the HPLC final fraction also showed that the band reacted with anti-human ApoC-1 antibody (data not shown).

A high serum ApoC-1 level is associated with a poor prognosis in pancreatic cancer patients

To examine the clinical significance of the ApoC-1 serum level, we assessed whether the ApoC-1 peak intensity level in the serum was clinically relevant in pancreatic cancer patients. Twenty patients were divided into two groups of 10 patients each based on the median value of the sum of 6630 and 6420 Da peak intensity levels of their preoperative serum: the low-level group (LL) had levels below the median value, and the high-level group (HL) had levels above the median value. The Kaplan–Meier analysis showed that the LL group patients had a significantly longer overall survival time than the HL group patients ($P=0.005$, log-rank test; Figure 2a). To confirm this result, we analysed the peak intensity levels of the 6630 and 6420 Da peptide in the preoperative serum samples from an independent group of 69 pancreatic cancer patients who had surgical resection of cancer (Table 1). The 69 patients were also divided into two groups based on the median value of the sum of 6630 and 6420 Da peak intensity preoperative serum levels. In this 69 patients group, the overall survival time was also longer in LL group patients than in HL group patients ($P=0.023$, log-rank test; Figure 2b).

We next compared the usefulness of the serum ApoC-1 peak intensity levels as a prognostic marker for pancreatic cancer with serum level of carbohydrate tumor-associated antigen 19-9 (CA19-9), which is most popular serum marker for pancreatic cancer. Again, these 69 patients were divided into two groups based on the median value of preoperative serum CA19-9 levels (median values: 175 U ml^{-1}; HL group, patients with CA19-9 level higher than 175 U ml^{-1}; LL group, patients with CA19-9 level lower than 175 U ml^{-1}). However, there was no statistically significant difference of the overall survival time between these two groups ($P=0.090$, log-rank test; Figure 2c). We also carried out the receiver-operator-characteristics (ROC) analysis between patients with survival time more and less than 2 years. The respective area under the ROC curve (AUC) was 0.66 for ApoC-1 peak intensity and 0.60 for CA19-9 level (Figure 2d). These results indicated that serum ApoC-1 peak intensity had the better ability as a prognostic marker than serum CA19-9 level.

On univariate analysis, tumor size ($\geqslant$30 mm vs $<$30 mm), the existence of lymph node metastasis, UICC classification stage (IIB, III vs I, IIA), and serum ApoC-1 peak intensity level (HL vs LL) were correlated with overall survival time. Furthermore, among these factors, only serum ApoC-1 peak intensity level was an independent prognostic factor on multivariate analysis (hazard ratio; 2.160, 95% confidence interval; 1.084–4.302, $P=0.0285$; Table 2).

Abundant expression of ApoC-1 in pancreatic cancer tissues

The decreased serum ApoC-1 level, which was found after curative surgery, suggested that ApoC-1 was overexpressed in cancerous tissue. To confirm this, we analysed the expression of ApoC-1 in pancreatic cancer tissues. Reverse transcription (RT)–PCR revealed that the expression level of ApoC-1 mRNA was much higher in pancreatic cancer tissues than in adjacent normal pancreatic tissue (Figure 3a). Quantitative RT–PCR using samples of resected pancreatic tissue obtained from 16 patients confirmed this finding; a significantly higher ApoC-1 mRNA level was found in pancreatic cancer tissues than in adjacent normal pancreatic tissue (11.49 ± 15.91- and 0.21 ± 0.18-fold ApoC-1/glyceraldehyde-3-phosphate dehydrogenase mRNA copy number, respectively, $P<0.0001$; Mann–Whitney U-test; Figure 3b).

Western blot analysis also confirmed that ApoC-1 protein was expressed in pancreatic cancer tissues. The cancerous tissues of the four cases that were examined were positive for ApoC-1 protein with a 6.6 Da band; in contrast, a same size band could not be found in adjacent normal pancreatic tissues (Figure 3c).

ApoC-1 is expressed in neoplastic epithelium of pancreatic cancer

To examine the localization of ApoC-1 in pancreatic cancer tissues, we carried out immunohistochemical staining for ApoC-1 in 66 invasive pancreatic ductal carcinoma tissues and in adjacent normal pancreatic tissues. In Figure 4a, hepatocytes from normal liver tissue, which are known to express ApoC-1 (Schaefer *et al*., 1978; Lauer *et al*., 1988), are clearly stained with anti-ApoC-1 antibody. As previously reported (Lauer *et al*., 1988), ApoC-1 expression was negative in pancreatic ductal cells that were located in adjacent normal pancreatic tissue (Figure 4b). On the other hand, ApoC-1 expression was found in carcinoma cells of 48 of 66 invasive pancreatic ductal carcinoma cases (72.7%; Figures 4c–f). The ApoC-1 expression was localized in the neoplastic epithelial cells and was not found in the stromal cells surrounding the ductal carcinoma cells. Moderately differentiated adenocarcinoma (Figures 4c and d) and poorly differentiated adenocarcinoma (Figures 4e and f) were almost equally stained with ApoC-1 antibody.

Furthermore, we analysed the correlation of ApoC-1 expression in carcinoma cells with serum ApoC-1 peak intensity level of SELDI in 66 pancreatic cancer patients. Interestingly, positive staining of ApoC-1 significantly correlated with high serum ApoC-1 peak intensity level ($P=0.036$; Mann–Whitney U-test).

ApoC-1 is secreted from pancreatic cancer cells

Next, we investigated whether ApoC-1 was also expressed in pancreatic cancer cell lines. In all four pancreatic cancer cell lines (MIA PaCa II, PanC-1, CFPAC-1 and AsPC-1), ApoC-1 expression was confirmed by RT–PCR (Figure 5a) and western blot

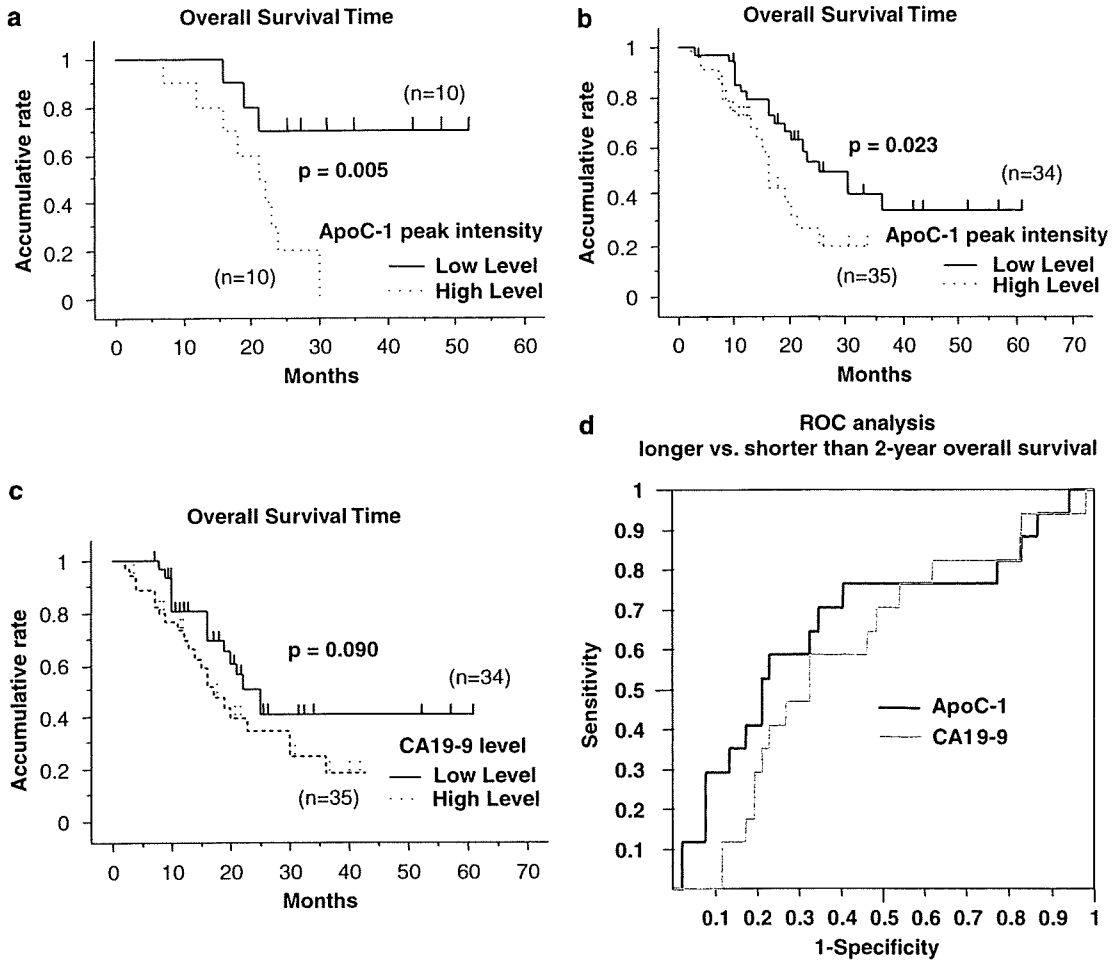

Figure 2 The Kaplan–Meier and receiver-operator-characteristics (ROC) analysis revealed that the peak intensity of apolipoprotein C-1 (ApoC-1) in the preoperative serum correlates with the overall survival of pancreatic cancer patients. (**a**) A total of 20 patients were divided into two groups ($n = 10$ for each group) based on the median value of their preoperative ApoC-1 peak intensity serum level (6630 + 6420 Da peak; LL, low-level group (peak intensity < median value); HL, high-level group (peak intensity > median value)). HL patients had a significantly shorter overall survival time than LL patients. (**b**) An independent group of 69 pancreatic cancer patients was divided into two groups based on their ApoC-1 peak intensity preoperative serum levels (6630 + 6420 Da peak; LL; $n = 34$, HL; $n = 35$, for each group). HL patients had a significantly shorter overall survival time than LL patients. (**c**) These 69 pancreatic cancer patients were divided into two groups based on their CA19-9 preoperative serum levels (cutoff level; 175 U ml^{-1} (median value of these patients), LL; $n = 34$, HL; $n = 35$, for each group). There was no significant difference in overall survival time between their two groups. (**d**) The ROC analyses were carried out for preoperative ApoC-1 peak intensity and CA19-9 serum levels between patients with longer and shorter than 2-year survival time. The respective AUCs were 0.66 for ApoC-1 peak intensity and 0.60 for CA19-9 level.

Table 2 Prognostic factors of 69 pancreatic cancer patients in Cox's proportional hazards model

Overall survival	*Univariate analysis*			*Multivariate analysis*		
Variables	*Hazard ratio*	*95% CI*	P	*Hazard ratio*	*95% CI*	P
Age (> 64 years per ⩽63 years)	1.251	0.654~2.394	0.4989			
Sex (F/M)	0.814	0.413~1.606	0.5526			
ApoC-1 peak intensity (H/L)	2.117	1.081~4.143	0.0286*	2.160	1.084~4.302	0.0285*
CA19-9 level (H/L)	1.739	0.901~3.358	0.0993			
N (+/–)	2.611	1.087~6.273	0.0318*			
Tumor size (⩾30 mm per <30 mm)	2.137	1.093~4.177	0.0264*			
UICC-stage (IIB, III/I, IIA)	2.871	1.117~7.377	0.0285*			

Abbreviations: F, female; M, male;*Significant value.

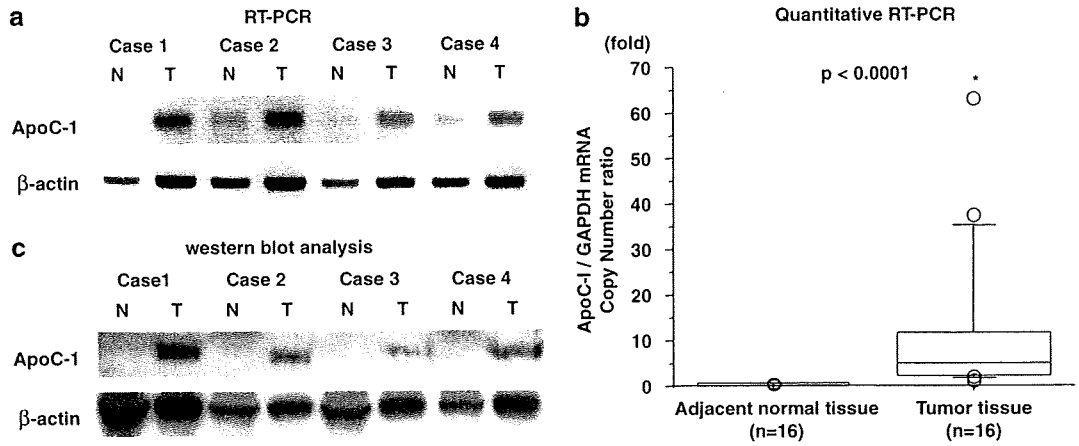

Figure 3 Pancreatic cancer tissues expressed more apolipoprotein C-1 (ApoC-1) mRNA and protein than adjacent normal pancreatic tissues. (**a**) Reverse transcription (RT)–PCR showed stronger ApoC-1 mRNA expression in pancreatic cancer tissues (T) than in adjacent normal pancreatic tissues (N) obtained from four patients. (**b**) Quantitative RT–PCR showed significantly higher ApoC-1 mRNA expression in pancreatic cancer tissues ($n = 16$) than in adjacent normal pancreatic tissues ($n = 16$). (**c**) Western blot analysis of ApoC-1. Western blot analysis showed abundant ApoC-1 protein expression in pancreatic cancer tissues (T), but not in adjacent normal pancreatic tissues (N) in all four cases examined.

analysis (Figure 5b). Furthermore, we investigated the possibility that ApoC-1 is secreted from pancreatic cancer cells. Western blot analysis showed that ApoC-1 protein with a 6.6 Da band was present in the medium with cultured MIA PaCa II cells, but not in the medium with no cultured cells (Figure 5c). These results indicated that the MIA PaCa II cells secreted ApoC-1.

Inhibition of ApoC-1 expression by siRNA suppresses cell proliferation of pancreatic cancer cells by inducing apoptotic cell death

The strong correlation of ApoC-1 serum level with poor clinical outcome, accompanied by expression in tumor cells, may indicate the involvement of this protein in cancer progression. For this reason, we next examined whether gene knockdown of this protein would affect the proliferation of pancreatic cancer cells. To specifically silence the ApoC-1 gene, two pancreatic cancer cell lines (MIA PaCa II and AsPC-1) were transfected with short interfering RNA (siRNA) targeting ApoC-1 mRNA (ApoC-1 siRNA1 and -2) or GL2siRNA as a negative control. The suppression of ApoC-1 mRNA level by transfection with 20 nM ApoC-1 siRNA1 and -2 were confirmed in both MIA PaCa II and AsPC-1 (Figure 5d). ApoC-1 protein levels were successfully reduced with ApoC-1 siRNA1 at 5–200 nM concentration 48 h after transfection, as confirmed by western blot analysis (Figure 5e). ApoC-1 siRNA2 also showed similar effects in reducing ApoC-1 protein expression level in MIA PaCa II (Figure 5f; 20 nM concentration of each siRNA).

Pancreatic cancer cells proliferation was comparatively determined by cell counting after the transfection with Mock, GL2siRNA, ApoC-1 siRNA1 and -2. MIA PaCa II and AsPC-1 cells were transfected with 20 nM siRNA, and total cell proliferation was counted 1–4 days after transfection. Cell proliferation was significantly suppressed by transfection with ApoC-1 siRNA1 and -2, compared with cells treated with GL2siRNA in both cell lines (Figure 6a). Interestingly, Trypan blue staining showed that the percentage of dead cells was significantly increased from 2 days after transfection with two ApoC-1 siRNA1 and -2, compared with cells transfected with GL2siRNA (Figure 6b). We also investigated the effect of ApoC-1 silencing on the invasion ability of cancer cells. The cell invasion ability was not affected by gene silencing by transfection of ApoC-1 siRNA1 and -2, compared with cells treated with GL2siRNA in both cell lines (see Supplementary data).

These results indicate that inhibition of ApoC-1 expression may induce apoptotic cell death in pancreatic cancer cells. To examine this, we investigated whether the rate of apoptotic cell death was increased by treatment with ApoC-1 siRNA in these pancreatic cancer cells. ApoC-1 siRNA1 treatment obviously increased the number of apoptotic cells, which were stained purple red, compared with cells treated with control GL2siRNA (Figure 7a). The proportion of apoptotic cells was significantly higher in MIA PaCa II cells treated with ApoC-1 siRNA1 than GL2siRNA at 24 h after transfection ($P<0.002$; Student's paired t-test; Figure 7b). We also investigated whether the inhibition of ApoC-1 led to activate the procaspase-3 in MIA PaCa II cells. As shown in Figure 7c, the western blot analysis showed that procaspase-3 was more cleaved to activate forms by transfection with both ApoC-1 siRNA1 and -2, compared with GL2siRNA, in MIA PaCa II cells. As well, the caspase-3 activity was significantly higher in MIA PaCa II cells treated with ApoC-1 siRNA1 than GL2siRNA at 24 h after transfection ($P<0.02$; Student's paired t-test; Figure 7d). These results confirmed that the inhibition of ApoC-1 expression induced apoptosis in pancreatic cancer cells.

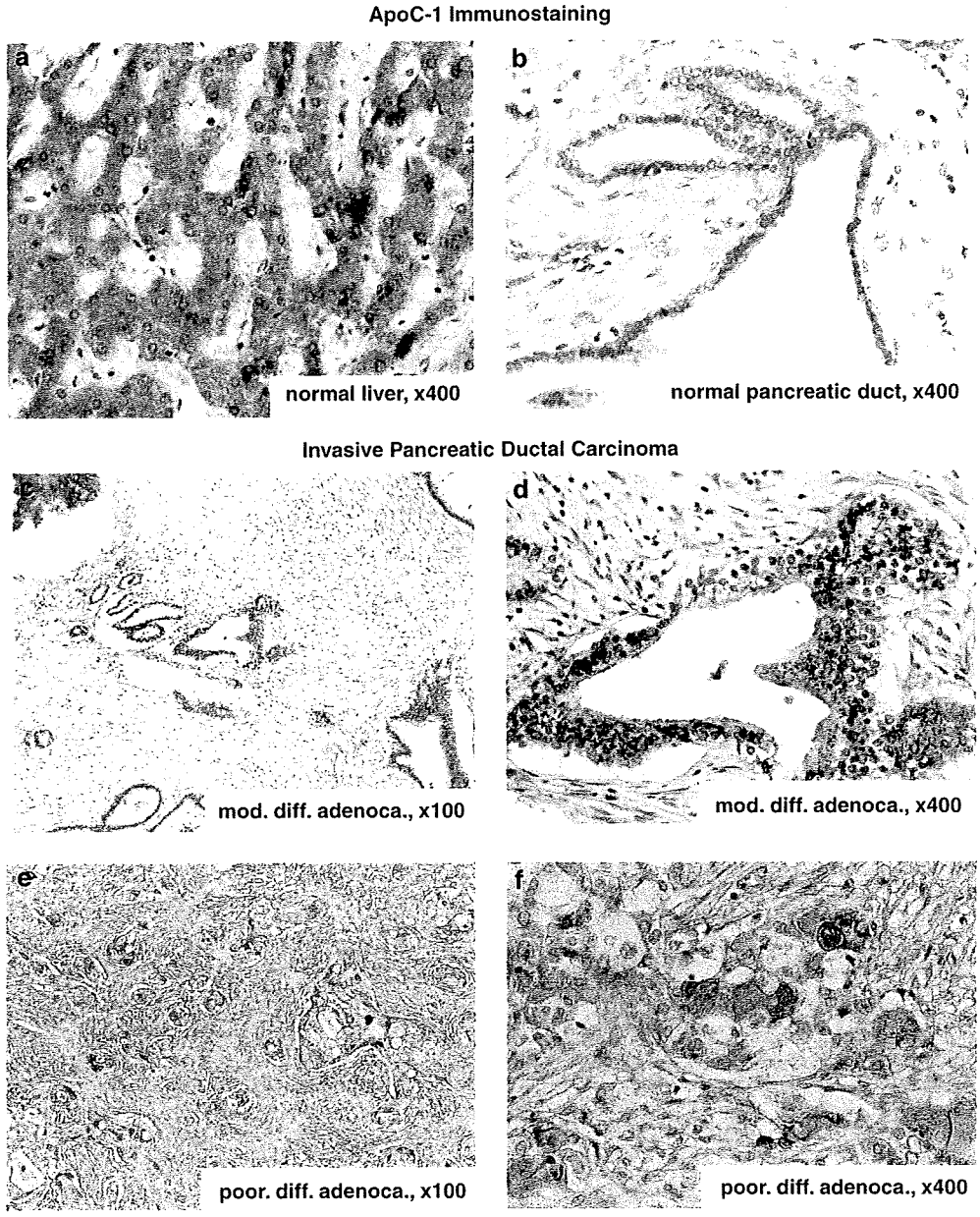

Figure 4 Immunostaining of apolipoprotein C-1 (ApoC-1) showed that ApoC-1 is specifically expressed in pancreatic cancer epithelium. (**a**) ApoC-1 staining in hepatocytes as a positive control (× 400). (**b**) Normal pancreatic ductal cells do not express ApoC-1 (× 400). (**c–f**) Pancreatic invasive ductal carcinoma cells express ApoC-1. Moderately differentiated adenocarcinoma (**c**, × 100; **d**, × 400) and poorly differentiated adenocarcinoma (**e**, × 100; **f**, × 400). Note that ApoC-1 expression is limited to the cancer epithelium, but is not found in stromal cells surrounding the cancer.

To analyse the effects of ApoC-1 gene knockdown in individual cells, immunofluorescence studies were carried out in MIA PaCa II cells treated with GL2siRNA (Figures 7e–g) and ApoC-1 siRNA (Figures 7h–j). Although the abundant expression of ApoC-1 could be found in cells treated with GL2siRNA (Figures 7e–g, red staining), many cells displayed only faint or no fluorostaining with anti-ApoC-1 antibody due to ApoC-1 siRNA treatment. These cells with faint ApoC-1 staining also showed typical apoptotic features, including dense chromatin condensation with 4,6-diamidino-2-phenylindole (DAPI) staining (Figure 7i, arrow; magnified figure is shown in Figure 7l), whereas the GL2siRNA treatment cells that still expressed high amount of ApoC-1 did not show the features (Figure 7f; magnified figure is shown in Figure 7k). These data also revealed that the inhibition of ApoC-1 resulted in apoptotic cell death in pancreatic cancer cells.

Discussion

To identify molecules related to pancreatic cancer progression, we used a new strategy based on the proteomic approach. We identified serum proteins that

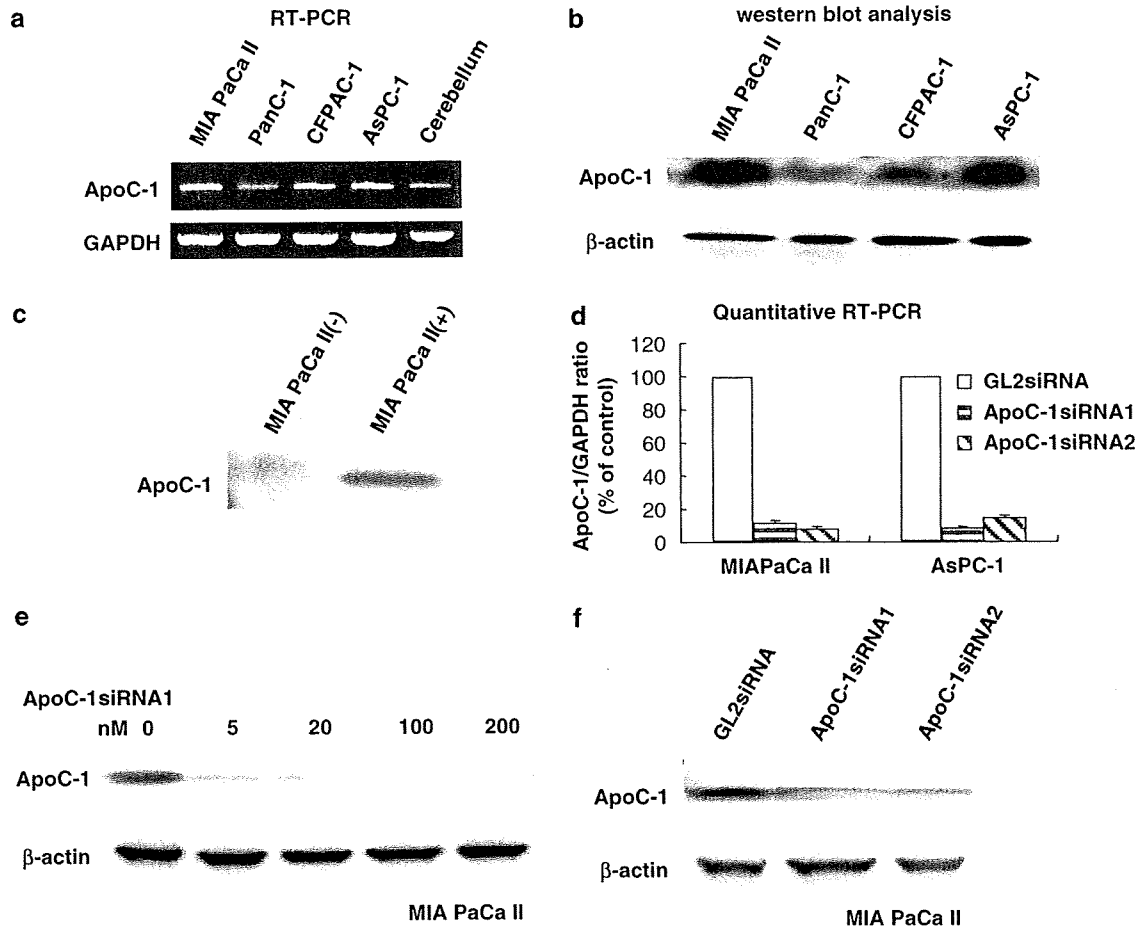

Figure 5 Apolipoprotein C-1 (ApoC-1) is expressed in pancreatic cancer cell lines and its expression is inhibited by short interfering RNA (siRNA) treatment. (**a** and **b**) ApoC-1 is expressed in all four pancreatic cancer cell lines (MIA PaCa II, PanC-1, CFPAC-1 and AsPC-1) analysed by reverse transcription (RT)–PCR (**a**) and western blot (**b**). (**c**) The supernatant of the medium in which no cells were cultured was used as a negative control (left lane). The supernatant of the medium in which MIA PaCa II cells were cultured expressed ApoC-1 protein with a 6.6 Da band (right lane). (**d**) ApoC-1 siRNA1 and -2 inhibit ApoC-1 mRNA expression in MIA PaCa II and AsPC-1. Cells are transfected with ApoC-1 siRNA1 and -2 at 20 nM for 24 h and mRNA levels normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA copy number are detected by quantitative RT–PCR. (**e**) ApoC-1 siRNA1 specifically inhibits ApoC-1 protein expression in MIA PaCa II in a dose-dependent manner. Western blot analysis of ApoC-1 and β-actin. Cells were transfected with ApoC-1 siRNA1 at the indicated concentration for 48 h. (**f**) Both ApoC-1 siRNA1 and -2 inhibit ApoC-1 protein expression in MIA PaCa II in contrast with GL2siRNA. Cells are transfected with siRNAs at 20 nM for 48 h.

were highly expressed in patients with pancreatic cancer using SELDI-TOF MS. The unique strength of SELDI-TOF MS is its ability to analyse proteins from a variety of crude samples with minimal sample consumption; this enables high-throughput analysis (Xiao *et al.*, 2005). In addition, SELDI-TOF MS has an advantage in resolving hydrophobic and low molecular weight proteins, as compared to conventional 2DE analyses. Thus, SELDI-TOF MS has been used extensively in cancer research and has led to the discovery of better serum markers for many cancers. When analysing human samples, it is important to analyse many samples to diminish individual background differences in protein expression. To minimize this, we compared serum samples obtained from the same patient before and after curative surgery. Using this strategy, we successfully identified proteins whose serum expression levels were reduced after curative surgery, based on the analysis of samples from a limited number of patients. Protein purification and amino-acid sequence analysis identified the proteins to be ApoC-1. Surprisingly, the SELDI peak intensity level of ApoC-1 in the preoperative serum was significantly correlated with patients' overall survival. This result was confirmed by a validation study involving 69 serum samples from an independent group of pancreatic cancer patients.

Serum CA19-9 level has been used as marker for the pancreatic cancer (Rhodes, 1999). Recently Ferrone *et al.* (2006) showed that perioperative CA19-9 levels predicted survival in patients with curative resection of pancreatic cancer. However, in our study, preoperative ApoC-1 peak intensity level of SELDI is better prognostic serum factor than serum CA19-9 levels in both analysis of Kaplan–Meier method and ROC curve.

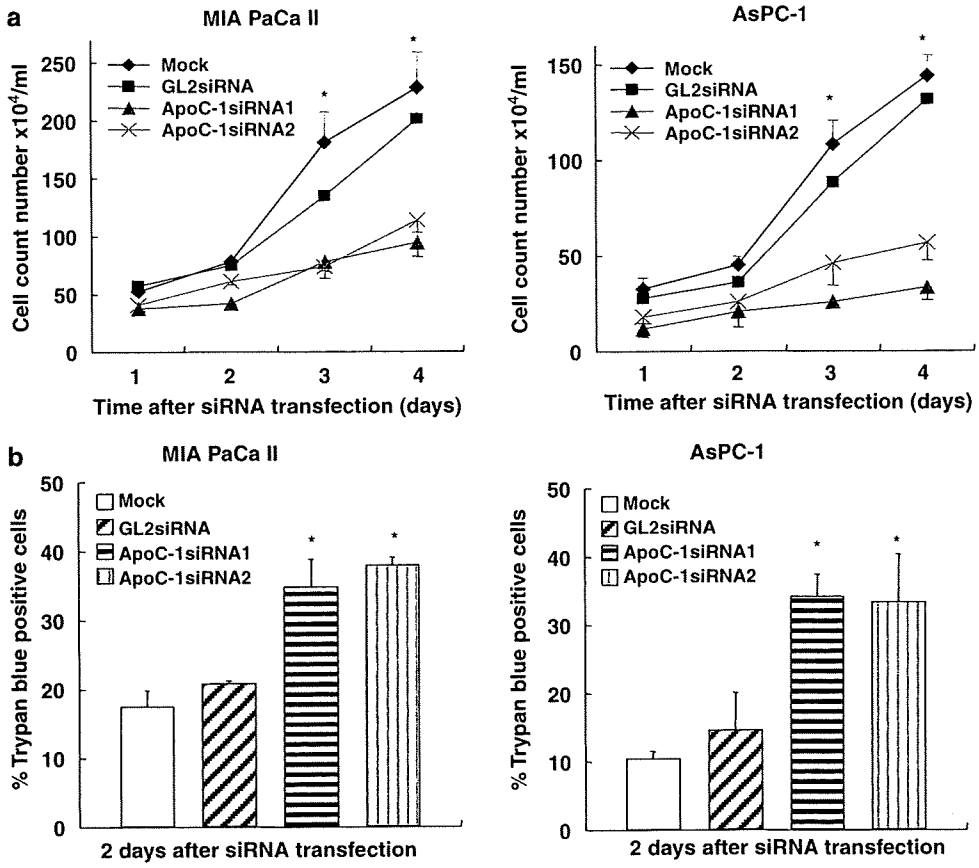

Figure 6 Inhibition of apolipoprotein C-1 (ApoC-1) suppresses cell proliferation and increases cell death. (**a**) Inhibition of ApoC-1 expression by ApoC-1 short interfering RNAs (siRNAs) significantly suppresses proliferation of pancreatic cancer cell lines (MIA PaCa II and AsPC-1). Cells were transfected with Mock, GL2siRNA, ApoC-1 siRNA1 and -2 and cultured for the indicated time (differences between GL2siRNA- and ApoC-1 siRNAs-treated cells; $*P<0.05$). (**b**) ApoC-1 siRNAs transfection increased the ratio of dead cells in MIA PaCa II and AsPC-1. Cell death was determined by the Trypan blue exclusion test at 2 days after siRNAs transfection. ApoC-1 siRNA transfected cells show significantly higher proportion of dead cells than GL2siRNA-transfected cells ($*P<0.05$).

This finding indicates the usefulness of the ApoC-1 serum level as a potentially prognostic marker for pancreatic cancer. In support of this, using serum protein profiling, several groups have recently reported increased serum levels of other apolipoproteins in patients with several types of cancer (Yu *et al.*, 2005; Goncalves *et al.*, 2006). On the other hand, Ehmann *et al.* (2007) recently found that serum levels of different apolipoproteins (ApoA-1 and -2) were decreased in pancreatic cancer patients compared with healthy volunteers. These facts may indicate that several apolipoproteins have different roles in pancreatic cancer development. Further analysis of the other peaks that were differentially expressed in the pre- and post-operative sera of pancreatic cancer patients will provide new and important information.

This is the first study that has found that ApoC-1 is highly expressed in pancreatic cancer cells but is faintly expressed in normal pancreatic ductal and stromal cells that surround cancerous cells. These findings are supported by studies indicating that ApoC-1 mRNA was highly expressed in pancreatic cancer tissues based on the serial analysis of gene expression analysis (Ryu *et al.*, 2001; Iacobuzio-Donahue *et al.*, 2002). We also found that ApoC-1 was expressed in the supernatant of medium used to culture pancreatic cancer cells. Based on these results, we consider that serum ApoC-1 protein is derived from cancer cells; ApoC-1 is overexpressed in the neoplastic epithelium of pancreatic cancer and is secreted into the blood, which results in elevated serum ApoC-1 levels. This is also supported by the correlation between ApoC-1 expression in cancer cells and high serum peak intensity in preoperative serum.

The correlation of ApoC-1 levels with overall survival in pancreatic cancer, together with its specific expression in cancer cells, may indicate that this protein is involved in cancer progression. This encouraged us to analyse whether ApoC-1 involves in cancer cell proliferation. Using siRNA, we showed that the inhibition of ApoC-1 expression suppressed cell proliferation of pancreatic cancer cell lines. Moreover, we found that this reduced cell proliferation was due to the increased rate of apoptotic cell death. These facts suggest that expression

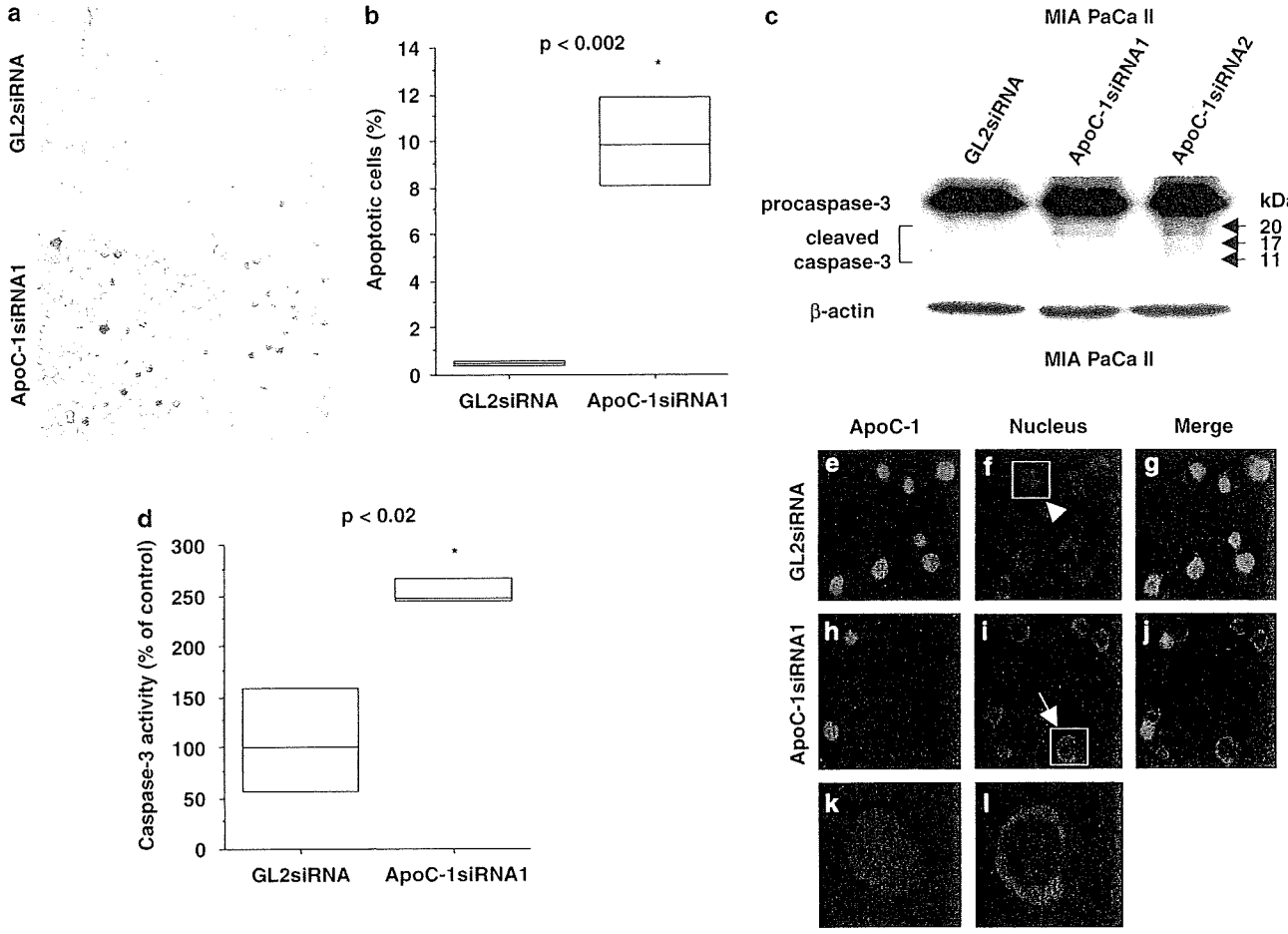

Figure 7 Apolipoprotein C-1 (ApoC-1) short interfering RNA (siRNA) treatment induces apoptotic cell death in MIA PaCa II. (**a**) APOPercentage assay is carried out at 24 h after siRNA transfection and representative results of GL2siRNA and ApoC-1 siRNA1-transfected cells are shown. Apoptotic cells are stained purple red. (**b**) The ratio of apoptotic cells is significantly higher in cells treated with ApoC-1 siRNA1 than with GL2siRNA. (**c**, **d**) ApoC-1 siRNAs induce the activation of procaspase-3 in MIA PaCa II. (**c**) Western blot analysis showed that the inhibition of ApoC-1 expression increases the cleaved caspase-3 (20, 17 and 11 Da; active form) compared to control (cells treated with GL2 siRNA). (**d**) Effect of the inhibition of ApoC-1 on caspase-3 activity in MIA PaCa II. Values represent percentage of control cells treated with GL2siRNA. ApoC-1 siRNA1-transfected cells show significantly higher proportion of caspase-3 activity than GL2siRNA-transfected cells at 24 h after transfection. (**e–l**) Immunofluorescence studies of ApoC-1 in MIA PaCa II cells. Cells treated with GL2 siRNA (**e–g**) and ApoC-1 siRNA1 (**h–j**) are shown. ApoC-1 staining with red color in the cytoplasm (**e**, **h**), nucleus with blue-stained by DAPI (**f**, **i**), and merge features (**g**, **j**). The nuclear morphology displayed apoptotic cell death with chromatin condensation by ApoC-1 siRNA treatment (arrow in (**i**); magnified picture showed in (**l**)), compared nonapoptotic cell treated with GL2 siRNA (arrow head in (**f**); magnified picture showed in (**k**)).

of ApoC-1 (secreted by autocrine manner) is essential for cancer cell survival by preventing from apoptosis, contributing to the malignant phenotype of pancreatic cancer. Supporting this, silencing expression of the apolipoprotein J gene, another apolipoprotein, in osteosarcoma and prostate cancer cells induced a significant reduction of cellular growth and high rates of spontaneous endogenous apoptosis (Trougakos *et al.*, 2004). Chen *et al.* (2005) also showed that the inhibition of apolipoprotein E, which is genetically linked closely with ApoC-1 (Lauer *et al.*, 1988), in ovarian cancer cells led to G2 cell-cycle arrest and apoptosis. In addition, RELN pathway through signaling via the VLDL receptor, to which ApoC-1 is known to bind, influences cell motility in pancreatic cancer (Sato *et al.*, 2006). It is very tempting to speculate that the inhibition of ApoC-1 expression in pancreatic cancer suppresses the tumor progression *in vivo*, and siRNA oligonucleotides against ApoC-1 may prove valuable agents for antipancreatic cancer therapy.

In conclusion, we found that serum levels of ApoC-1, which appears to be secreted by cancer cells, can predict the prognosis of pancreatic cancer patients. We also found an unexpected role of ApoC-1 in regulating cancer cell proliferation by avoiding spontaneous apoptotic cell death. Further research to determine the molecular mechanisms whether ApoC-1 inhibits apoptosis in cancer cells is warranted and will likely lead to the discovery of new therapies for pancreatic cancer using ApoC-1 as a therapeutic target.

Materials and methods

Patient samples and cell lines

To identify novel serum markers, serum samples were collected pre- and postoperatively (3–4 weeks after surgery, when the serum levels of C-reactive protein returned to the normal range) from 20 pancreatic cancer patients who had curative surgery. For the validation study, blood samples were obtained preoperatively from 69 patients diagnosed with primary invasive pancreatic ductal carcinoma who had surgery in the Department of General Surgery, Chiba University Hospital, Chiba, Japan, from June 2001 to April 2006. All blood samples were processed according to a standardized protocol, and serum samples were immediately frozen in aliquots at −80 °C until the proteomic study was done. In all patients, the diagnoses of carcinoma were confirmed histologically. Patient characteristics are summarized in Table 1 (Sobin and Wittekind, 2002). None of the patients received any additional therapies, such as radiation or chemotherapy, pre- or postoperatively, until serum samples were collected. The excised pancreatic tissue samples were placed in liquid nitrogen and stored at −80 °C until use. The ethics committee of our institute approved the protocol. Written informed consent was obtained from all patients. The four human pancreatic cancer cell lines, MIA PaCa II and PanC-1 (American Type Culture Collection, Manassas, VA, USA), CFPAC-1 and AsPC-1 (DS Pharma Biomedical Co., Ltd., Japan) that were used in this study were cultured in the appropriate medium and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

SELDI-TOF MS analysis

To discover the candidate protein, an aliquot of the stored 20-paired pre- and postoperative serum samples was used for SELDI-TOF MS analysis with a weak cationic exchanger 2 (WCX2; Ciphergen Biosystems, Fremont, CA, USA; see Supplementary methods for detail). Each analysis was carried out in duplicate. Peak detection was carried out using ProteinChip Software 3.1 (Ciphergen).

For the validation study, the peak intensity of 6630 and 6420 Da proteins was measured using the SELDI-TOF MS analysis on CM10 ProteinChip arrays (Ciphergen) in 69 preoperative sera of pancreatic cancer patients; the measurements were done in duplicate under the same urea buffer condition as the WCX2 expériment. To reduce the coefficient of variation for peak intensities, we used a robot, Biomek 3000 Laboratory Automation Workstation (Beckman Coulter Inc., Fullerton, CA, USA) in this validation study. Mass accuracy was calibrated externally with an all-in-one-peptide molecular mass standard (Ciphergen).

Isolation and identification of the target proteins

The candidate proteins were purified, isolated and identified (see Supplementary methods for detail). After purification, the target protein was identified by N-terminal amino-acid sequence analysis. The immunodepletion assay and western blot were carried out for the confirmation of the identified protein.

RT–PCR and quantitative RT–PCR

Total RNA was extracted with the RNeasy Mini Kit (Qiagen Tokyo, Japan) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA with the T-Primed First-Strand Kit for RT–PCR (Amersham Biosciences, Buckinghamshire, UK). Human brain (cerebellum) total RNA (BD Bioscience Clontech, Takaka Bio company, Shiga, Japan) was used as positive control of ApoC-1 (Lauer *et al.*, 1988). Quantitative RT–PCR was carried out as previously described (Mitsuhashi *et al.*, 2003) using LightCycler with LightCycler-Fast Start DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany; see Supplementary Methods for detail).

Western blot analysis

The extracted proteins from frozen tissue samples, the cultured cells and the supernatant of the cultured cells were subjected to western blot analysis using mouse anti-human ApoC-1 monoclonal antibody (CHEMICON International, Temecula, CA, USA) and rabbit anti-human procaspase-3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; see Supplementary Methods for detail).

Immunohistochemistry

Paraffin-embedded tissues were cut in 4-μm-thick serial sections and were deparaffinized. These slides were placed in citric acid buffer (10 mmol l^{-1}, pH 6.0) with 0.2% Tween 20 and boiled in a microwave oven (2 × 6 min) to retrieve the antigen. The slides were then rinsed and blocked in 10% H_2O_2 solution with methanol for 10 min. Next, they were incubated with mouse anti-human ApoC-1 monoclonal antibody (CHEMICON International) at 1:200 dilution overnight at 4 °C. They were then rinsed in phosphate-buffered saline (PBS), and incubated for 60 min with secondary antibody labeled with streptoavidin-biotin-peroxidase (DAKO LSAB2 kit, DakoCytomation, Glostrup, Denmark). The bound complex was visualized using diaminobenzidine liquid chromogen and counterstained with hematoxylin. Mouse-monoclonal IgG2a (X0943, DAKO) was used as a negative control at an optimal dilution.

Gene knockdown using siRNA

Short interfering RNA (Hannon and Rossi, 2004) that specifically targeted ApoC-1 mRNA was used to reduce ApoC-1 expression. The target sequences for ApoC-1 RNA interference were as follows; ApoC-1 siRNA1: 5′-CTGGAG GACAAGGCTCGGGAA-3′, ApoC-1 siRNA2: 5′-CTGAAG GAGTTTGGAAACACA-3′. Double-stranded synthetic siRNA1 and 2, and luciferase (GL2) siRNA as a negative control, were purchased from Qiagen. *In vitro* transfection was carried out using Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions (see Supplementary Methods for detail).

Cell proliferation assay and Trypan blue exclusion test

A total of 20×10^4 MIA PaCa II and AsPC-1 human pancreatic cancer cell lines in six-well plates, were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% heat-inactivate fetal bovine serum (FBS), and were cultured in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) with 20% FBS, respectively, incubated in a humidified atmosphere containing 5% CO_2 at 37 °C for 24 h. After washing with PBS, these cells were transfected with siRNA (20 nM final concentration). Both attached and floating cells were corrected with trypsinization. After staining with Trypan blue, the total cell number and the number of Trypan blue positive cells were counted on days 1–4 after transfection. All of these experiments were carried out in triplicate for three times, independently. The medium was changed every 2 days.

Invasion assay

A total of 1×10^5 MIA PaCa II or AsPC-1 cells in Opti-MEM I Reduced Serum Medium were plated onto BD BioCoat

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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References

Bardeesy N, Depinho RA. (2002). Pancreatic cancer biology and genetics. *Nat Rev* **2**: 897–909.

Burris III HA, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR *et al.* (1997). Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* **152**: 2403–2413.

Chen X, Lin J, Kanekura T, Su J, Lin W, Xie H *et al.* (2006). A small interfering CD147-targeting RNA inhibited the proliferation, invasiveness, and metastatic activity of malignant melanoma. *Cancer Res* **66**: 11323–11330.

Chen YC, Pohl G, Wang TL, Morin PJ, Risberg B, Kristensen GB *et al.* (2005). Apolipoprotein E is required for cell proliferation and survival in ovarian cancer. *Cancer Res* **65**: 331–337.

Ehmann M, Felix K, Hartmann D, Schnolzer M, Nees M, Vorderwulbecke S *et al.* (2007). Identification of potential markers for the detection of pancreatic cancer through comparative serum protein expression profiling. *Pancreas* **34**: 205–214.

Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* **148**: 2207–2216.

Ferrone CR, Finkelstein DM, Thayer SP, Muzikansky A, Fernandez-delCastillo C, Warshaw AL. (2006). Perioperative CA19-9 levels can predict stage and survival in patients with respectable pancreatic adenocarcinoma. *J Clin Oncol* **24**: 2897–2902.

Goncalves A, Esterni B, Bertucci F, Sauvan R, Chabannon C, Cubizolles M *et al.* (2006). Postoperative serum proteomic profiles may predict metastatic relapse in high-risk primary breast cancer patients receiving adjuvant chemotherapy. *Oncogene* **25**: 981–989.

Hannon GJ, Rossi JJ. (2004). Unlocking the potential of the human genome with RNA interference. *Nature* **431**: 371–378.

Iacobuzio-Donahue CA, Ryu B, Hruban RH, Kern SE. (2002). Exploring the host desmoplastic response to pancreatic carcinoma. *Am J Pathol* **160**: 91–99.

Jemal A, Siegel R, Ward E, Murray T, Xu J, Sumigal C *et al.* (2006). Cancer statistics, 2006. *CA Cancer J Clin* **56**: 106–130.

Lauer SJ, Walker D, Elshourbagy NA, Reardon CA, Wilson BL, Taylor JM. (1988). Two copies of the human apolipoprotein C-I gene are linked closely to the apolipoprotein E gene. *J Biol Chem* **263**: 7277–7286.

Melle C, Ernst G, Schimmel B, Bleul A, Thieme H, Kaufmann R *et al.* (2005). Discovery and identification of á-defensins as low abundant, tumor-derived serum markers in colorectal cancer. *Gastroenterology* **129**: 66–73.

Mitsuhashi N, Shimizu H, Ohtsuka M, Wakabayashi Y, Ito H, Kimura F *et al.* (2003). Angiopoietins and tie-2 expression in angiogenesis and proliferation of human hepatocellular carcinoma. *Hepatology* **37**: 1105–1113.

Moore MJ, Goldstein D, Hamm J, Figer A, Hechet JR, Gallinger S *et al.* (2007). Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* **25**: 1942–1952.

Mutaguchi K, Yasumoto H, Mita K, Matsubara A, Shiina H, Igawa M *et al.* (2003). Restoration of insulin-like growth factor binding

protein-related protein 1 has a tumor-suppressive activity through Induction of apoptosis in human prostate cancer. *Cancer Res* **63**: 7717–7723.

Nomura F, Tomonaga T, Sogawa K, Ohashi T, Nezu M, Sunaga M *et al.* (2004). Identification of novel and downregulated biomarkers for alcoholism by surface enhanced laser desorption/ionization-mass spectrometry. *Proteomics* **4**: 1187–1194.

Paradis V, Degos F, Dargere D, Pham N, Belghiti J, Degott C *et al.* (2005). Identification of a new marker of hepatocellular carcinoma by serum protein profiling of patients with chronic liver diseases. *Hepatology* **41**: 40–47.

Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM *et al.* (2002). Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* **359**: 572–577.

Rhodes J. (1999). Usefulness of novel tumor markers. *Ann Oncol* **10**: 118–121.

Rosty C, Christa L, Kuzdzal S, Baldwin WM, Zahurak ML, Carnot F *et al.* (2002). Identification of hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein I as a biomarker for pancreatic ductal adenocarcinoma by protein biochip technology. *Cancer Res* **62**: 1868–1875.

Ryu B, Jones J, Hollingsworth MA, Hruban RH, Kern SE. (2001). Invasion-specific genes in malignancy: serial analysis of gene expression comparisons of primary and passaged cancers. *Cancer Res* **61**: 1833–1838.

Sato N, Fukushima N, Chang R, Matsubayashi H, Goggins M. (2006). Differential and epigenetic gene expression profiling identifies frequent disruption of the RELN pathway in pancreatic cancers. *Gastroenterology* **130**: 548–565.

Schaefer EJ, Eisenberg S, Levy RI. (1978). Lipoprotein apoproteins metabolism. *J Lipid Res* **19**: 667–687.

Shida T, Furuya M, Nikaido T, Hasegawa M, Koda K, Oda K *et al.* (2006). Sonic hedgehog-Gli1 signaling pathway might become an effective therapeutic target in gastrointestinal neuroendocrine carcinomas. *Cancer Biol Ther* **5**: 1530–1538.

Sobin LH, Wittekind CH. (2002). *International Union Against Cancer (UICC): TNM Classification of Malignant Tumours*, 6th edn. Wiley: New York.

Tomonaga T, Matsushita K, Yamaguchi S, Oh-Ishi M, Kodera Y, Maeda T *et al.* (2004). Identification of altered protein expression and post-translational modifications in primary colorectal cancer by using agarose two-dimensional gel electrophoresis. *Clin Cancer Res* **10**: 2007–2014.

Trougakos IP, So A, Jansen B, Gleave ME, Gonos ES. (2004). Silencing expression of the clusterin/apolipoprotein J gene in human cancer cells using small interfering RNA induces spontaneous apoptosis, reduced growth ability, and cell sensitization to genotoxic and oxidative stress. *Cancer Res* **64**: 1834–1842.

Wroblewski MS, Wilson-Grady JT, Martinez MB, Kasthuri RS, McMillan KR, Flood-Urdangarin C *et al.* (2006). A functional polymorphism of apolipoprotein C1 detected by mass spectrometry. *FEBS J* **273**: 4707–4715.

Xiao Z, Adam BL, Cazares LH, Clements MA, Davis JW, Schellhammer PF *et al.* (2001). Quantitation of serum prostate-specific membrane antigen by a novel protein biochip immunoassay discriminates benign from malignant prostate disease. *Cancer Res* **61**: 6029–6033.

Xiao Z, Prieto D, Conrads TP, Veenstra TD, Issaq HJ. (2005). Proteomic patterns: their potential for disease diagnosis. *Mol Cell Endocrinol* **230**: 95–106.

Yu KH, Rustgi AK, Blair IA. (2005). Characterization of proteins in human pancreatic cancer serum using differential gel electrophoresis and tandem mass spectrometry. *J Proteome Res* **4**: 1742–1751.

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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

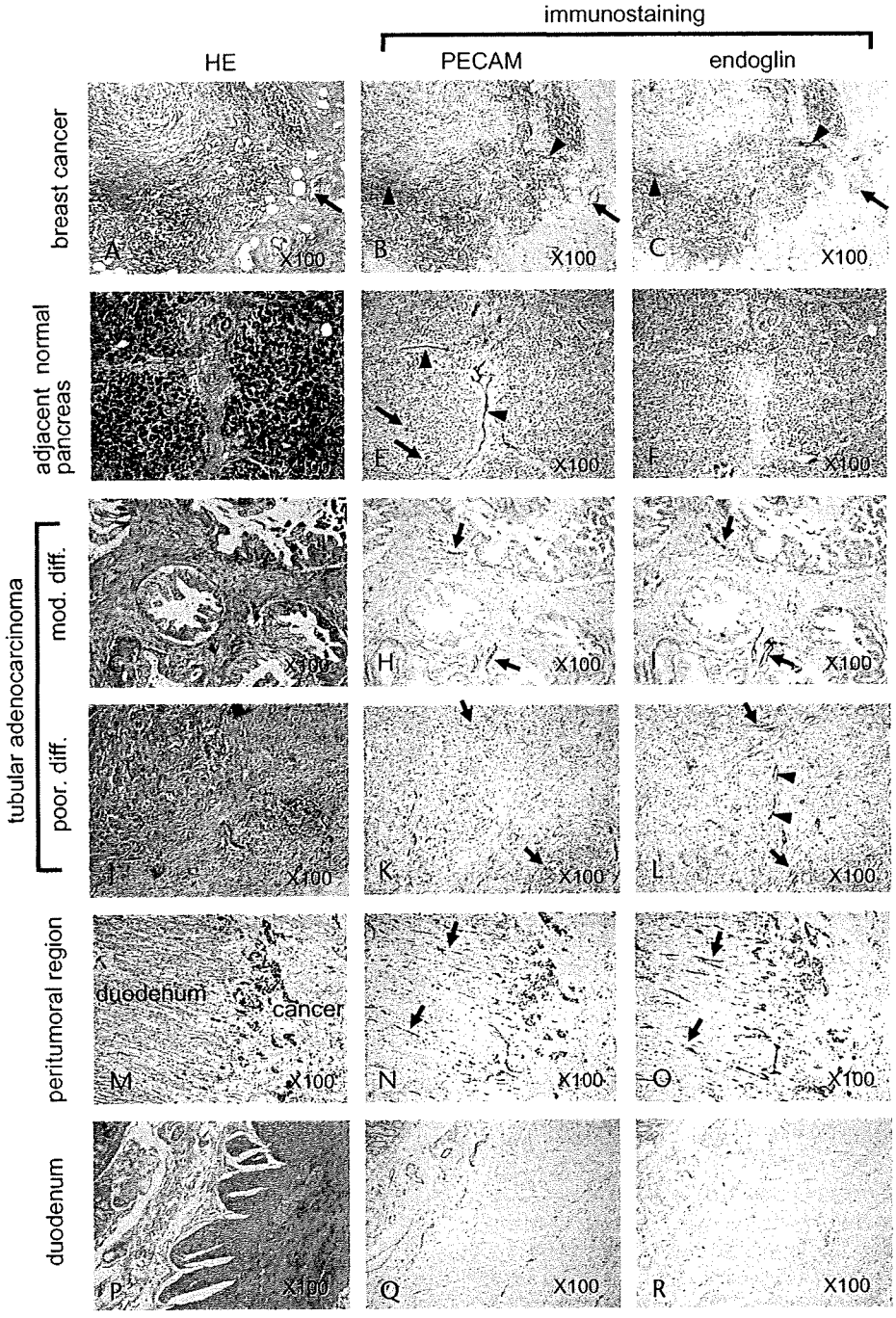

FIGURE 1. Endoglin is expressed in peritumoral and intratumoral vessels in pancreatic cancer. Sequential sections were subjected to HE staining (A, D, G, J, M, P) and immunostaining with anti-PECAM antibody (B, E, H, K, N, Q) and anti-endoglin antibody (C, F, I, L, O, R). A–C, Endothelial cells of blood 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.

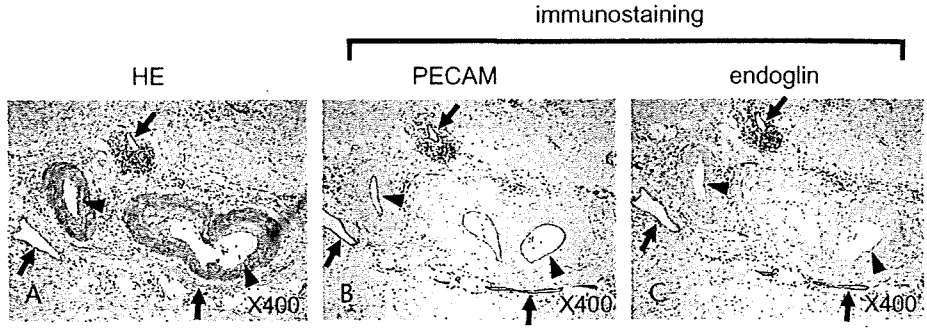

FIGURE 2. Endoglin is expressed only in endothelial cells of small vessels with thin walls and not in arterial vessels. Sequential sections of pancreatic cancer tissues were subjected to HE staining (A) and immunostaining with anti-PECAM (B) and anti-endoglin (C) antibodies. Endothelial cells of arteries 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

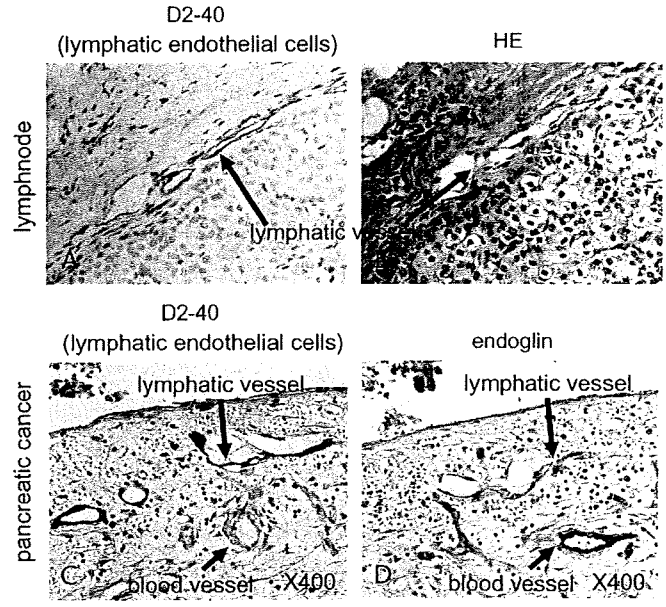

FIGURE 3. Endoglin is expressed in small lymphatic vessels in pancreatic cancer tissue. A and B, Lymphatic vessels in lymph node are stained with anti–D2-40 antibody (A). B, HE staining of the sequential section. C and D, Immunostaining with D2-40 (C) and anti-endoglin (D) antibodies. The endothelial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

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

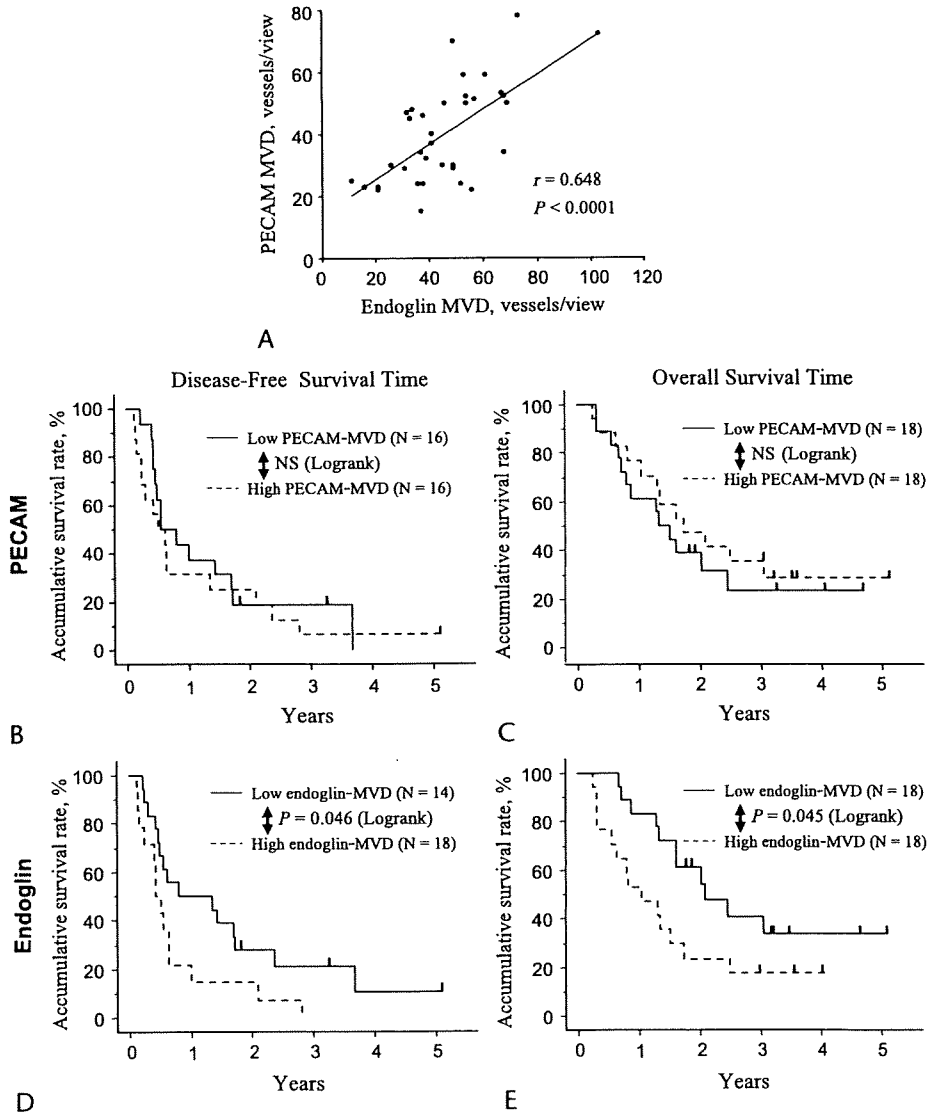

FIGURE 4. Microvessel density (MVD) assessed by endoglin immunostaining (endoglin-MVD) is significantly correlated with disease-free and overall survival, but MVD assessed by PECAM immunostaining (PECAM-MVD) does not show these relationships. A, Endoglin-MVD and PECAM-MVD were strongly correlated ($P < 0.0001$). 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.

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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REFERENCES

1. Fennelly D, Kelsen DP. The role of chemotherapy in the treatment of adenocarcinoma of the pancreas. *Hepatogastroenterology.* 1996;43: 356–362.
2. Yamanaka Y, Friess H, Buchler M, et al. Overexpression of acidic and basic fibroblast growth factors in human pancreatic cancer correlates with advanced tumor stage. *Cancer Res.* 1993;53:5289–5296.
3. Itakura J, Ishikawa T, Friess H, et al. Enhanced expression of vascular endothelial growth factor in human pancreatic cancer correlates with local disease progression. *Clin Cancer Res.* 1997;3:1309–1316.
4. Ikeda N, Adachi M, Taki T, et al. Prognostic significance of angiogenesis in human pancreatic cancer. *Br J Cancer.* 1999;79:1553–1563.
5. Seo Y, Baba H, Fukuda T, et al. High expression of vascular endothelial growth factor is associated with liver metastasis and a poor prognosis for patients with ductal pancreatic adenocarcinoma. *Cancer.* 2000; 88:2239–2245.
6. Baker CH, Solorzano CC, Fidler IJ. Blockade of vascular endothelial growth factor receptor and epidermal growth factor receptor signaling for therapy of metastatic human pancreatic cancer. *Cancer Res.* 2002; 62:1996–2003.
7. Bockhorn M, Tsuzuki Y, Xu L, et al. Differential vascular and transcriptional responses to anti-vascular endothelial growth factor antibody in orthotopic human pancreatic cancer xenografts. *Clin Cancer Res.* 2003;9:4221–4226.
8. Buchler P, Reber HA, Ullrich A, et al. Pancreatic cancer growth is inhibited by blockade of VEGF-RII. *Surgery.* 2003;134:772–782.
9. Kindler HL, Friberg G, Singh DA, et al. Phase II trial of bevacizumab plus gemcitabine in patients with advanced pancreatic cancer. *J Clin Oncol.* 2005;23:8033–8040.
10. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature.* 2000;407:249–257.
11. Arap W, Pasqualini R, Ruoslahti E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science.* 1998;279: 377–380.
12. Cheifetz S, Bellon T, Cales C, et al. Endoglin is a component of the transforming growth factor-b receptor system in human endothelial cells. *J Biol Chem.* 1992;267:19027–19030.
13. Lebrin F, Goumans MJ, Jonker L, et al. Endoglin promotes endothelial cell proliferation and TGF-b/ALK1 signal transduction. *EMBO J.* 2004;23:4018–4028.
14. Li DY, Sorensen LK, Brooke BS, et al. Defective angiogenesis in mice lacking endoglin. *Science.* 1999;284:1534–1537.
15. Wang JM, Kumar S, Pye D, et al. A monoclonal antibody detects heterogeneity in vascular endothelium of tumors and normal tissues. *Int J Cancer.* 1993;54:363–370.
16. Akagi K, Ikeda Y, Sumiyoshi Y, et al. Estimation of angiogenesis with anti-CD105 immunostaining in the process of colorectal cancer development. *Surgery.* 2002;131:S109–S113.
17. Ho JW, Poon RT, Sun CK, et al. Clinicopathological and prognostic implications of endoglin (CD105) expression in hepatocellular carcinoma and its adjacent non-tumorous liver. *World J Gastroenterol.* 2005; 11:176–181.
18. Yao Y, Kubota T, Takeuchi H, et al. Prognostic significance of microvessel density determined by an anti-CD105/endoglin monoclonal antibody in astrocytic tumors: comparison with an anti-CD31 monoclonal antibody. *Neuropathology.* 2005;25:201–206.
19. Fonsatti E, Altomonte M, Nicotra MR, et al. Endoglin (CD105): a powerful therapeutic target on tumor-associated angiogenetic blood vessels. *Oncogene.* 2003;22:6557–6563.
20. Tan GH, Wei YQ, Tian L, et al. Active immunotherapy of tumors with a recombinant xenogeneic endoglin as a model antigen. *Eur J Immunol.* 2004;34:2012–2021.
21. Weidner N, Semple JP, Welch WR, et al. Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. *N Engl J Med.* 1991;324:1–8.
22. Vecchi A, Garlanda C, Lampugnani MG, et al. Monoclonal antibodies specific for endothelial cells of mouse blood vessels. Their application in the identification of adult and embryonic endothelium. *Eur J Cell Biol.* 1994;63:247–254.
23. Kahn HJ, Bailey D, Marks A. Monoclonal antibody D2-40, a new marker of lymphatic endothelium, reacts with Kaposi's sarcoma and a subset of angiosarcomas. *Mod Pathol.* 2002;15:434–440.
24. Li D, Xie K, Wolff R, et al. Pancreatic cancer. *Lancet.* 2004;363: 1049–1057.
25. Hirakawa S, Hong Y, Harvey N, et al. Identification of vascular lineage-specific genes by transcriptional profiling of isolated blood vascular and lymphatic endothelial cells. *Am J Pathol.* 2003;162: 575–586.
26. Salven P, Mustjoki S, Alitalo R, et al. VEGFR-3 and CD133 identify a population of CD34+ lymphatic/vascular endothelial precursor cells. *Blood.* 2003;101:168–172.
27. Alitalo K, Tammela T, Petrova TV. Lymphangiogenesis in development and human disease. *Nature.* 2005;438:946–953.
28. Achen MG, Mann GB, Stacker SA. Targeting lymphangiogenesis to prevent tumor metastasis. *Br J Cancer.* 2006;94:1355–1360.
29. Barbara NP, Wrana JL, Letarte M. Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor-b superfamily. *J Biol Chem.* 1999;274:584–594.
30. Li C, Hampson IN, Hampson L, et al. CD105 antagonizes the inhibitory signaling of transforming growth factor b1 on human vascular endothelial cells. *FASEB J.* 1999;14:55–64.
31. Seon BK, Matsuno F, Haruta Y, et al. Long-lasting complete inhibition of human solid tumors in SCID mice by targeting endothelia cells of tumor vasculature with antihuman endoglin immunotoxin. *Clin Cancer Res.* 1997;3:1031–1044.
32. Matsuno F, Haruta Y, Kondo M, et al. Induction of lasting complete regression of preformed distinct solid tumors by targeting the tumor vasculature using two new anti-endoglin monoclonal antibodies. *Clin Cancer Res.* 1999;5:371–382.
33. Tabata M, Kondo M, Haruta Y, et al. Antiangiogenic radioimmunotherapy of human solid tumors in SCID mice using 125I-labeled anti-endoglin monoclonal antibodies. *Int J Cancer.* 1999;82:737–742.
34. Takahashi N, Haba A, Matsuno F, et al. Antiangiogenic therapy of established tumors in human skin/severe combined immunodeficiency mouse chimeras by anti-endoglin (CD105) monoclonal antibodies, and synergy between anti-endoglin antibody and cyclophosphamide. *Cancer Res.* 2001;61:7846–7854.
35. Tsujie M, Uneda S, Tsai H, et al. Effective anti-angiogenic therapy of established tumors in mice by naked anti-human endoglin (CD105) antibody: Differenced in growth rate and therapeutic response between tumors growing at different sites. *Int J Oncol.* 2006;29:1087–1094.

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.**

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.

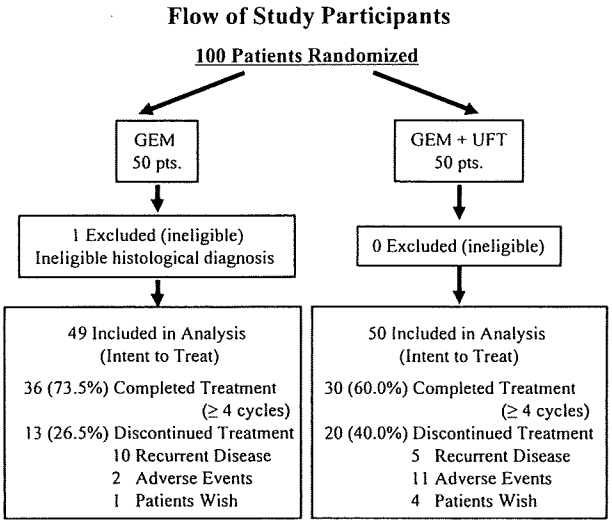

FIGURE 1. The flow of study participants is depicted. GEM indicates gemcitabine alone; UFT, tegafur/uracil.

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