Matrigel Invasion Chamber (8 μm pore size; BD Biosciences) and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 4 h. After transfected with each siRNA (20 nM final concentration; see Supplementary Methods for detail), cells were incubated for 32 h in appropriated medium (DMEM or RPMI 1640 with FBS), noninvading cells on the upper chamber were scraped with a cotton swab. The relative number of invading cells that penetrated the Matrigel-coated membrane were quantified by colorimetric cell proliferation assay using the Cell Counting Kit-8 (DOJINDO, Kumamoto, Japan) according to the manufacturer's instructions (Chen *et al*., 2006; Shida *et al*., 2006). All of these experiments were carried out in quadruplicate for three times, independently.

Apoptosis assay
A total of 2×10^4 MIA PaCa II and AsPC-1 cells were plated in 96-well plates, incubated for 24 h and transfected with siRNA (20 nM final concentration). After 24 h, apoptotic cells were stained using the APOPercentage Kit (Biocolor Ltd, Newtownabbey, Northern Ireland, UK) according to the manufacturer's instructions (Fadok *et al*., 1992; Mutaguchi *et al*., 2003). Purple-red stained cells were identified as apoptotic cells, and counted manually in each four different random positions in blinded fashion. All experiments were carried out for three times.

Caspase-3 assay
The caspase-3 activity assay was carried out according to the manufacturer's protocol (BD ApoAlert Caspase-3 Colorimetric Assay Kit, BD Biosciences Clontech, Mountain View, CA, USA). In brief, a total of 2×10^6 MIA PaCa II cells were plated in six-well plate, transfected with siRNA (20 nM final concentration) and harvested at 24 h after transfection. After counting cell number, cells were centrifuged and resuspended in 50 μl of chilled Cell Lysis Buffer following incubation for 10 min on ice. Cell lysates were mixed with equal amount of with Reaction Buffer/DTT Mix containing 50 μM DEVD-pNA (*p*-nitroaniline) substrate and incubated at 37°C for 2 h. Enzyme-catalysed release of pNA was monitored using a Bio-Rad Microplate Reader at 405 nm wavelength. These experiments were carried out for three times.

Immunofluorescence
The cultured cells transfected with siRNA were fixed on slide glasses with acetone for 10 min at 4°C. After three washes with PBS, the nonspecific binding of antibodies was blocked with blocking buffer (10% FBS/PBS) for 1 h. Samples were incubated for 1 h with mouse anti-human ApoC-1 monoclonal antibody (CHEMICON International) diluted 1:500. After a wash with PBS, samples were incubated with 1:3000 diluted Alexa Fluor 568-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes, Eugene, OR, USA) for 1 h. DNA was counterstained with DAPI III Counterstain (Vysis, Abbott Park, IL, USA). Samples were observed with a fluorescence microscope (Leica QFISH; Leica Microsystems, Tokyo, Japan).

Statistical analysis
Statistical analyses were carried out using the appropriate tests as indicated. *P*-values <0.05 were considered statistically significant.

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Specific Expression of Endoglin (CD105) in Endothelial Cells of Intratumoral Blood and Lymphatic Vessels in Pancreatic Cancer

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Objectives: Endoglin, a component of the transforming growth factor β receptor expressed in embryonic vascular endothelial cells, is expressed in vascular endothelial cells in several types of cancer tissues and is involved in tumor angiogenesis. The aim of this study was to analyze the expression pattern of endoglin in pancreatic cancer and assess the involvement of this molecule in cancer progression.

Methods: Pancreatic cancer and adjacent normal tissues obtained from 36 patients were subjected to immunostaining with anti-endoglin antibody, and the microvessel density (MVD) was assessed based on the number of endoglin-positive vessels.

Results: Endoglin was expressed in endothelial cells of small capillary-like vessels in pancreatic cancer tissues from all 36 patients, and lymphatic endothelial cells in the tumors also expressed endoglin. In contrast, endothelial cells of vascular and lymphatic vessels in normal pancreatic tissue did not express endoglin. Patients with a higher MVD of endoglin-positive vessels had shorter disease-free and overall survival.

Conclusions: Endoglin is specifically expressed in endothelial cells of small vascular and lymphatic vessels in cancer tissues. The MVD of endoglin-positive vessels may also be a useful prognostic marker in pancreatic cancer patients.

Key Words: endoglin (CD105), endothelial cell, pancreatic cancer, angiogenesis, lymphangiogenesis

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Pancreatic cancer has a high mortality rate, and the prognosis remains poor despite recent advances in treatment, with a 5-year survival rate of less than 5%. The poor prognosis is in part due to the high chemoresistance of most pancreatic malignancies.[1] Therefore, there is an urgent need for improved understanding of the biological mechanisms that regulate the progression of pancreatic cancer, such that new therapeutic strategies can be developed for this disease.

It is widely accepted that angiogenesis is involved in cancer growth and metastasis. In pancreatic cancer, overexpression of angiogenic factors such as acidic fibroblast growth factor, basic fibroblast growth factor, and vascular endothelial growth factor (VEGF)[2–5] correlates with disease progression. Inhibition of signals evoked by these angiogenic factors is effective in suppressing tumor progression in animal models of pancreatic cancer,[6–8] and several antiangiogenic agents are in clinical use. For example, bevacizumab, a monoclonal antibody against VEGF, has shown encouraging results in treatment of pancreatic cancer in phase II study.[9] However, administration of bevacizumab may increase the risk of serious adverse events such as arterial thrombosis and hypertension due to the wide expression and multiple functions of VEGF in noncancerous tissues.[9] This indicates the importance of understanding the specific mechanisms regulating tumor angiogenesis.

Tumor vessels are developed by sprouting or intussusception from preexisting vessels through stimulation by angiogenic factors. Tumor vasculature has different characteristics from those of normal tissues: tumor vessels are tortuous, dilated, uneven in diameter, excessively branched, and leaky with numerous openings.[10] These characteristics indicate expression of specific proteins in tumor vessels, and such molecules may be candidates as targets of tumor-specific antiangiogenic therapy.[11]

Endoglin (CD105) is a transmembrane glycoprotein and a component of the transforming growth factor (TGF)-β receptor system in endothelial cells.[12] Endoglin promotes proliferation of endothelial cells in vitro by modulating TGF-β signaling,[13] and mice lacking endoglin die at the midgestation stage as a result of defective vascular development,[14] suggesting that endoglin has roles in endothelial proliferation and vascular development, that is, angiogenesis and vasculogenesis. Several groups have shown that endoglin is mainly expressed in peritumoral and intratumoral blood vessels, which were small and probably immature, in brain, prostate, breast, colorectal, and hepatic cancers.[15–18] The specific expression pattern of endoglin in tumoral vessels indicates that it may be a target molecule in antiangiogenic therapy in cancer,[19] and Tan et al[20] have investigated this possibility in an animal model using immunotherapy.

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In this study, we analyzed the expression pattern of endoglin in vessels of pancreatic cancer tissues using immunohistochemistry and determined whether the density of endoglin-expressing vessels correlates with tumor malignancy based on clinical outcome. According to these analyses, we discuss the possible use of endoglin as a new target for pancreatic cancer therapy.

MATERIALS AND METHODS

Tissues

Cancer tissues and adjacent normal tissues were obtained from 36 patients with pancreatic cancer who underwent pancreatectomy in the Department of General Surgery, Chiba University Hospital, Japan, between April 2001 and March 2004. All patients were histologically diagnosed with primary invasive pancreatic ductal carcinoma. Only patients with complete patient records, including sex, age, clinical manifestation, tumor size and localization, and presence of metastasis were included in the study (Table 1). Written informed consent was obtained from each patient for approval of use of surgical specimens.

Immunohistochemistry

Tissues were fixed in formalin, embedded in paraffin, and sectioned at 4 μm using standard procedures. Serial sections were immunostained using anti-platelet endothelial cell adhesion molecule (PECAM), anti-endoglin antibody, and anti–D2-40 antibody and also subjected to hematoxylin and eosin (HE) staining. After deparaffinization and rehydration, sections were treated with boiling citrate buffer (10 mM sodium citrate, pH 6.0) in a microwave 5 times for 5 minutes each, and endogenous peroxidase activity was then blocked by immersing the sections in 0.3% H_2O_2 in methanol for 30 minutes. After washing in phosphate-buffered saline, sections were reacted with the following primary antibodies at 4°C overnight: rabbit polyclonal antihuman CD105/endoglin (used as obtained; Lab Vision, Fremont, Calif), mouse monoclonal anti-CD31/PECAM (1:40 dilution; DAKO, Denmark), and mouse monoclonal antihuman D2-40 (dilution 1:100; DAKO, Denmark). After washing in phosphate-buffered saline, the primary antibodies were detected with the LSAB2 System, Peroxidase (DAKO, Denmark), using diaminobenzidine as the chromogen (DAKO, Carpinteria, Calif). Sections were counterstained with hematoxylin.

TABLE 1. Characteristics of Patients With Resected Pancreatic Cancer

	Total	Low Endoglin-MVD (n = 18)	High Endoglin-MVD (n = 18)	
Age (mean ± SD), yr	62.9 ± 9.5	63.2 ± 10.0	62.1 ± 9.2	NS
Sex				
Male	21	9	12	NS
Female	15	9	6	
TNM classification stage				
IA	2	2	0	NS
IB	0	0	0	
IIA	5	0	5	
IIB	22	12	10	
III	1	1	0	
IV	6	3	3	
Histology				
Tubular adenocarcinomas				
Well differentiated	0	0	0	NS
Moderately differentiated	24	14	10	
Poorly differentiated	10	3	7	
Adenosquamous carcinoma	1	1	0	
Anaplastic carcinoma	1	0	1	
Lymph node metastasis				
Negative	5	2	3	NS
Positive	31	16	15	

NS indicates not significant.

Quantification of Microvessel Density

Intratumoral microvessels were highlighted by immunostaining of endothelial cells for endoglin or PECAM. After comparison with HE-stained sequential sections, the microvessel density (MVD) in the tumor-bearing area was quantified. Large and small microvessels and single brown immunostained endothelial cells separated from adjacent microvessels and stromal structures were included in the microvessel count, as previously described.[21] Areas of higher MVD were identified, and vessel counts per field (100× magnification) were assessed by 2 investigators (H.Y. and S.K.) without knowledge of patient outcome. Multiple fields were analyzed, and the mean of the 2 fields with the highest counts was taken as the final count.

The relationship between endoglin-MVD and PECAM-MVD was examined using Pearson correlation. Patients were divided into groups using the median values for endoglin-MVD (low and high endoglin-MVD groups) or PECAM-MVD (low and high PECAM-MVD groups), and relationships with patient characteristics were analyzed by χ^2 test and Student t test. Survival curves were calculated using the Kaplan-Meier method and analyzed by log-rank test. Statistical significance was defined as $P < 0.05$.

RESULTS

Endoglin Is Specifically Expressed in Endothelial Cells of Peritumoral and Intratumoral Capillaries of Pancreatic Cancers

The expression pattern of endoglin was examined by immunostaining of surgically resected tissues from 36 patients (Table 1) with invasive ductal carcinoma. Serial sections from each tissue sample were also subjected to HE staining and immunostaining with PECAM (CD31) as a marker for vascular endothelial cells.[22]

It is already reported that endoglin is expressed in endothelial cells of blood vessels in tumor tissues of breast cancer.[15] First, we examined immunostaining of the breast cancer tissue as a positive control. Anti-PECAM antibody stained endothelial cells of blood vessels with thick wall [Figs. 1A (HE

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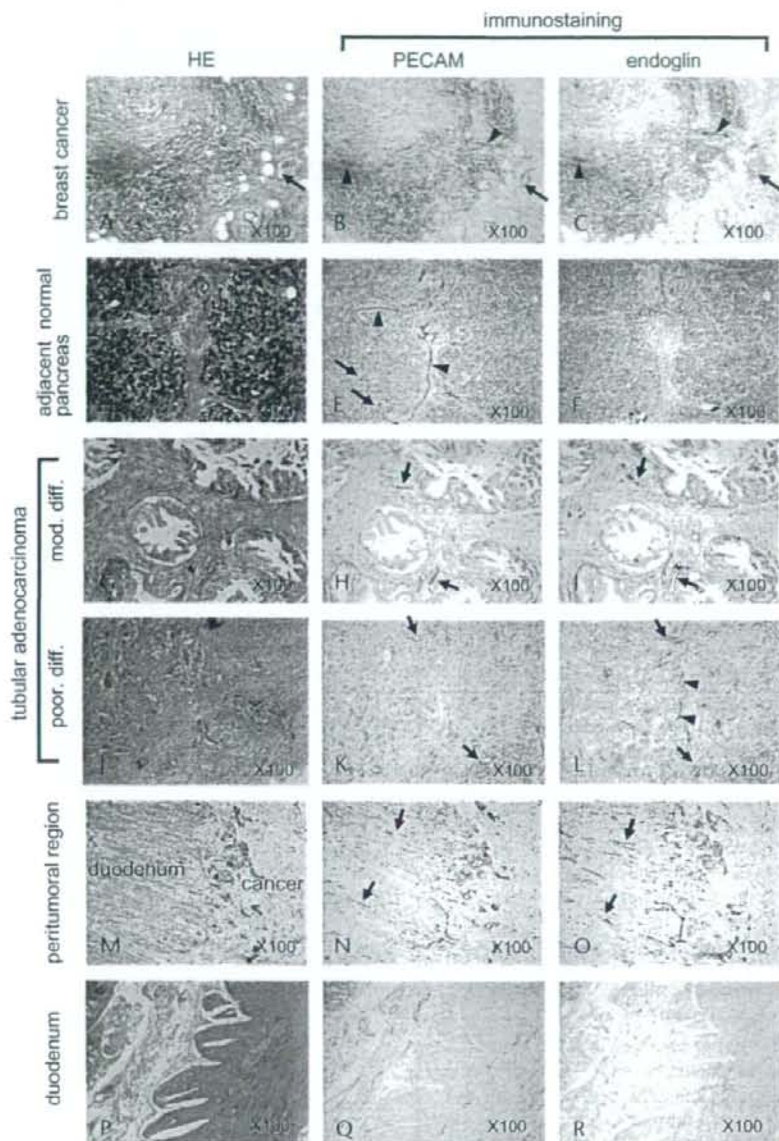

FIGURE 1. Endoglin is expressed in peritumoral and intratumoral vessels in pancreatic cancer. Sequential sections were subjected to HE staining (A, D, G, J, M, P) and immunostaining with anti-PECAM antibody (B, E, H, K, N, Q) and anti-endoglin antibody (C, F, I, L, O, R). A–C, Endothelial cells of blood vessels in breast cancer were stained with anti-PECAM and endoglin antibodies, as positive control staining. D–F, In normal pancreatic tissue, endothelial cells of intralobular (arrows) and interlobular (arrow heads) lobular vessels, marked with PECAM staining, did not express endoglin. G–L, In pancreatic cancer tissues of moderately differentiated (mod. diff.) (G–I) and poorly differentiated (poor. diff.) (J–L) tubular adenocarcinoma, endothelial cells of small capillary-like vessels in the tumor were stained with both anti-PECAM and anti-endoglin antibodies (arrows). Some vessel structures were endoglin-positive but PECAM-negative (arrow heads). M–O, In duodenal tissue with pancreatic cancer invasion, endothelial cells of vessels in the peritumoral region also expressed PECAM and endoglin (arrows). P–R, In normal duodenum, only few endoglin-positive vessels were present in the muscular layer.

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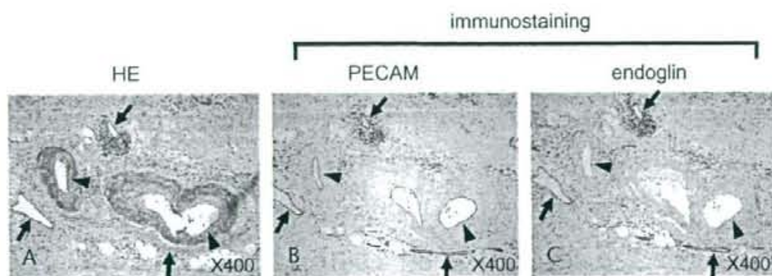

FIGURE 2. Endoglin is expressed only in endothelial cells of small vessels with thin walls and not in arterial vessels. Sequential sections of pancreatic cancer tissues were subjected to HE staining (A) and immunostaining with anti-PECAM (B) and anti-endoglin (C) antibodies. Endothelial cells of arteries with thick tunica media walls did not express endoglin (arrow heads), whereas endothelial cells of small capillary-like vessels showed expression of endoglin (arrows).

staining), B (PECAM); arrow] as well as small capillary-like vessels [Figs. 1A (HE staining), B (PECAM); arrowheads] in breast cancer tissues. These endothelial cells, especially those of capillary-like small vessels (Fig. 1C; arrowheads), were also stained by anti-endoglin antibody. These results confirmed the immunoreactivity of antibodies against PECAM and endoglin with endothelial cells of blood vessels.

In normal pancreatic tissues (Fig. 1D), anti-PECAM antibody stained endothelial cells of small intralobular capillaries (Fig. 1E, arrows) and vessels in interlobular connective tissues (Fig. 1E, arrowheads), but anti-endoglin antibody failed to stain these vessels, showing that neither intralobular nor interlobular vascular vessels expressed endoglin (Fig. 1F). In cancer tissues (Fig. 1G), endothelial cells of intratumoral small vessels stained by anti-PECAM antibody (Fig. 1H, arrows) were also positive for endoglin (Fig. 1I, arrows), and endoglin-positive capillary structures were found in cancer tissues in all 36 cases, regardless of histological characteristics or stage.

Results obtained for moderately and poorly differentiated tubular adenocarcinomas are shown in Figures 1G–I and J–L, respectively. Both tissue types included many intratumoral vessels with endothelial cells positive for PECAM and endoglin (Figs. 1H, I, K, and L; arrows). Endoglin-positive capillaries were also present in the peritumoral area. Sequential sections of the peritumoral area at a site of pancreatic cancer invasion of the duodenum are shown in Figs. 1M–O, with staining for HE, PECAM, and endoglin, respectively. PECAM staining occurred in small vessels in the muscular layer of the duodenum (Fig. 1N, arrow), and these capillaries were also endoglin-positive (Fig. 1O, arrow); in contrast, the muscle layer of a normal duodenum (Figs. 1P–R) contained few endoglin-positive capillaries (Fig. 1R). These data show that endothelial cells of intrapancreatic and peripancreatic tumoral small vessels express endoglin, in contrast to vessels in normal pancreatic tissues.

PECAM-positive and endoglin-negative vessels were also found in the intratumoral area (Figs. 2B, C; arrow heads). These vessels usually had larger diameters than endoglin-positive vessels (Figs. 2B, C; arrow) and were surrounded by a thick tunica media, as seen in HE staining (Fig. 2A), suggesting that they were arteries. This observation shows that endoglin is expressed only in endothelial cells of small vessels, such as capillaries, in pancreatic tumors.

Endoglin Is Expressed in Endothelial Cells of Lymphatic Vessels

With careful observation, we realized that there were a few cells that expressed endoglin, but did not express PECAM, in the tumor (Figs. 1K, L, arrowheads). These cells formed vessel-like structures, and the staining results suggested that these structures might be lymphatic vessels. To examine this possibility, sequential sections were immunostained

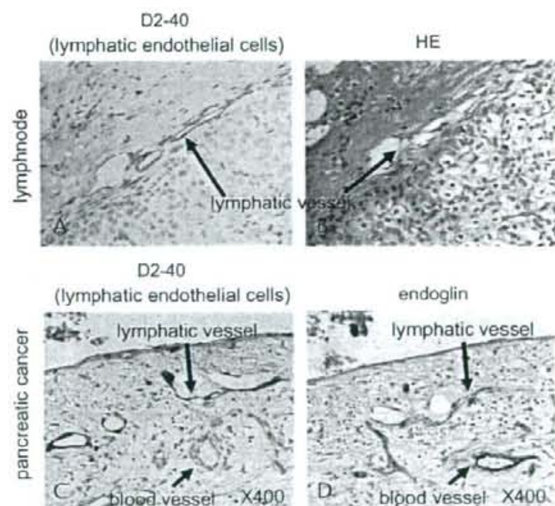

FIGURE 3. Endoglin is expressed in small lymphatic vessels in pancreatic cancer tissue. A and B, Lymphatic vessels in lymph node are stained with anti–D2-40 antibody (A). B, HE staining of the sequential section. C and D, Immunostaining with D2-40 (C) and anti-endoglin (D) antibodies. The endothelial cells of lymphatic vessels, which are stained with D2-40 antibody, also expressed endoglin, whereas endoglin-positive and D2-40-negative endothelial cells were present in blood vessels.

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with anti-endoglin antibody and a second antibody, anti–D2-40 antibody, that identifies a 40-kD O-linked sialoglycoprotein and specifically reacts with lymphatic endothelium.[23] Figure 3A shows immunostaining of lymph node using this antibody. Anti–D2-40 antibody clearly stained endothelial cells of lymphatic vessels existing in lymph node [compare with HE staining (Fig. 3B)]. Immunostaining with D2-40 antibody stained lymphatic vessel structures in pancreatic cancer tissues (Fig. 3C), and these vessels were also stained with anti-endoglin antibody (Fig. 3D). In contrast, the endothelial cells of blood vessels were endoglin-positive but D2-40–negative (Figs. 3C, D). These data show that endoglin is expressed in endothelial cells of intratumoral lymphatic vessels in pancreatic cancer, in addition to its expression in blood vessels.

MVD of Endoglin-Positive Vessels Is a Prognostic Predictor in Pancreatic Cancer

Increased angiogenesis in tumors is correlated with high malignancy in several types of cancer, and consequently, a high density of microblood vessels in the tumor is correlated with prognosis. To examine whether the MVD in pancreatic cancer tissues also correlated with prognosis, we determined the MVD in endoglin-positive (endoglin-MVD) and PECAM-positive (PECAM-MVD) vessels. There was a strong correlation between endoglin-MVD and PECAM-MVD, as

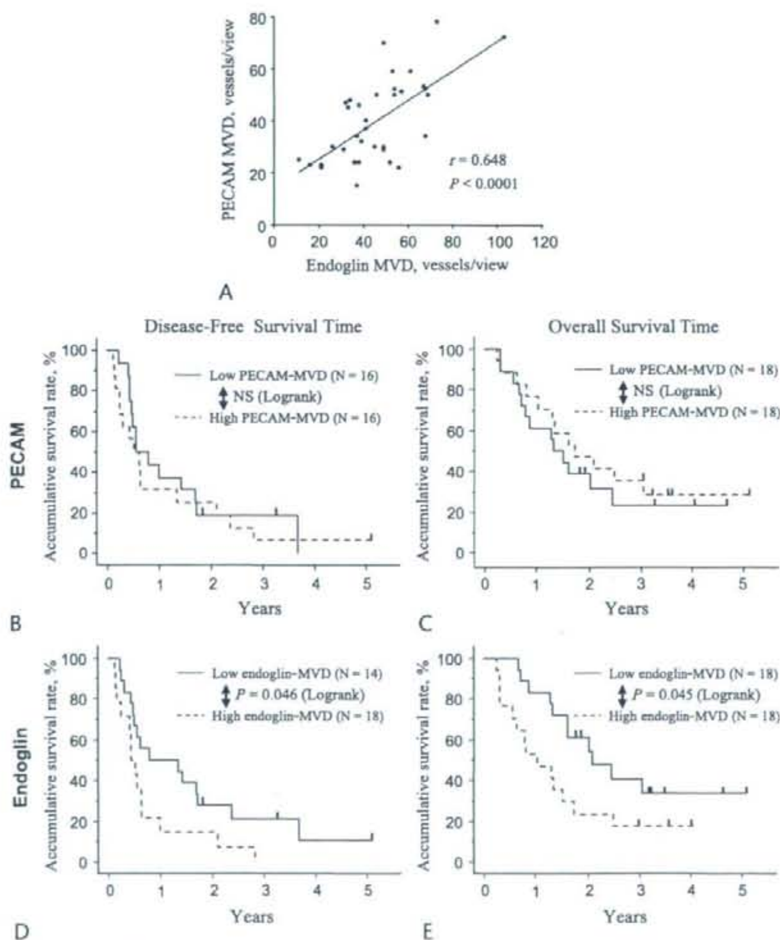

FIGURE 4. Microvessel density (MVD) assessed by endoglin immunostaining (endoglin-MVD) is significantly correlated with disease-free and overall survival, but MVD assessed by PECAM immunostaining (PECAM-MVD) does not show these relationships. A, Endoglin-MVD and PECAM-MVD were strongly correlated ($P < 0.0001$). B and C, Kaplan-Meier analysis of disease-free and overall survival in groups with low and high PECAM-MVD, based on the median value (35.5 vessels per field) in 36 pancreatic cancer patients. NS indicates not significant. D and E, Kaplan-Meier analysis of disease-free and overall survival in groups with low and high endoglin-MVD, based on the median value (45.5 vessels per field) in the same 36 patients.

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expected (Fig. 4A). The patients were divided into 2 groups using the median endoglin-MVD (>45.5 and <45.5 vessels per fields, n = 18 in each group) or the median PECAM-MVD (>35.5 and <35.5 vessels per fields, n = 18 in each group). There were no statistically significant differences in age, sex, tumor stage, tumor histology, and presence of lymph node metastasis between the high and low endoglin-MVD groups (Table 1) or between the high and low PECAM-MVD groups (data not shown). There were no significant differences between the low and high PECAM-MVD groups in disease-free (2-year disease-free survival rate, 18.8% and 25.0%, respectively) and overall survival (2-year overall survival rate, 47.1% and 38.9%, respectively) (Figs. 4B, C), but disease-free and overall survival were both significantly longer in the low endoglin-MVD group compared with the high endoglin-MVD group (2-year disease-free survival rate, 27.8% and 14.3%, respectively; 2-year overall survival rate, 54.3% and 23.5%, respectively). These data show that a high MVD of endoglin-positive vessels in the tumor correlates with a poor prognosis in pancreatic cancer patients.

DISCUSSION

Pancreatic cancer remains as a major health problem, and conventional cancer treatment has had little impact on the disease.[24] Therefore, there is an urgent need for better understanding of the underlying molecular mechanisms to facilitate development of new therapies. In this study, we focused on angiogenesis in pancreatic cancer and showed that endoglin is expressed specifically in the intratumoral and peritumoral blood vessels in the cancer tissue. Endoglin has been shown to be expressed in similar blood vessels in several malignancies, including brain, prostate, breast, colorectal, and hepatic tumors,[15–18] but to our knowledge, this is the first report showing specific expression of endoglin in tumor-related blood vessels in pancreatic cancer.

Interestingly, we found that endoglin is only expressed in capillary-like vessels with thin vascular walls. Tumor vessels induced by angiogenic factors are structurally abnormal, with a loose connection of endothelial cells and a few pericytes resulting in a thin vascular wall.[10] This indicates that endoglin-positive endothelial cells are mainly found in vessels induced by tumor angiogenesis. Strongly endoglin-positive vessels were also found in the muscle layer of the duodenum at sites of tumor invasion, but only faint staining was present in the muscle layer of normal duodenum, again suggesting that the endoglin-positive endothelial cells were induced by tumor angiogenic factors. In contrast, endothelial cells of intratumoral arteries with thick tunica media walls (normal arterial structure) did not express endoglin. These vessels, which differ structurally from newly formed tumor vessels, are probably preexisting arteries in cancer tissues. Therefore, these results suggest that endoglin is specifically expressed in endothelial cells of vessels induced by tumor angiogenesis.

In pancreatic cancer tissue, we also found endoglin expression in lymphatic endothelial cells, which were identified by staining with D2-40 antibody.[23] Hirakawa et al[25] reported that mature lymphatic endothelial cells in human skin do not express endoglin and, interestingly, also found cells expressing both endoglin and prox-1, a lymphatic endothelial cell marker, during embryonic development. In addition, Salven et al[26] reported that lymphatic and vascular endothelial precursor cells in embryonic liver express endoglin, as well as CD34, CD133, and VEGF receptor 3. By contrast, D2-40 and endoglin double-positive endothelial cells in tumor tissues were not found in adjacent normal pancreatic tissue (data not shown). These results suggest that endoglin-expressing lymphatic vessels in pancreatic tumors consist of immature endothelial cells induced by tumor lymphangiogenesis.

A higher MVD of endoglin-positive vessels was associated with shorter disease-free and overall survival in patients with pancreatic cancer in the current study. In contrast, the MVD of PECAM-positive vessels did not show a correlation with prognosis, despite the strong correlation between endoglin-MVD and PECAM-MVD. Because endoglin is only expressed in small capillary-like vessels, which may be induced by tumor factors, whereas PECAM is also expressed in preexisting vessels associated with the tumor; the density of endoglin-positive vessels more precisely represents the level of tumor angiogenesis and/or lymphangiogenesis, and these activities may reflect the malignant potential for tumor invasion. In support of this hypothesis, there is emerging evidence showing that enhanced expression of angiogenic factors, such as fibroblast growth factors and VEGF, in pancreatic cancer cells correlates with disease progression.[2,3,5] In addition, it is reported that tumor lymphangiogenesis also plays an important role in cancer progression, including metastasis.[27,28] These studies and our data emphasize the importance of angiogenesis and lymphangiogenesis in pancreatic cancer progression, and the MVD of endoglin-positive vessels may be a valuable prognostic marker in pancreatic cancer patients.

Endoglin is an accessory protein in the TGF-β signaling receptor complex,[29] and TGF-β signaling has important roles in endothelial cell proliferation.[13,30] Moreover, mice lacking endoglin die from defective vascular development in utero,[14] indicating that endoglin may have a role in endothelial cell proliferation of tumor vessels induced by tumor angiogenesis or lymphangiogenesis. These data may indicate that endoglin is a candidate molecule as a target for a new antitumor therapy for the pancreatic cancer based on inhibition of angiogenesis and lymphangiogenesis, and this idea has been validated experimentally in mouse models of several different solid tumors, using anti-endoglin antibody.[31–35]

In conclusion, our results show that endoglin is specifically expressed in endothelial cells of vascular and lymphatic vessels in pancreatic cancer, and MVD assessed by endoglin immunostaining may be a useful prognostic marker in this disease. Further analysis is needed to obtain a better understanding of endoglin function in pancreatic cancer.

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A Randomized Phase II Trial of Adjuvant Chemotherapy With Uracil/Tegafur and Gemcitabine Versus Gemcitabine Alone in Patients With Resected Pancreatic Cancer

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BACKGROUND. There have been few randomized studies of adjuvant chemotherapy using gemcitabine (GEM) in patients with resected pancreatic cancer.

METHODS. Patients with invasive ductal pancreatic cancer who underwent radical surgery were enrolled and assigned to receive uracil/tegafur (UFT) and GEM together (GU) or GEM alone (G). GEM was administrated at a dosage of 1 g/m^2 intravenously weekly 3 of 4 weeks and UFT at a dosage of 200 mg/day orally continuously. Eligibility included resection status 0 or 1, and no previous chemo- or/ and radiation therapy. The primary endpoint was disease-free survival (DFS), and secondary endpoints included overall survival (OS) and toxicity.

RESULTS. Between 2002 and 2005, 100 patients were randomized into the 2 arms of the trial (50 patients to GU and 50 to G). One patient in the G group was found to be ineligible. Baseline characteristics were well balanced between the 2 groups. With a median observation period of 21 months, the 1- and 3-year DFS rates were 50.0% and 17.7% in the GU group and 49.0% and 21.6% in the G group, respectively. The median OS was 21.2 months in the GU group and 29.8 months in the G group. Toxicity was minor and acceptable, less than grade 4 in both groups.

CONCLUSIONS. Postoperative GEM-based adjuvant chemotherapy was safe and well tolerated. However, addition of UFT with GEM did not improve DFS as compared with GEM alone. Further clinical trial resources for adjuvant chemotherapy should address other combinations and novel agents. ***Cancer*** **2008;113:2448–56.** *© 2008 American Cancer Society.*

KEYWORDS: pancreatic cancer, adjuvant therapy, gemcitabine, uracil/tegafur.

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Pancreatic cancer is 1 of the most lethal human malignancies and continues to be a major unsolved health problem. It is the fourth leading cause of death from cancer in the United States[1] and the fifth in Japan.[2] Despite the recent advances in the management of the disease, long-term survival remains poor, with a 5-year survival rate of about 5%.[1,2]

Surgery is the only means to obtain a cure for patients with pancreatic cancer. Even with advanced cancer, the best survival rates are achieved after surgical resection.[3,4] However, because of the high incidence of recurrences, the 5-year survival rate of patients who undergo resection remains low, approximately 20%.[3,5] Extended resections do not improve survival, as demonstrated in several randomized trials.[6-8] These facts indicate that, to achieve long-term disease control in patients with pancreatic cancer, it is important to develop an effective multidisciplinary therapy, a combination of surgery with other nonsurgical therapies such as radiation and chemotherapy.

In fact, it has been clearly shown that adjuvant chemotherapy prolongs postoperative survival in several types of malignancies, including breast,[9] colorectal,[10] and gastric cancer.[11] To date, several randomized studies of adjuvant therapy have been conducted in patients with resected pancreatic cancer. The Gastrointestinal Tumor Study Group (GITSG) first reported, in a multicenter randomized-controlled study, that adjuvant chemoradiation therapy prolonged the postoperative survival of patients with resected pancreatic cancer.[12] However, the results of several subsequent trials, in which 5-fluorouracil (5-FU)-based chemotherapy was applied, were inconsistent.[13-17] Although Stocken et al showed by meta-analysis that 5-FU-based chemotherapy is an effective adjuvant treatment in pancreatic cancer, the survival rate of patients with adjuvant chemotherapy was still poor, with a median survival time of only 19.0 months.[18]

Since Burris et al first reported an improvement in survival and clinical benefits with gemcitabine, an analog of deoxycytidine, compared with 5-FU for advanced pancreatic cancer,[19] gemcitabine has become a major first-line reagent for patients with unresectable pancreatic cancer. The same year, when they published their paper showing the benefits of gemcitabine therapy, the German group started a randomized controlled trial (CONKO-001) to estimate the benefits of adjuvant chemotherapy with gemcitabine for patients with resected pancreatic cancer, and recently reported that it significantly delayed the development of recurrent diseases.[20] However, there exist only a few other trials of adjuvant chemotherapy with gemcitabine for patients with resected pancreatic cancer.

With this background, we planned a similarly randomized trial to evaluate the survival benefit of gemcitabine adjuvant therapy in combination with another reagent. For this purpose, we used tegafur/uracil (UFT). UFT is an oral fluoropyrimidine agent composed of tegafur and uracil at 1:4 fixed molar ratio to increase the tumor concentration and antineoplastic activity of 5-FU.[21] In vitro experiments showed that pretreatment with 5-FU increased the cell intensity and toxicity of gemcitabine by synergistic activity.[22] Furthermore, the combination of gemcitabine and UFT has already shown a high tumor response rate in patients with lung cancer.[23,24] It has also been shown that a combination of capecitabine, another prodrug of 5-FU, and gemcitabine increased the survival rate of patients with unresectable pancreatic cancer with good performance status compared with those produced by gemcitabine treatment alone.[25]

In 2002, we initiated a multicenter randomized controlled phase II trial to estimate the possible efficacy of a UFT combination with gemcitabine, compared with gemcitabine alone, for adjuvant chemotherapy in patients with resected pancreatic cancer.

MATERIALS AND METHODS

Patients and Design

Patient recruitment for the multicenter randomized phase II trial was begun in May 2002 and was closed in December 2005 in 19 Japanese institutions. Patients who had pancreatic cancer histologically verified as invasive ductal carcinoma and who had undergone macroscopic complete resection were enrolled. Patients with carcinoma in situ were excluded. Patients with prior radiation or neoadjuvant chemotherapy or with distant metastasis except minimal para-aortic lymph node metastasis were excluded from this study. Other eligibility criteria included: being aged 20 years or older and 79 years or younger at the time of registration; absence of active infection, significant cardiac disease, brain disease, and/or active malignancies other than pancreatic cancer; and adequate hematologic, renal, and hepatologic function (leukocytes $\geq 4000/mm^3$, hemoglobin ≥ 9.0 g/dL, platelets $\geq 1 \times 10^5/mm^3$, creatinine $\leq 1.5\times$ upper limit of normal [ULN], total bilirubin $\leq 3 \times$ ULN, transaminase $\leq 2.5 \times$ ULN). The protocol was approved by the institutional review board at each study site, and all patients provided written informed

consent. The patients were registered within 10 weeks of surgery and were then randomly assigned to 1 of 2 groups: adjuvant chemotherapy with a gemcitabine alone (GEM) group and a gemcitabine + UFT (GEM + UFT) group. All patients were diagnosed as free of recurrences by computed tomography postoperatively before enrollment. Randomization was performed at the coordinating center of the trial using a computer-generated procedure. Standard surgical procedures were used depending on the extent of tumor involvement and according to institutional policy. Handling and histological examination of the resected specimens were carried out according to the recommendations of the Japan Pancreatic Society.[26] During the study, vital signs and complete blood counts were obtained weekly. Additional 4-week assessments included serum biochemistry and adverse events. Imaging by computed tomography or ultrasound was carried out every 3 months. Diagnosis of recurrence was made based on the imaging findings. Treatment after recurrence was not defined.

Adjuvant Chemotherapy

Chemotherapy was started within 1 week of randomization. Patients in the GEM group received adjuvant chemotherapy of at least 4 cycles of gemcitabine every 4 weeks. Each chemotherapy cycle consisted of 3 weekly infusions of gemcitabine at 1000 mg/m^2 given by intravenous infusion during a 30-minute period, followed by a 1-week pause. Patients in the GEM + UFT group received UFT at 200 mg/day continuously in addition to gemcitabine with the same protocol as the GEM group. Patients who received 4 cycles of treatment were considered to have completed the therapy. Patients were allowed to continue the same therapy after 4 cycles. Toxicity was assessed according to National Cancer Institute Common Terminology Criteria for Adverse Events versions 2.0 (~2004) and 3.0 (~2004). If the patient showed grade 3 or worse hematologic adverse events, serum transaminase level >2.5-fold ULN, serum total bilirubin level >3.0 mg/dL, or other adverse clinical events of grade 2 or worse, chemotherapy was stopped until recovery from these criteria. The dose of gemcitabine was reduced to 800 mg/m^2 in the following cycles and to 600 mg/m^2 if additional adverse events occurred. In the GEM + UFT group, UFT was stopped if adverse events occurred even after a reduction of gemcitabine to 800 mg/m^2, with gemcitabine further reduced to 600 mg/m^2 in the following cycles. Chemotherapy was discontinued if adverse events within these criteria occurred regardless of whether the gemcitabine dose had been reduced to 600 mg/m^2.

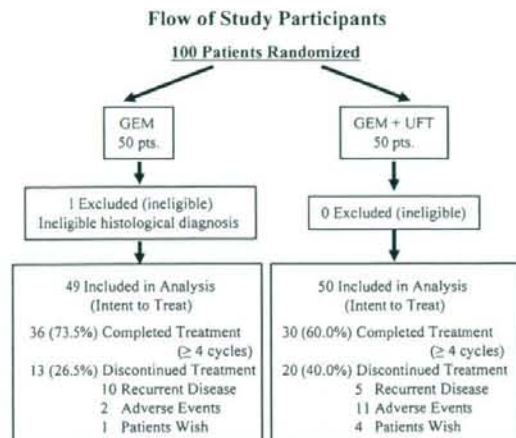

FIGURE 1. The flow of study participants is depicted. GEM indicates gemcitabine alone; UFT, tegafur/uracil.

Statistics

The primary endpoint of the study was the 1-year disease-free survival rate. Secondary endpoints included toxicity and overall survival. The duration of disease-free and overall survival was calculated from the date of surgery to the date of recurrence and death, respectively. Efficacy analyses were performed according to the intention-to-treat principle. Survival curves were drawn using the Kaplan-Meier technique, and the log-rank test was used to assess differences in survival estimates among the groups. The univariate analysis was done using the Cox proportional hazards model. Assuming a 1-year disease-free survival rate of 40% in the GEM arm, the present study was designed to enroll more than 89 patients to detect an absolute increase of at least 15% in the GEM + UFT arm, with a significance level of 5% with 90% power, and taking into consideration a dropout rate of 25%. Data analysis was performed using StatView version 5.0 (SAS Institute Inc. Cary, NC).

RESULTS

Patients

Between May 2002 and December 2005, 100 patients were recruited into the study from 19 institutions in Japan. The patients were randomized to the GEM group (n = 50) and the GEM + UFT group (n = 50) (Fig. 1). One patient in the GEM group was rated ineligible because of a histological diagnosis

TABLE 1
Characteristics of Eligible Patients

	Gemcitabine	Gemcitabine+UFT	P
Patients, n	49	50	
Age, median (range), y	63 (38-78)	63 (38-78)	.35
Sex, women/men	31 / 18	33 / 17	.78
Period from surgery to randomization, median (IQR), d	30.0 (26.0-37.0)	26.5 (20.25-35.75)	.41
Period from surgery to start of adjuvant chemotherapy, median (IQR), d	35.0 (27.0-41.0)	30.0 (23.25-42.50)	.48
Operative procedure, PD/DP/TP	38/8/3	38/9/3	.98
UICC stage, IA/IB/IIA/IIB/III/IV	0/1/13/26/2/7	1/1/10/33/1/4	.64
JPS stage, I/II/III/IVa/IVb	0/2/16/22/9	1/2/21/17/9	.68
Primary tumor status, 1/2/3/4	0/2/45/2	2/4/23/1	.39
Nodal status, 0/1	15/34	13/37	.61
Distant metastasis, 0/1	42/7	46/4	.32
Resection status, 0/1	32/17	41/9	.06
Histology			.88
Tubular adenocarcinoma, well/mod/poor	3/34/6	6/33/7	
Papillary adenocarcinoma	1	1	
Adenosquamous carcinoma	1	1	
Anaplastic carcinoma	1	1	
Invasive carcinoma derived from intraductal tumor	3	1	

UFT indicates tegafur/uracil; IQR, interquartile range; PD, pancreaticoduodenectomy; DP, distal pancreatectomy; TP, total pancreatectomy; UICC, International Union Against Cancer; well, well-differentiated type, mod, moderately differentiated type; poor, poorly differentiated type. JPS, Japan Pancreas Society.

(undifferentiated carcinoma) determined after randomization. The remaining 99 patients were included in the analysis. The baseline characteristics of the patients in the 2 groups were comparable (Table 1). There was no statistical difference between the 2 groups in the median time from surgery to the start of chemotherapy of 35.0 days in the GEM group and 30.0 days in the GEM + UFT group.

Treatment Data

All patients received at least 1 dose of gemcitabine. The median number of the gemcitabine administrations for a patient was 12 times for the GEM group and 14 times for the GEM + UFT group. Median relative dose intensity within the first 4 cycles was 89.1% for the GEM group and 87.4% for the GEM + UFT group; there was no statistical difference between the 2 groups (Table 2). Median duration of UFT administration in the GEM + UFT group was 5 months, and the median relative dose intensity within the first 4 cycles was 100% (Table 2).

Thirty-six patients (73.5%) in the GEM group and 30 patients (60.0%) in the GEM + UFT group com-

TABLE 2
Total Dose and Relative Intensity

	Gemcitabine, median (range)	Gemcitabine+UFT, median (range)	P
Gemcitabine			
Total amount, g	19.2 (3.9-76.5)	19.2 (2.5-113.1)	.86
Administrations, n	12 (3-76)	14 (2-81)	.67
Relative dose intensity, %	89.1 (22.5-100)	87.4 (13.5-100)	.69
UFT			
Total amount, g	—	27.8 (1.2-158.0)	
Duration of administration, mo	—	5 (1-26)	
Relative dose intensity, %	—	100 (5.4-100)	

UFT indicates tegafur/uracil.

pleted 4 or more cycles of treatment. The reasons for treatment discontinuation within 4 cycles in the GEM group were recurrent disease (10 patients, 76.9%), adverse events (2 patients, 15.4%), and patient's wish (1 patient, 7.7%). In the GEM + UFT group, the reasons were recurrent disease (5 patients, 25.0%), adverse events (11 patients, 55.0%), and patient's wish (4 patients, 20.0%).

Toxicity

Although the majority of the patients, especially those in the GEM + UFT group, experienced minor toxicity, no grade 4 or higher toxicities were observed in either group (Table 3). Fifteen (30.6%) patients in the GEM group and 12 (24.0%) patients in the GEM + UFT group experienced grade 3 toxicity, mainly leukocytopenia. Two patients in the GEM group and 11 patients in the GEM + UFT group discontinued treatment within 4 cycles because of repeated toxicities despite dose modification. All toxicities were reversible and resolved with conservative treatment alone in all patients.

Efficacy

With a median observation period of 21 months (range, 3 months to 57 months), recurrent disease developed at comparable rates of 73.5% in the GEM group (36 of 49 patients) and 78% in the GEM + UFT group (39 of 50 patients). The sites of recurrence were similar in both groups (GEM group and GEM + UFT group); the local recurrence was observed in 75.0% and 69.2% of patients, respectively. The number of patients with local recurrence alone was 13 (36.1%) in the GEM group and 17 (43.6%) in the GEM + UFT group. The most frequent primary site of distant metastasis was the liver, with 12 (33.3%) patients of the GEM group and 13 (33.3%) patients of the GEM + UFT group. The estimated

TABLE 3
Summary of Toxicities

	Gemcitabine			Gemcitabine+UFT		
	Any Grade	Grade 3	Grade 4	Any Grade	Grade 3	Grade 4
Total	29 (59.2%)	15 (30.6%)	0 (0.0%)	45 (90.0%)	12 (24.0%)	0 (0.0%)
Hematologic						
Leukocytes	26 (53.1%)	11 (22.4%)	0 (0.0%)	36 (72.0%)	9 (18.0%)	0 (0.0%)
Hemoglobin	20 (40.8%)	4 (8.2%)	0 (0.0%)	17 (34.0%)	2 (4.0%)	0 (0.0%)
Platelets	13 (26.5%)	3 (6.1%)	0 (0.0%)	11 (22.0%)	0 (0.0%)	0 (0.0%)
Nonhematologic						
Nausea/vomiting	10 (20.4%)	0 (0.0%)	0 (0.0%)	12 (24.0%)	0 (0.0%)	0 (0.0%)
Anorexia	9 (18.4%)	1 (2.0%)	0 (0.0%)	14 (28.0%)	1 (2.0%)	0 (0.0%)
Biochemical						
AST/ALT	11 (22.4%)	0 (0.0%)	0 (0.0%)	11 (22.0%)	1 (2.0%)	0 (0.0%)
Glucose intolerance	1 (2.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (2.0%)	0 (0.0%)

AST indicates aspartate aminotransferase; ALT, alanine aminotransferase.

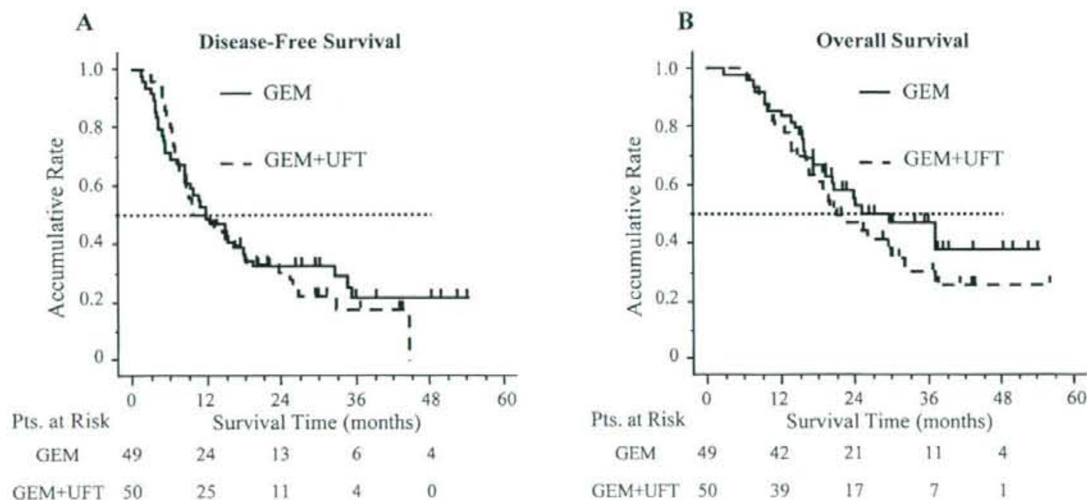

FIGURE 2. Disease-free (A) and overall (B) survival are shown. GEM indicates gemcitabine alone; Pts., patients; UFT, tegafur/uracil.

1- and 3-year disease-free survival rates were 49.0% and 21.6% in the GEM group and 50.0% and 17.7% in the GEM + UFT group, respectively. The median disease-free survival time was also comparable to 12.0 months in the GEM group and 12.3 months in the GEM + UFT group (log-rank, $P = .67$, Fig. 2A).

In the randomized patients, 57 patients (26 in the GEM group and 31 in the GEM + UFT group) died because of recurrent disease; there were no deaths attributed to any other causes in the observation period. The median overall survival time was 29.8 months in the GEM group and 21.2 months in the GEM + UFT group. The estimated survival rates at 1 and 3 years were 85.7% and 46.9% in the GEM group and 80.0% and 30.4% in the GEM + UFT group, respectively. There was no statistical difference between the overall survival times of the GEM group and the GEM + UFT group (log-rank, $P = .28$, Fig. 2B).

Prognostic Factors for Patients With Adjuvant Chemotherapy

We analyzed the clinical outcomes of all patients in this study to estimate the efficacy of adjuvant chemotherapy using gemcitabine for patients with resected pancreatic cancer. The median disease-free

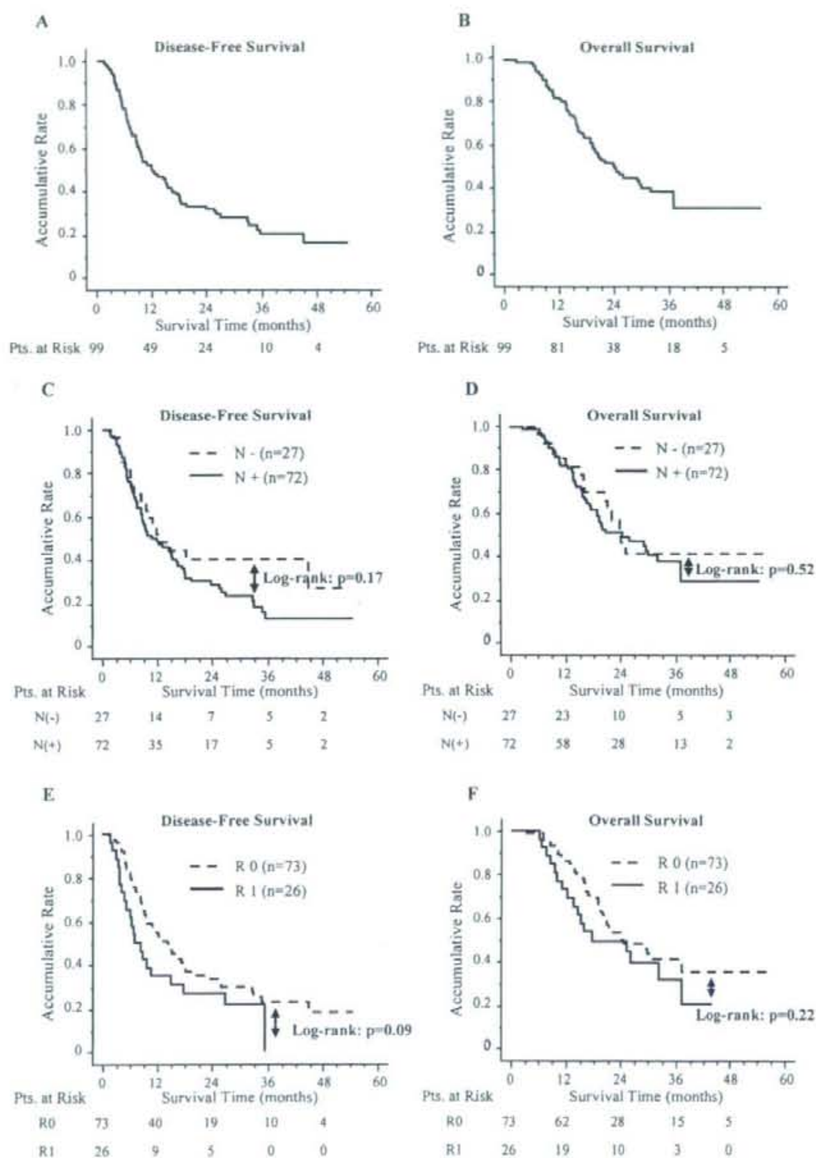

FIGURE 3. Disease-free and overall survival of all patients (Pts.) are shown: (A) disease-free survival; (B) overall survival; (C, D) disease-free (C) and overall (D) survival of the patients categorized by nodal status (solid line indicates lymph node positive [N+]; dotted line: lymph node negative [N−]); (E, F) disease-free (E) and overall (F) survival of the patients categorized by resection status (R) (solid line indicates R1; dotted line, R0).

survival time of all 99 patients in this study was 12.0 months, and the estimated 1- and 3-year disease-free survival rates were 49.5% and 19.5%, respectively (Fig. 3A). The median overall survival time of those patients was 24.1 months, and the estimated 1- and 3-year survival rates were 82.8% and 38.8%, respectively (Fig. 3B).

To assess the influence of prognostic factors, the relationships between the survival outcomes and the following variables were investigated: sex, age (≤63

years/>63 years), tumor location (head/body or tail), International Union Against Cancer stage (IA-IIA/IIB-IV), Japan Pancreas Society (JPS) stage (I-III/IVa, b), operation time (≤444 minutes/>444 minutes), blood loss during operation (≤920 mL/>920 mL), tumor size (≤2 cm/>2 cm), nodal status (negative/positive), para-aorta lymph node metastasis (negative/positive), tumor histology (poorly differentiated tubular adenocarcinoma/other), and resection status (R0/R1). We performed univariate analysis of these factors for disease-free and overall survival times. Among these factors, only the JPS stage showed a significant value for disease-free survival time (hazard ratio, 0.473; 95% confidence interval; 0.288-0.775; $P = .003$), and no factors exerted a significant influence on overall survival time.

Because factors that are known to have prognostic value for survival such as nodal status and resection status did not show significant values on univariate analysis, we performed a Kaplan-Meier analysis of the disease-free and overall survival times for all 99 patients, and categorized the outcome by these factors. As shown in univariate analysis, there were no significant differences between patients with and without lymph node metastasis with respect to disease-free and overall survival times (Fig. 3C, D). Moreover, there were no significant differences between patients with R0 and R1 resection with respect to disease-free and overall survival times (Fig. 3E, F).

DISCUSSION

For the treatment of patients with pancreatic cancer, even with advanced cancer, the best survival rates are achieved after surgical resection.[3,4] However, because of the high recurrence rate, the prognosis of the patient remains poor even after curative surgery. This indicates that it is important to establish an effective multidiscipline therapy for pancreatic cancer. In this study, we aimed to estimate the efficacy of adjuvant chemotherapy using gemcitabine and UFT for patients with resected pancreatic cancer.

Burris et al first reported in 1997 that gemcitabine improved the survival of patients with advanced pancreatic cancer, with a median survival time of 5.65 months compared with 4.41 months for patients treated with 5-FU.[19] Since then, gemcitabine has become the first-line chemotherapy for patients with pancreatic cancer. Meanwhile, several randomized studies have shown that 5-FU-based adjuvant chemotherapy can improve the survival of patients with resected pancreatic cancer.[13,27] Therefore, we strongly expected that adjuvant chemotherapy using gemcitabine would improve the survival of our patients. We thus attempted in our phase II study to optimize the efficacy of adjuvant chemotherapy by combination with gemcitabine and other agents.

We selected UFT as the agent to use in combination with gemcitabine. There were several reasons. First, in vitro analysis showed that pretreatment of pancreatic cancer cells with 5-FU increased the intracellular concentration of gemcitabine, suggesting that UFT, a prodrug of 5-FU, might have the potential to supplement the therapeutic benefits of gemcitabine.[22] Second, UFT, an oral fluoropyrimidine, might be more convenient to administer than continuous 5-FU infusion, especially in an adjuvant setting. In addition, several groups had shown favorable results from the use of a combination of gemcitabine and UFT as a treatment for advanced pancreatic cancer.[28,29]

Our study showed that adjuvant chemotherapy with gemcitabine, with or without UFT, could be carried out with acceptable safety. No grade 4 toxicities were observed in any patients in either group, and no patient died because of toxic events related to adjuvant therapy. Grade 3 hematologic toxicities were observed in about 30% of the patients in both groups. Leukocytopenia was most frequently observed, as shown in the CONKO-001 study.[20] Although a high incidence of leukocytopenia from gemcitabine has also been reported in the treatment of nonresected pancreatic cancer with 9.7% grade 3 leukocytopenia,[19] the frequency in this study was relatively high. Onoue et al have reported as well that severe leukocytopenia induced by gemcitabine administration developed readily in patients who had undergone surgical resection.[30] This suggests that it is very important to observe patients closely, especially in adjuvant chemotherapy, to avoid fatal toxicities. Nevertheless, as no serious adverse events were observed in this study, we concluded that the adjuvant chemotherapy using gemcitabine with or without UFT can be carried out safely.

Unfortunately, this study failed to show any additional benefit in using UFT in concert with gemcitabine for patients with resected pancreatic cancer. Disease-free survival was similar in both groups, with a 1-year disease-free survival rate of 49.0% in the GEM group and a 50.0% rate in the GEM + UFT group. Moreover, the overall survival rate was slightly worse among the patients of the GEM + UFT group than among those of the GEM group, with median survival time of 21.2 months and 29.8 months, respectively. Although the observation period was short, we concluded from our data that other combinations with gemcitabine must be considered as

future trials for adjuvant chemotherapy for resected pancreatic cancer.

Although UFT did not induce any survival benefits, the patients of both groups who received gemcitabine adjuvant chemotherapy after surgery experienced relatively longer survival times. The median disease-free survival time of the total of 99 patients in this study was 12.0 months, and the median overall survival time was 24.1 months. The overall median survival time is usually reported as about 10 months to 20 months for patients with resected pancreatic cancer who did not received adjuvant chemotherapy.[12-17,20] In addition, survival in this study was favorable compared with studies of adjuvant therapy for pancreatic cancer. The median overall survival time of patients with adjuvant therapy was reported as 20.0 months in the GITSG study[12] and 20.1 months in the ESPAC-1 study.[17] Also, as expected, our data were almost equivalent with the CONKO-001 study, in which the median survival time of patients with gemcitabine adjuvant chemotherapy was 22.1 months. These results support the use of gemcitabine as adjuvant chemotherapy in resectable pancreatic cancer.

The involvement of radiation as the adjuvant therapy for patients with resected pancreatic cancer has been discussed. The GITSG study showed the efficacy of chemoradiation as the adjuvant therapy.[12] Conversely, the recent ESPAC-1 study failed to show the efficacy of this therapy on postoperative survival, in contrast to chemotherapy alone.[17] In this study, although the local recurrence was most frequently observed, many of these patients also showed other types of recurrences at the same time. This may indicate that the effect of radiation therapy as the adjuvant therapy might be limited regarding the survival of patients with resected pancreatic cancer.

Interestingly, in this study there were no significant differences in disease-free and overall survival rates between N− and N+ patients and between R0 and R1 patients. Nodal status (N) and resection status (R) are usually considered prognostic factors for pancreatic cancer after resection.[31-33] However, the ESPAC-1 study also reported that patients with R1 resection also benefited from adjuvant chemotherapy.[31] These results may indicate that patients with N+ or R1 status benefit more from adjuvant chemotherapy using gemcitabine.

In conclusion, the present study did not demonstrate the efficacy of a UFT and gemcitabine combination as an adjuvant therapy for patients with resected pancreatic cancer compared with gemcitabine alone. It did, however, add further evidence that an adjuvant therapy using gemcitabine can produce favorable effects on the prognosis without any severe toxicity. This strongly suggests that further clinical trial resources for adjuvant chemotherapy should be addressed through the use of other combinations of agents with gemcitabine.

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ORIGINAL ARTICLE

High intratumoral dihydropyrimidine dehydrogenase mRNA levels in pancreatic cancer associated with a high rate of response to S-1

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Abstract

Purpose Although the prognosis in patients with pancreatic cancer has been poor, we recently reported unusually high response rate and survival benefit of S-1 treatment in patients with pancreatic cancer. The aim of this study was to reveal genetic background of this unique activity of S-1 against pancreatic cancer. S-1 is a novel oral fluoropyrimidine derivative consisting of Tegafur (FT) and dihydropyrimidine dehydrogenase (DPD) inhibitor (5-chloro-2,4-dihydroxypyridine; CDHP). Accordingly, intratumoral DPD mRNA expression level was measured to reveal whether the level in pancreatic cancer was different from other GI cancer and whether it was relevant to chemosensitivity.

Methods Thirty-three recurrent pancreatic cancer patients treated with S-1 were studied. We obtained 15 responders and 13 non-responders according to the change of serum CA19-9. The mRNA was extracted from paraffin-embedded surgical specimens using laser captured microdissection, and relative expression levels of each DPD/β-actin were measured using a quantitative reverse transcription polymerase chain reaction (RT-PCR) (Taqman) system. Forty-four colorectal cancer patients and 20 gastric cancer patients treated with S-1 were enrolled as control groups. Thymidylate synthase (TS) mRNA expression levels were also measured.

Results Intratumoral DPD mRNA expression level was significantly higher in pancreatic cancer than that in colorectal cancer ($P = 0.0003$; median level, 1.38 vs. 0.44) and gastric cancer ($P = 0.0061$; 1.38 vs. 0.82). No difference in TS mRNA expression levels was observed among cancer types. DPD expression among responded pancreatic cancer was significantly lower than non-responded. ($P = 0.012$, Mann–Whitney U test).

Conclusions Intratumoral DPD mRNA expression level in pancreatic cancer was significantly higher than the other malignancies. This result may elucidate possible reasons for the high effectiveness of S-1 in pancreatic cancer.

Keywords Pancreatic cancer · DPD · S-1 · Gene expression

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Introduction

The outcomes of patients with pancreatic cancer remain very poor. Even after curative resection, the 5-year survival rate is only 7% [19]. 5-Fluorouracil (5-FU) had been the mainstay of treatment for pancreatic cancer, although, its response rate as a single agent is less than 20% [9]. Clinical trials have shown that 5-FU-based combination chemotherapy is no more effective than single-agent treatment, with greater toxicity [4, 5, 7]. In recent studies, gemcitabine showed a survival benefit over 5-FU [3] and is now used as a standard treatment for pancreatic cancer.

S-1 is a novel oral fluoropyrimidine derivative consisting of tegafur (FT) and two modulators, 5-chloro-2,4-dihydoxypyridine (CDHP) and potassium oxonate (Oxo) [24]. Antitumor effect is provided by the 5-FU prodrug FT. CDHP competitively inhibits the 5-FU degradative enzyme dihydropyrimidine dehydrogenase (DPD), resulting in prolonged active concentrations of 5-FU in blood [12, 26]. Nowadays, S-1 is widely used to treat many types of cancer, including gastric cancer [23], colorectal cancer [20], breast cancer [22], and head and neck cancer [18].

We have used S-1 in patients with pancreatic cancer and obtained a high response rate and prolonged survival [10]. Although, our previous study was small and retrospective, the response rate was 20% in patients given S-1 alone, as compared with 57.1% in those given S-1 plus cisplatin. To elucidate possible reasons for the high effectiveness of S-1 in pancreatic cancer, which is generally resistant to 5-FU-based therapy, we studied intratumoral DPD expression. We hypothesized that DPD expression levels were higher in pancreatic cancer than in other types of gastrointestinal cancers. We measured the DPD gene expression levels of pancreatic cancers by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) and compared the results with the levels in colorectal cancer and gastric cancer. Since thymidylate synthase (TS) is the other key enzyme for 5-FU metabolism, TS gene expression levels were also measured in these samples.

Patients and samples

Thirty-three patients with recurrent pancreatic cancer were studied (21 men and 11 women; median age 61.5 years, range 37–80). All patients had undergone surgical resection between 1998 and 2001 at the Department of Gastroenterology, Tokyo Women's Medical University, Tokyo, Japan. Diagnoses were individually confirmed by histopathological examination. All patients received S-1 and cisplatin after confirmation of recurrence. S-1 was given orally twice daily for 21 days, and cisplatin 30 mg/m^2 was given on days 1 and 8, followed by a 2 week period of no treatment. The dose of S-1 was based on body surface area (BSA) as follows: BSA < 1.25 m^2, 40 mg; BSA > 1.25 but < 1.5 m^2, 50 mg; and BSA > 1.5 m^2, 60 mg. All of the patients were Japanese, and written informed consent was obtained from each patient according to institutional regulations. No patient had preoperatively received neoadjuvant chemotherapy. Serum CA19–9 tumor marker levels were measured every 2 weeks during chemotherapy. Forty-four patients with advanced colorectal cancer and 20 with advanced gastric cancer were studied as controls.

Microdissection

FFPE tumor specimens were cut into serial sections 10 μm in thickness. For pathological diagnosis, one slide was stained with hematoxylin and eosin and evaluated by a pathologist. Other sections were stained with nuclear fast red (NFR, American MasterTech Scientific Inc., Lodi, CA) to facilitate visualization of histologic features. All tumor samples underwent laser capture microdissection (P.A.L.M. Microlaser Technologies AG, Munich, Germany) to ensure that only tumor cells were dissected.

RNA isolation and cDNA synthesis

RNA was extracted and cDNA was prepared from each sample as described previously [15, 16].

Reverse transcription-PCR

Quantification of TS, DPD and an internal reference gene (β-actin) was done using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System [Taqman]; Applied Biosystems, Foster City, CA) as described previously [11]. The primers and probe sequences used are listed in Table 1. The PCR reaction mixture consisted of 1,200 nM of each primer, 200 nM probe, 0.4 U of AmpliTaq Gold Polymerase, 200 nM each of dATP, dCTP, dGTP, and dTTP, 3.5 mM $MgCl_2$ and 1× Taqman Buffer A containing a reference dye, to a final volume of 20 μl (all reagents from PE Applied Biosystems, Foster City, CA, USA). Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 46 cycles at 95°C for 15 s and 60°C for 1 min. Gene expression values (relative mRNA levels) are expressed as ratios (differences between the Ct values) between the gene of interest (TS, DPD) and an internal reference gene (β-actin), which provides a normalization factor for the amount of RNA isolated from a specimen.

Statistical analysis

Median DPD mRNA levels were compared among pancreatic, colorectal, and gastric cancers by the Mann–Whitney *U* test, and multiple testing was corrected using the Benjamini and Hochberg False Discovery Rate. Median DPD mRNA levels were compared between responders and non-responders with the use of Mann–Whitney's *U* test. TS mRNA levels were compared by the Kruskal–Wallis test. *P*-values of less than 0.05 were considered to indicate statistical significance. All values were two-sided.

Table 1 Primers and probes

Thymidylate synthase (TS)		
Gen bank accession: NM_001071		
Forward primer	TS-764F	5′-GCCTCGGTGTGCCTTTCA-3′
Reverse primer	TS-830R	5′-CCCGTGATGTGCGCAAT-3′
Probe	TS-785T	5′-TCGCCAGCTACGCCCTGCTCA-3′
Dihydropyrimidine dehydrogenase (DPD)		
Gen bank accession: NM_000110		
Forward primer	DPD-51F	5′-AGGACGCAAGGAGGGTTTG-3′
Reverse primer	DPD-134R	5′-GTCCGCCGAGTCCTTACTGA-3′
Probe	DPD-71Tc	5′-CAGTGCCTACAGTCTCGAGTCTGCCAGTG-3′
β-actin		
Gen bank accession: NM_001101		
Forward primer	β-actin-592F	5′-TGAGCGCGGCTACAGCTT-3′
Reverse primer	β-actin-651R	5′-TCCTTAATGTCACGCACGATTT-3′
Probe	β-actin-611T	5′-ACCACCACGGCCGAGCGG-3′

Results

DPD mRNA expression levels in pancreatic, gastric, and colorectal cancers are shown in Table 2 and Fig. 1. The median DPD mRNA level in pancreatic cancer was significantly higher than the levels in colorectal cancer ($P = 0.0003$; median level, 1.38 vs. 0.44) and gastric cancer ($P = 0.0061$; 1.38 vs. 0.82). The median DPD mRNA level also significantly differed between gastric cancer and colorectal cancer ($P = 0.0025$; 0.82 vs. 0.44). No significant difference in TS mRNA levels was observed among pancreatic, colorectal, and gastric cancer ($P = 0.10$) (Table 2).

The serum CA19-9 tumor marker level was above the upper limit of normal (37 U/ml) in 28 of the 33 patients with pancreatic cancer. During chemotherapy the serum CA19-9 level fell by at least 50% in 18 patients (64.3%, responders) and either decreased by less than 50% or increased in 10 (35.7%, non-responders). The median DPD mRNA level in the responders was significantly lower than that in the non-responders ($P = 0.02$; 1.25 vs. 2.20) (Fig. 2).

None of the demographic and clinicopathological variables were significantly associated with the tumor response to S-1 chemotherapy at a P-value of 0.10 on Fisher's exact test (data not shown).

Table 2 Intratumoral DPD mRNA levels in various types of cancer

	Cancer type		
	Gastric	Pancreatic	Colorectal
Number of samples	20	33	44
DPD mRNA levels (median)	0.82	1.38	0.44
Range	0.17–2.21	0.21–4.16	0–2.64
TS mRNA levels (median)	3.26	2.38	2.47
Range	1.69–10.99	0.04–6.68	0.49–19.23

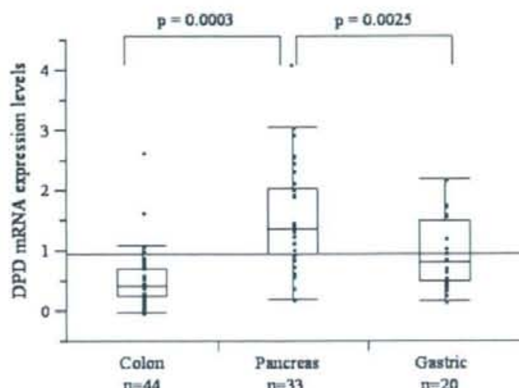

Fig. 1 DPD mRNA expression levels in pancreatic, colorectal, and gastric cancer. DPD expression in pancreatic cancer was significantly higher that that in colorectal [16] and gastric cancer [3]

Discussion

In our study, the DPD mRNA expression levels was significantly higher in pancreatic cancer than in colorectal cancer or gastric cancer, although no difference was seen in TS mRNA expression levels. This difference in DPD expression may explain the discrepancy between the high resistance to 5-fluorouracil and the high sensitivity to S-1 in patients with pancreatic cancer. In patients with colorectal cancer, treatment with 5-fluorouracil alone or a combination of 5-fluorouracil and leucovorin is somewhat effective [1, 6, 8, 21] and had been used as standard therapy before the advent of irinotecan. The response rate of pancreatic cancer is only 7% with 5-fluorouracil alone [5] and is not much higher with 5-fluorouracil plus leucovorin [4]. Ohtsu et al. [20] reported that S-1 had a response rate of 35% in