve the survival. The importance of an adequate resectional margin has been advocated in numerous series on bile duct cancer.[1,3-5,9-12] But the status of *rm* and *hm* has never been discussed individually, to the best of our knowledge, although our series is too small to make a solid, statistically justified statement. Ductal extension of bile duct cancer can be classified into superficial spread along the mucosal lining or submucosal infiltration,[13] the significance of both of which should be further evaluated in the future.

Although several authors have reported that middle bile duct cancer has a worse prognosis than hilar or distal bile duct cancer,[22-24] others do not concur.[3,4] Our clinical impression was that middle bile duct cancer is more serious than distal bile duct cancer because (1) middle bile duct cancer is nearer to the hepatic hilum, which makes it difficult to obtain a negative ductal margin during resection; (2) middle bile duct cancer can easily infiltrate the hepatoduodenal ligament, occasionally invade the vascular structures, and easily extend to the serosal surface; (3) selection of the appropriate surgical procedure is confusing in the case of middle bile duct cancer, but for distal bile duct cancer, either PD or PPPD is the inevitable and standard procedure. In our experience, the surgical procedure for middle bile duct cancer (EHBR, PD, PPPD, Hx, or HPD) has been determined according to the location of the tumor and the preference of the surgeon. Contrary to our expectations, neoplastic invasion was deeper in distal bile duct cancer than in middle bile duct cancer, primarily due to pancreatic invasion (Table III); nevertheless, there were no survival differences between patients with middle and distal bile duct cancers (Fig 3). These results may be related to the difference in resectability rates between the two groups and the size of our samples, which affect the power of the statistical analyses. In practice, the surgical procedure for middle and distal bile duct cancer should be determined primarily by focusing on the *rm*, not the ductal margin, and taking into consideration the need to avoid blood transfusion. When the *rm* is apparently positive for cancer, HPD to secure a negative ductal margin would not appear to offer long-term survival. Although Miyagawa et al[14] reported favorable results of HPD using preoperative biliary drainage and portal vein embolization technique in patients with biliary malignancy, the reported mortality of HPD still remains high,[15,16] and performance of HPD to secure a negative ductal margin should be considered very carefully.

It has been reported that blood transfusion is associated with a poor outcome in multiple other malignancies, such as colorectal,[25] breast,[26] gastric,[27] periampullary,[7,28] and hepatocellular carcinoma.[29] The immunosuppressive effect of transfusion has been proposed as a possible reason.[30] Park et al observed a poorer outcome in transfused patients than in nontransfused patients among patients with distal bile duct cancer (n = 141); however, the difference did not reach statistical significance.[28] Blood transfusion might reflect the extensiveness of the operation, and an extensive surgery, not blood transfusion itself, may be related to a poor outcome. However, the quantitative analysis of surgical extensiveness is difficult, and the essential cause remains unclear. The upshot was the conclusion that all efforts should therefore be used to avoid whole-blood transfusion.

In most reported series, the nodal status has been described as an important prognostic factor in the treatment of hilar, middle, and distal bile duct cancer.[1-9,11,12,23-24,31] In our analysis, node-positive patients had worse survival than node-negative patients, but the difference did not reach statistical significance. This finding may be explained in part by the strong impact of another factor (eg, depth of invasion or positive *rm*) on survival, concealing the prognostic influence of nodal involvement, and in part by the small size of the sample analyzed, limiting the power of our statistical analyses. Interestingly, in a previous study of 58 patients with hilar bile duct cancer, we reported that regional lymph node metastasis was not a significant prognostic factor.[9] We supposed that this result was due to systematic nodal dissection, which may have overcome the impact of regional nodal metastases. On the other hand, the clinical significance of nodal dissection still remains debatable. Yoshida et al[5] insisted on the necessity of a radical extended lymphadenectomy and adjuvant chemotherapy for the treatment of distal bile duct cancer in a retrospective study of 27 patients. On the other hand, Yeo et al[32] recently described the negative impact of extended lymphadenectomy for periampullary adenocarcinoma based on a prospective randomized trial. However, only 16% of the subject patients had distal bile duct cancer, and more than half of the patients had pancreatic cancer. The clinical significance of lymphadenectomy for the treatment of bile duct cancer should be further evaluated in a multicenter study including a large number of patients.

In conclusion, the depth of neoplastic invasion including pancreatic invasion and blood transfusion were important prognostic factors in the surgical treatment of middle and distal bile duct cancer. It is of great importance to obtain a negative *rm* against radial or vertical tumor-infiltration, although it may be less beneficial to obtain a negative *hm* against longitudinal extension. Surgeons should thus spare no effort to secure a negative *rm* and avoid blood transfusion.

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