Prognostic Significance of Tissue Factor in Pancreatic Ductal Adenocarcinoma

Nobuhiro Nitobi,1,5 Yoshiinori Ino,1 Yukihiro Nakanishi,1 Tesshi Yamada,2 Kazufumi Honda,2 Kazuyoshi Yanagihara,3 Tomoo Kosuge,4 Yae Kanai,1 Masaki Kitajima,5 and Setsuo Hirohashi1

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
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 (IgG1, 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 IgG1k 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 Ca2+/Mg2+-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 beads (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 2X 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 tryptic 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-β-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-44PE (scirrhous gastric carcinoma cell line; Immunogen). Forty micrograms of whole cell lysate were applied to each lane and separated by SDS-PAGE under reducing conditions. 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 immunoprecipitations 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 pancreatectoduodenectomy in 20 patients, and pancreatoduodenectomy 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, overnight at 4°C, then incubated sequentially with biotinylated goat anti-mouse IgG and avidin-FITC (Vector Laboratories) under fluorescence microscopy. 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 (siRNA489) and 653 to 673 (siRNA653), numbered from the start codon, and the siRNAs were purchased from Dharmacon, Inc. (Lafayette, CO). Control experiments were done using two unrelated siRNAs, siRNA489 was Cy3 labeled siRNA directed against Luciferase mRNA (Dharmacon) and siRNA653 (mock) was Nonspecific Control Duplex X (Dharmacon) sequence of the latter (5’-NNATTCATACTACCGTGAC-3’) was confirmed to have no homology with any known mRNA by a BLAST search; however, it had the same GC content as siRNA489.

At first, we examined transfection efficiencies among the TF-positive cell lines BxPC-3, STI 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 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.

www.aacrjournals.org 2533 Clin Cancer Res 2005;11 (7) April 1, 2005
Downloaded from clincancerres.aacrjournals.org on May 31, 2017. © 2005 American Association for Cancer Research.
Table 1. Association between TF expression and clinicopathologic variables

<table>
<thead>
<tr>
<th>TF expression</th>
<th>Low TF (25%, n = 63)</th>
<th>Weak TF (0%, n = 50)</th>
<th>High TF (≥ 25%, n = 50)</th>
<th>P value (Low vs. High)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variables</td>
<td>Negative TF (0%, n = 13)</td>
<td>Weak TF (0%, n = 50)</td>
<td>High TF (≥ 25%, n = 50)</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>&lt;65</td>
<td>8</td>
<td>26</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>≥65</td>
<td>5</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>7</td>
<td>31</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>6</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>Extent of the primary tumor spread</td>
<td>pT1</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>pT2</td>
<td>3</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>pT3</td>
<td>4</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>pT4</td>
<td>3</td>
<td>18</td>
<td>29</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>pN0</td>
<td>2</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>pN1a</td>
<td>10</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>pN1b</td>
<td>1</td>
<td>23</td>
<td>34</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>pM0</td>
<td>9</td>
<td>42</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>pM1</td>
<td>4</td>
<td>8</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>M1 (LYM)(^c)</td>
<td>2</td>
<td>7</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>M1 (HEP)(^c)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>M1 (PER)(^c)</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Stage</td>
<td>I</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>5</td>
<td>23</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>IVA</td>
<td>3</td>
<td>13</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>IVB</td>
<td>4</td>
<td>8</td>
<td>22</td>
</tr>
<tr>
<td>Histopathologic tumor grade</td>
<td>G1</td>
<td>7</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>5</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>G3</td>
<td>1</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Lymphatic invasion(^b)</td>
<td>Negative</td>
<td>4</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>9</td>
<td>45</td>
<td>42</td>
</tr>
<tr>
<td>Vascular invasion(^b)</td>
<td>Negative</td>
<td>8</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>5</td>
<td>36</td>
<td>38</td>
</tr>
<tr>
<td>Perineural invasion(^b)</td>
<td>Negative</td>
<td>2</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>11</td>
<td>44</td>
<td>46</td>
</tr>
<tr>
<td>Growth pattern(^b)</td>
<td>Expansive + intermediate</td>
<td>9</td>
<td>30</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Infiltrative</td>
<td>4</td>
<td>20</td>
<td>29</td>
</tr>
<tr>
<td>Surgical margin</td>
<td>Negative</td>
<td>10</td>
<td>34</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>3</td>
<td>16</td>
<td>20</td>
</tr>
</tbody>
</table>

\(^b\)Significant.  
\(^c\)LYM, lymphatic metastasis; HEP, hepatic metastasis; PER, peritoneal metastasis.
\(^\dagger\)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, Becton Dickinson InVitro 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% CO2/95% 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 χ2 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 immunoprecipitation 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
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}$, has been ascertained under fluorescence microscopy by immunocytochemistry (Fig. 4A, middle and bottom). Densitometric analysis (Fig.4B) and invasion assays (Fig.4C) showed that transfection with either siRNA$_{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
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 G3 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 Duke’s 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. siRNA489 and siRNA653 are directed against TF. Control experiments were done with a Cy3-labeled siRNA directed against an unrelated mRNA (Luciferase; siRNAnc; used as a mock-transfectant). Transfection efficiency was confirmed by using Cy3-labeled siRNAnc 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 siRNA653 against TF (A, middle), whereas control siRNAnc 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 siRNA489 or siRNA653 significantly reduced TF compared with mock-transfected cells (siRNAnc), whereas transfection with a siRNA targeted to an unrelated mRNA (siRNAnc) 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 to 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), resveratrol (40), vitamin D3 (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.

Acknowledgments

The authors are grateful to A. Miura and F. Kaiya for their expert technical assistance. N. Nitiri is a recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research in Japan.
References
Prognostic Significance of Tissue Factor in Pancreatic Ductal Adenocarcinoma

Nobuhiro Nitori, Yoshinori Ino, Yukihiro Nakanishi, et al.