from 5.0 to 14.0 months, which seem to be much higher than in previous trials using gemcitabine alone (22-25). Gemcitabine combined with other agents may provide other potential options of building on the activity of gemcitabine. Therefore, we thought that a further synergy effect was obtained by using triple agent chemotherapy for advanced biliary carcinoma.

In this trial, we did not experience an impressive response such as that stated in the previous report. It is difficult to compare directly the response rate in different trials because of the relatively small sample size and the heterogeneous patient populations of their series. However, in this trial, both response rates and median survival was relatively favorable compared with other reports. Furthermore, a relatively high disease-control rate was observed. This combination of triple agents was relatively well tolerated. While non-hematological toxicity of grade 3/4 was not observed through this trial, the relatively high incidence of leucocytopenia in our patients compared to the previous report is an important concern. Therefore, dose modification was needed in particular this morbid patients. However, there were no serious outcomes of adverse events in this trial.

Interestingly, according to the tumor site, the difference of tumor response was experienced in this trial. While similar response rates were observed in gallbladder (25.0%) carcinoma than that in biliary tract carcinoma (20.0%), over all survival time (median: 6.0 months *v.s.* 9.6 months) and TTP (median: 2.2 months *v.s.* 6.0 months) was better in biliary tract carcinoma than in gallbladder. Moreover, the disease control period in gallbladder carcinoma was within about 2 months, and this period was very much shorter than that of the biliary tract carcinoma. A reason for this is that carcinomas of biliary and gallbladder may be biologically sufficiently different as to lead to different sensitivities to chemotherapy. In other several reports, response rates of patients with gallbladder carcinoma were higher compared with biliary tract carcinoma, though there was no significant difference in overall survival rates between gallbladder carcinoma and biliary tract carcinoma (26). However, the relationship between chemosensitivity and cellular origin of biliary carcinoma, as well as the differential activity of gemcitabinem, cisplatin and 5-FU on advanced biliary carcinoma, remains to be clarified.

In conclusion, our trial indicates that a regimen of gemcitabine combined with 5-fluorouracil and cisplatin is an active and relatively well tolerated new therapeutic option as first line chemotherapy for patients with advanced biliary tract carcinoma. Furthermore, this regimen warrants further evaluation in detail to determine whether biological activity according to the tumor site of the biliary carcinoma reflects chemosensitivity in a stratification strategy.

FOOTNOTES:

The authors have no conflict of interest to declare regarding the contents of this manuscript.

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Tumor–stromal interactions with direct cell contacts enhance proliferation of human pancreatic carcinoma cells

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Pancreatic ductal adenocarcinoma is often characterized by an abundant desmoplastic stroma that is partially induced by activated pancreatic stellate cells (PSCs). Indirect co-culture has often been used to investigate the effects of cancer–stromal interactions on the proliferation of cancer cells, but the effects of cell–cell adhesion and juxtacrine signaling between cancer and stromal cells cannot be evaluated using this method. This study aimed to establish a simplified direct co-culture system that could be used to quantify populations of cancer cells in co-culture with PSCs, and to evaluate the effects of direct cell contact on the proliferation of cancer cells. We established three green fluorescent protein (GFP)-expressing pancreatic cancer cell lines and were able to quantify them with high reliability and reproducibility, even when co-cultured directly with PSCs, using a color plate reader. We assessed the differential effects of direct and indirect co-culture with PSCs on the proliferation of cancer cells, and found that the proliferation of GFP-expressing pancreatic cancer cell lines was dramatically enhanced by direct co-culture with PSCs, compared with the indirect co-culture system. We also found that direct co-culture of cancer cells and PSCs activated the Notch signaling pathway in both cell types. Direct cell contact between cancer cells and PSCs plays an important role in the control of cancer cell proliferation, and is essential to the understanding of tumor–stromal interactions. (*Cancer Sci* 2009; 100: 2309–2317)

Pancreatic ductal adenocarcinoma (PDAC) is often characterized by an abundant desmoplastic stroma,[1–3] which is defined as a proliferation of fibrotic tissue with an altered extracellular matrix (ECM) that is conductive to tumor growth and metastasis.[4–7] The host's desmoplastic reaction is characterized by complex interactions between normal host epithelial cells, invading tumor cells, stromal fibroblasts, inflammatory cells, proliferating endothelial cells, the altered ECM, and growth factors, which activate oncogenic signaling pathways by autocrine and paracrine mechanisms.[7–10] Recently, a pronounced increase in the number of α-smooth muscle actin (α-SMA)-positive myofibroblasts was reported in PDAC.[3] In addition, other studies have demonstrated that pancreatic stellate cells (PSCs) are associated with tumor desmoplasia.[1,8,11] Although the desmoplastic reaction was initially regarded as a host barrier against tumor invasion, it has become evident that pancreatic cancer cells induce fibrosis by activating PSCs to synthesize excessive ECM.[1,3,12] The ECM influences the growth, differentiation, survival, and motility of cells by both providing a physical scaffold and acting as a reservoir for soluble mitogens.[5,6,9,10] PSCs have also been reported to inhibit apoptosis[13,14] and enhance the migration and invasion of pancreatic cancer cells.[14,15] The tumor-supportive microenvironment is thus a dynamic environment that promotes tumor growth and invasion.

Several models have been established to investigate tumor–stromal interactions, including *in vivo* xenograft models,[16–20] *in vitro* three-dimensional co-culture models,[21–24] *in vitro* two-chamber co-culture models using culture inserts,[10,18,25–29] and *in vitro* direct co-culture models.[30–32] The two-chamber co-culture models, which are often used for *in vitro* experiments, are not suitable for investigating the effects of direct cell contacts between stromal cells and cancer cells on tumor biology. In contrast, the *in vivo* xenograft and *in vitro* direct co-culture models can be used to evaluate the effects of cell–cell adhesion and juxtacrine signaling, but simple and reproducible quantitative assessment of cell populations using these methods remains problematic. Krtolica *et al.*[33,34] established a method for quantifying a population of epithelial cells directly co-cultured with fibroblasts using fluorescence imaging of 4,6-diamidino-2-phenylindole (DAPI) and green fluorescent protein (GFP). In the present study, we modified and simplified this method to investigate the parameters affecting cell growth with high sensitivity, high reproducibility, and ease of handling, which are difficult to achieve with other available methods. We quantified the population of GFP-expressing cells using a color plate reader,[35–38] and were able to quantitatively detect GFP-expressing cancer cells, even in direct co-culture with PSCs. We compared the use of direct and indirect co-culture systems for investigating the effects of cell interactions with PSCs on the proliferation of GFP-expressing pancreatic cancer cell lines. Furthermore, to investigate the effects of the juxtacrine mechanism, we assessed the associations of the Notch signaling pathway with these two co-culture systems.

Materials and Methods

Establishment of cell lines constitutively expressing GFP. We used three pancreatic cancer cell lines in our study (Table 1). SUIT-2 and Panc-1 were generously provided by Dr H. Iguchi, (National Shikoku Cancer Center, Matsuyama, Japan), and MIA PaCa-2 was obtained from the Japanese Cancer Resource Bank (Tokyo, Japan). Cells were maintained as previously described.[18] A pAcGFP1-N1 vector (Clontech, Palo Alto, CA, USA) encoding GFP was used to create stable GFP-expressing cell lines (GFP-SUIT-2, GFP-Panc-1, and GFP-MIA PaCa2). The pAcGFP1-N1 vector was electroporated into SUIT-2, Panc-1, and MIA PaCa-2 cell lines using Nucleofector (Amaxa Biosystems, Koln, Germany), according to the manufacturer's instructions. SUIT-2 cells electroporated with pAcGFP1-N1 were selected for neomycin resistance (G418, 800 μg/mL) in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical

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Table 1. Cell lines

Cell line	Tissue origin	Diagnosis	Doubling time (h)
Pancreatic cancer cell lines constitutively expressing GFP			
GFP-SUIT-2	Liver metastasis	PDAC	19.56 ± 0.73
GFP-Panc-1	Pancreas	PDAC	22.24 ± 1.10
GFP-MIA PaCa-2	Pancreas	PDAC	16.81 ± 0.15
Fibroblast cell line			
MRC5	Human embryonic lung		27.75 ± 1.37
Primary cultured myofibroblasts			
NPF-1	Normal pancreas	Benign endocrine tumor	24.26 ± 2.48
NPF-2	Normal pancreas	Bile duct carcinoma	53.28 ± 2.47
PCF-1	Pancreatic cancer	PDAC	40.60 ± 0.75
PCF-2	Pancreatic cancer	PDAC	21.74 ± 2.18
MCF-1	Metastatic tumor of abdominal wall	PDAC	30.84 ± 3.15

GFP, green fluorescence protein; PDAC, pancreatic ductal adenocarcinoma.

Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL), and penicillin (100 U/mL). Green colonies were isolated and grown in the absence of selective pressure for several months. Panc-1 and MIA PaCa-2 cells electroporated with pAcGFP1-N1 were sorted using a cell sorter (Epics Altra; Beckman Coulter, Fullerton, CA, USA), according to the manufacturer's instructions.

Fibroblasts and myofibroblasts including PSCs. We used a human fibroblast cell line MRC5 (Riken, Tokyo, Japan). Two cultures of PSCs derived from normal pancreases without pancreatitis (NPF-1, from a patient with a benign endocrine tumor of the pancreas, and NPF-2, from a patient with bile duct cancer), two cultures of PSCs derived from pancreatic cancer tissues of patients with PDAC (PCF-1 and PCF-2), and a culture of myofibroblasts derived from a metastatic tumor of the abdominal wall in a patient with PDAC (MCF-1) were also used in this study (Table 1). All primary cultures of myofibroblasts were isolated using the outgrowth method, as described previously.[39] Cells were maintained as described previously.[18]

Propidium iodide (PI) assay. To calculate the doubling time of each cell line, cells were seeded in 24-well plates (Becton Dickinson Labware, Bedford, MA, USA) at a density of 1×10^4 cells/well, using cell numbers previously counted using a particle distribution analyzer (CDA 500; Sysmex, Kobe, Japan). Cell populations were evaluated by measuring the fluorescence intensity of PI at specified times, as described previously.[40]

GFP fluorescence measurements. The fluorescence of cells in multiwell plates was quantified in triplicate using a Cytofluor II (Perseptive Biosystems, Framingham, MA, USA) at gain 80, with filter settings of excitation at 485 nm with a bandwidth of 20 nm and emission at 530 nm with a bandwidth of 25 nm, as described previously.[35–38] Fluorescence intensity was calculated in relative fluorescence units (RFU). The nonspecific signal of wells containing cell-free medium or PSCs alone (blank value) was subtracted from the results to give the fluorescence signal of the GFP-expressing cells. To obtain sensitive and reproducible measurements, we used DMEM without phenol red.

***In vitro* direct co-culture system.** For proliferation assays, 1×10^4 GFP-expressing cancer cells were mixed with 1×10^4 stromal cells. Each cell mixture was seeded in a 24-well plate (1×10^4 cancer cells/well) in triplicate, and cultured in DMEM supplemented with 1% or 10% FBS. The fluorescence signals of each well were detected at specified times. To analyze GFP expression, 5×10^4 GFP-SUIT-2 cells were mixed with 5×10^4 PSCs, seeded in a six-well plate in triplicate, and cultured in DMEM supplemented with 10% FBS for 3 days. After harvesting the cells, the total cell number was determined using the CDA 500. The PSC/GFP-SUIT-2 cell proportion was determined using a cell sorter (Epics Altra) based on the GFP fluorescence as described previously,[30,32] and we isolated GFP-SUIT-2 cells and GFP-negative PSCs according to the manufacturer's instructions.

***In vitro* indirect co-culture system.** For proliferation assays, 1×10^4 GFP-expressing cancer cells were seeded in triplicate into the lower wells of a transwell cell culture system (24-well type, fluoroblock membrane with 3-μm pores; Becton Dickinson, San Jose, CA, USA) and 1×10^4 PSCs were seeded into the upper chambers (cell culture inserts), and cultured in DMEM supplemented with 1% or 10% FBS. The fluorescence signals of each well were detected at specified times. To analyze GFP expression, 5×10^4 GFP-SUIT-2 cells were seeded in triplicate into the lower wells of a transwell cell culture system (six-well type, 3-μm pores; Becton Dickinson) and 5×10^4 PSCs were seeded into the upper chambers, and cultured in DMEM supplemented with 10% FBS for 3 days. After harvesting the cells, the total cell number was determined using the CDA 500.

Immunoblot analysis for α-smooth muscle actin (α-SMA). Immunoblot analysis for α-SMA was performed as described previously.[18] Briefly, whole-cell lysates were fractionated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was incubated with 1:500 dilutions of monoclonal mouse antihuman α-SMA antibody (Dako, Glostrup, Denmark) overnight at 4°C, and then probed with antimouse IgG conjugated with horseradish peroxidase (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. Immunoblots were detected using the enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, UK) and visualized with a Molecular Imager (Chemi-Doc XRS System; Bio-Rad Laboratories, Hercules, CA, USA). The membrane was stripped and probed with anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), as an internal control.

Immunofluorescence staining of α-SMA. PSCs (5×10^4) were seeded on six-well plates and cultured in DMEM supplemented with 10% FBS for 24 h. Cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100, blocked with blocking solution (1% FBS and 1% BSA in PBS), and incubated with 1:500 dilutions of monoclonal mouse antihuman α-SMA antibody (Dako) for 2 h at room temperature. The cells were then incubated for 1 h with Alexa 546-conjugated antimouse IgG (Molecular Probes, Eugene, OR, USA) and 0.05 μg/mL DAPI. A TE-2000U inverted microscope (Nikon, Tokyo, Japan) was used for immunofluorescence microphotography and images were managed using VB-Viewer software (Keyence, Osaka, Japan).

Flow cytometry. Cellular expression of α-SMA was examined by flow cytometry (Epics Altra) using a phycoerythrin (PE)-conjugated monoclonal mouse antihuman α-SMA antibody (R&D

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Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Non-specific mouse IgG (Miltenyi Biotec, Auburn, CA, USA) was used as a negative control.

Quantitative real-time reverse transcription–polymerase chain reaction (qRT–PCR). Total RNA was extracted from cultured cells using a High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol. qRT–PCR was performed using a QuantiTect SYBR Green RT–PCR Kit (Qiagen, Tokyo, Japan) with Opticon4 (Bio-Rad Laboratories), as described previously.[41] Briefly, the reaction mixture was first incubated at 50°C for 15 min to allow reverse transcription. PCR was initiated with one cycle at 95°C for 10 min to activate modified Taq polymerase, followed by 45 cycles at 94°C for 15 s, 55°C for 20 s, and 72°C for 10 s, and one cycle at 95°C for 0 s, 65°C for 15 s, and +0.1°C/s to 95°C for melting analysis. Each sample was run in triplicate. The 10% deviation was calculated from the concentrations determined from the calibration curve. The level of mRNA expression was calculated from a standard curve constructed using total RNA from MRC5 cells. We designed specific primers (Table 2), and screened a database with BLASTN to confirm the specificity of these primers. Primers for *Snail* were designed by Takara Bio (primer set ID: HA075019; Ohtsu, Shiga, Japan). Expression of each mRNA was normalized to that of *18S rRNA*.

Statistical analysis. Statistical analyses and graph presentations were carried out using JMP 7 (SAS Institute, Cary, NC, USA). Values were expressed as the mean ± SD. Comparisons between two groups were performed using Student's *t*-test. The level of statistical significance was set at $P < 0.05$. Correlations between two groups were statistically evaluated by regression analysis and by calculating Spearman's rank-correlation coefficient.

Results

Correlation between the number of cells and fluorescence intensity of GFP. We established three pancreatic cancer cell lines constitutively expressing GFP, as described in the Materials and Methods (Fig. 1a). These clones were confirmed by flow cytometry to be >99% GFP-positive in comparison with the non-GFP-expressing parental cell lines (Fig. 1b). In the first series of experiments, we evaluated the efficiency of GFP fluorescence for the determination of cell numbers (Fig. 1c). Regression analysis confirmed that the fluorescence intensity of total GFP-SUIT-2 cells was correlated with the cell numbers counted within the range from 5×10^3–1×10^6 cells/well (Fig. 1d; Spearman's rank-correlation coefficient: 0.995, $P < 0.0001$). Similarly, there were significant correlations between fluorescence intensity and the numbers of GFP-Panc1 and GFP-MIA PaCa-2 cells (data not shown). In addition, we found a significant correlation between GFP fluorescence and PI fluorescence calculated by PI assay as another method for evaluating cell proliferation (Supporting Information Fig. S1a,b; Spearman's rank-correlation coefficient: 0.998, $P < 0.0001$). The lower limit of detection for GFP fluorescence was in the order of 1000 cells/well for these cell lines (data not shown). At $\geq 1 \times 10^6$ cells/well, cells became confluent in flat-bottomed 24-well plates; thus, 1×10^4–5×10^5 cells were used in subsequent experiments.

α-SMA expression in myofibroblasts. To elucidate tumor–stromal interactions between pancreatic cancer cells and PSCs, we isolated bulky lines of myofibroblasts from resected normal pancreas, pancreatic cancer tissue, and a metastatic tumor from a patient with PDAC using the outgrowth method, as described previously.[39] To confirm that PSCs expressed high levels of α-SMA and collagen type I (COL1),[1,12,39,42] we analyzed the expression levels of α-*SMA* and *COL1* mRNAs in these myofibroblast cultures. All myofibroblast cultures expressed higher levels of α-*SMA* and *COL1* mRNA than MRC5 and cancer cells (Fig. 2a,b). Interestingly, MCF-1 myofibroblasts derived from a metastatic tumor also expressed high levels of α-*SMA* and *COL1* mRNAs. Immunoblot analysis and immunofluorescence staining revealed that these myofibroblast cultures expressed α-SMA protein (Fig. 2c,d). We further found that >80% of PSCs and myofibroblasts expressed α-SMA by flow cytometry (Fig. 2e,f). We used these four PSC cultures and one myofibroblast culture to establish a simplified direct co-culture system using GFP-expressing cancer cells in the following experiments.

Effects of co-culture on cell morphology. Indirect co-culture has often been used to investigate the effects of cancer–stromal interactions on the proliferation of cancer cells, because of its easy evaluation (Fig. 3a). However, the effects of cell–cell adhesion and juxtacrine signaling between cancer and stromal cells cannot be evaluated by this method. To evaluate these effects, we established a direct co-culture system using GFP-expressing cells (Fig. 3a). Initially, we assessed the effects of direct co-culture with PSCs on the morphology of cancer cells. Monocultured GFP-SUIT-2 cells were almost round in shape (Fig. 3b), whereas indirectly and directly co-cultured cells exhibited a fibroblastoid morphology (Fig. 3b). These findings suggest that co-culture with PSCs promoted the epithelial–mesenchymal transition (EMT)[43] of GFP-SUIT-2 cells. We evaluated the effects of the morphological alterations on the GFP expression levels in GFP-SUIT-2 cells by flow cytometry and found that there was no significant difference between monocultured and co-cultured cells (Fig. 3c). To confirm the induction of the EMT in co-cultured GFP-SUIT-2 cells, we isolated them using a cell sorter (Fig. 3d), and quantified the mRNA levels of EMT markers, including *Snail*, *Vimentin*, and *N-cadherin* (Fig. 3e).

Table 2. Primer sequences and product size

Primer	Forward	Reverse	Product size
	Sequence 5′–3′	Sequence 5′–3′	
α-*SMA*	ccgggagaaaatgactcaaa	gcgtccagaggcatagagag	97
COL1	acgtgatctgtgacgagacc	agcaaagtttcctccgaggc	250
Snail	Takara Bio (primer set ID: HA075019)		
Vimentin	tgcccttaaaggaaccaatg	gcttcaacggcaaagttctc	72
N-cadherin	aggatcaaccccatacacca	tggtttgaccacggtgacta	125
Notch-1	tccaccagtttgaatggtca	cgcagagggttgtattggtt	80
Hes-1	ccaaagacagcatctgagca	tcagctggctcagactttca	91
Jagged-1	ctgcctctctgatccctgtc	tggggaacactcacactcaa	76
18S rRNA	gtaacccgttgaaccccatt	ccatccaatcggtagtagcg	151

α-SMA, α-smooth muscle actin; COL1, collagen type I; Hes-1, hairy and enhancer-of-split homolog-1.

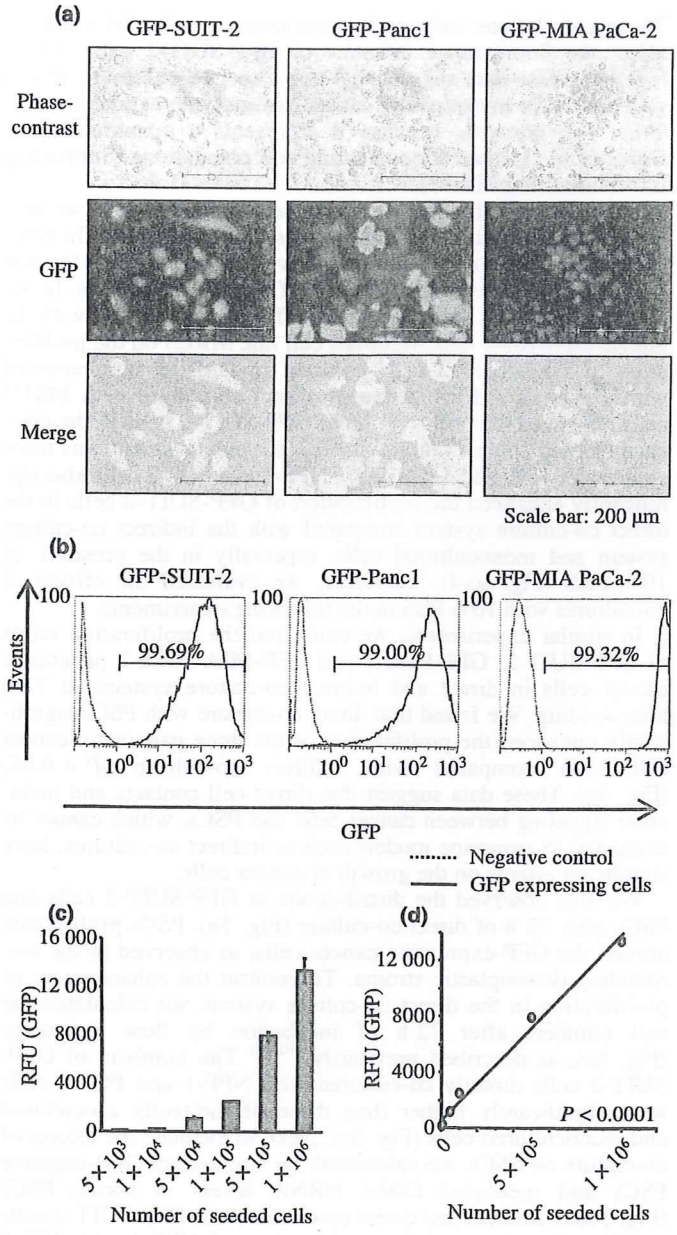

Fig. 1. (a) Microphotographs of three pancreatic cancer cell lines constitutively expressing green fluorescent protein (GFP). (b) The GFP-SUIT-2, GFP-Panc-1, and GFP-MIA PaCa-2 clones used for subsequent experiments were confirmed by flow cytometry to be >99% GFP-positive in comparison with the non-GFP-expressing parental cell lines. (c) GFP fluorescence intensity of GFP-SUIT-2 cells. (d) Regression analysis confirmed that the fluorescence intensity of GFP-SUIT-2 cells was correlated with the number of cells counted within the range from 5×10^3–1×10^6 cells/well (Spearman's rank-correlation coefficient: 0.994, $P < 0.0001$).

Directly co-cultured GFP-SUIT-2 cells expressed significantly higher levels of these mRNAs than monocultured cells.

Proliferation of cancer cells in direct and indirect co-culture systems. In our preliminary study, we evaluated the effects of co-culture on the fluorescence intensity of GFP-SUIT-2 cells. Both types of GFP-SUIT-2 (5×10^4) co-cultures expressed similar levels of fluorescence intensity to monocultured cells, regardless of the number of co-cultured PSCs (Supporting Information Fig. S1c), and the fluorescence intensity of GFP-SUIT-2 cells (1×10^4–2×10^5) was correlated with the number of cells,

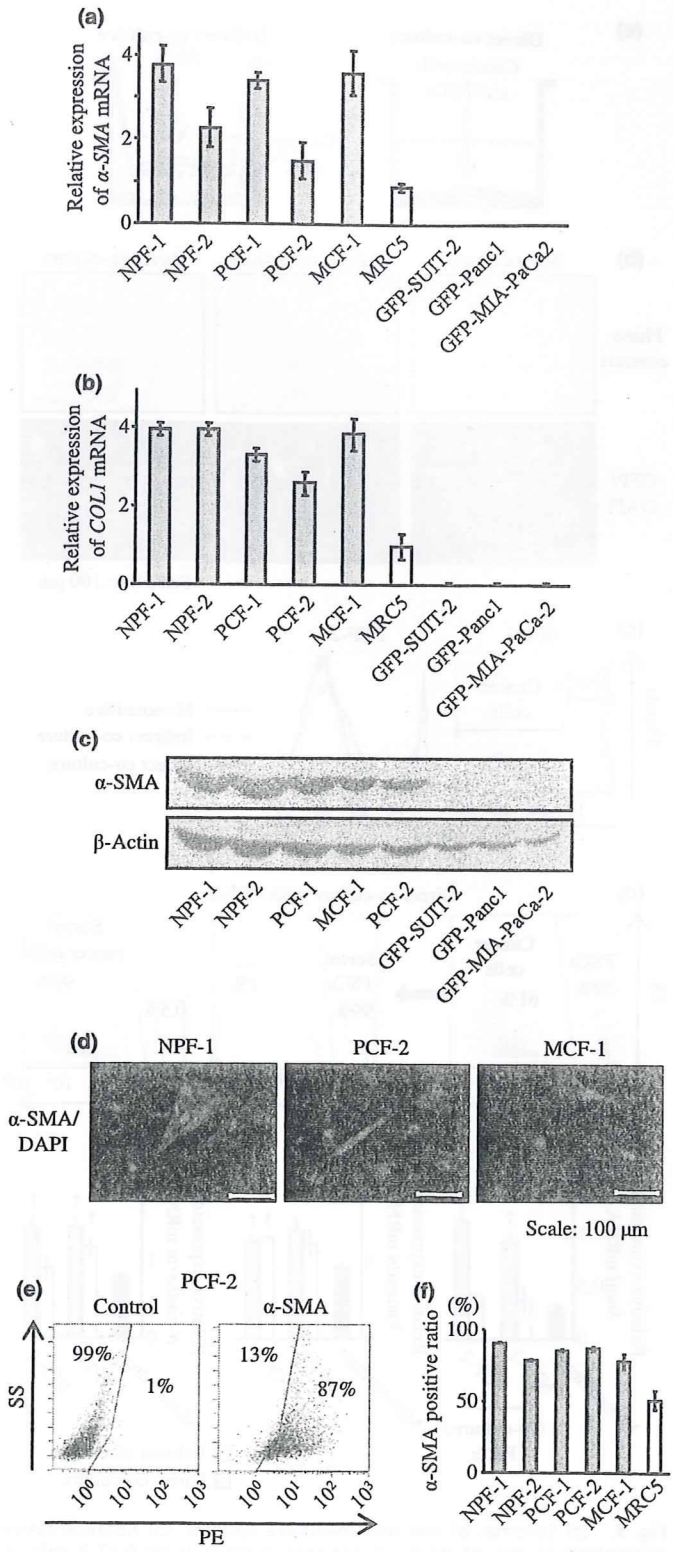

Fig. 2. (a,b) Pancreatic stellate cells (PSCs) expressed higher levels of *α-smooth muscle actin* (*α-SMA*) and *collagen type I* (*COL1*) mRNAs than MRC5 and cancer cells. (c,d) Immunoblot analysis and immunofluorescence staining revealed that these myofibroblasts expressed α-SMA protein. (e,f), Flow cytometry demonstrates that >80% of the PSCs and myofibroblasts expressed α-SMA.

despite the coexistence of PSCs (4×10^4) and their EMT-like morphological changes (Supporting Information Fig. S1d,e; Spearman's rank-correlation coefficient: 0.993, $P < 0.0001$).

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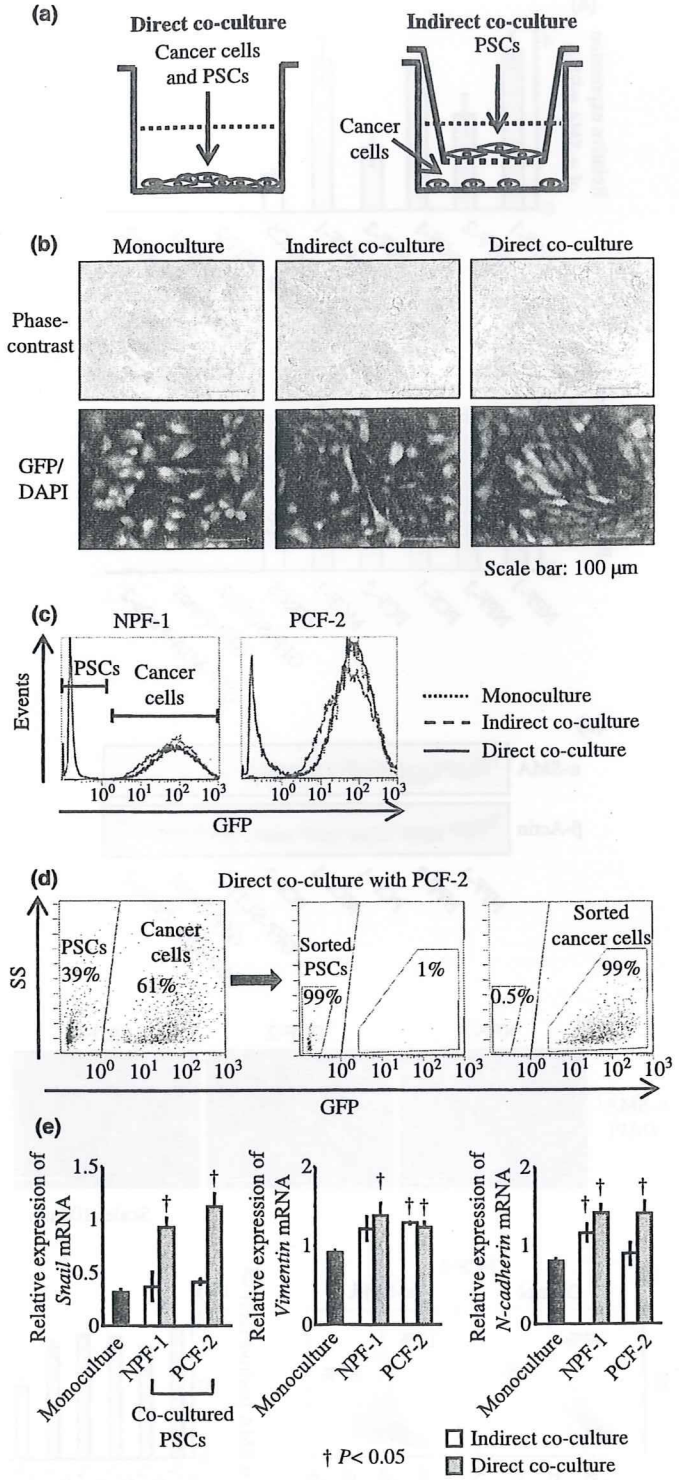

Fig. 3. (a) Schemas of the two co-culture systems. (b) Representative microphotographs of green fluorescent protein (GFP)-SUIT-2 cells in monoculture (left), indirect co-culture with PCF-2 cells (center), and direct co-culture with PCF-2 cells (right). Monocultured GFP-SUIT-2 cells were almost round in shape, whereas co-cultured cells exhibited a fibroblastoid morphology. (c) There was no significant difference in the GFP expression levels between monocultured and co-cultured cells. (d) The PSC/GFP-SUIT-2 cell proportion was determined, and GFP-expressing cancer cells and GFP-negative PSCs were isolated using a cell sorter. (e) Directly co-cultured GFP-SUIT-2 cells expressed significantly higher mRNA levels for the epithelial–mesenchymal transition (EMT) markers *Snail*, *Vimentin*, and *N-cadherin*, compared with monocultured cells.

These correlations indicate that coexistence with PSCs did not affect the fluorescence intensity of GFP-SUIT-2 cells. Taken together, these data suggest that this direct co-culture system is well suited for investigating cancer–stromal interactions through direct cell contacts, because it represents a quantitative and reproducible method for evaluating cell populations (Supporting Information Fig. S1a–e).

Using *in vitro* direct and indirect co-culture systems, we analyzed the proliferation of GFP-expressing cancer cells. In order to compare both co-culture systems, we assessed the proliferation ratios and compared them with that of monocultured cells. In our preliminary study, we assessed the effects of co-culture with the human embryonic lung fibroblast cell line MRC5 on the proliferation of GFP-SUIT-2 cells cultured in DMEM supplemented with 1% or 10% FBS. Although direct co-culture with MRC5 cells enhanced the proliferation of GFP-SUIT-2 cells in the presence of both concentrations of FBS, the enhancement was more evident for 10% FBS (Fig. 4a,b). NPF-1 and PCF-2 cells also significantly enhanced the proliferation of GFP-SUIT-2 cells in the direct co-culture system compared with the indirect co-culture system and monocultured cells, especially in the presence of 10% FBS (Fig. 4c–f). Therefore, we evaluated the effects of co-cultures with 10% FBS in the following experiments.

In similar experiments, we compared the proliferation ratios of GFP-SUIT-2, GFP-Panc1, and GFP-MIA PaCa-2 pancreatic cancer cells in direct and indirect co-culture systems at 72 h after seeding. We found that direct co-culture with PSCs significantly enhanced the proliferation of the three pancreatic cancer cell lines compared with indirect co-culture ($P < 0.05$) (Fig. 4g). These data suggest that direct cell contacts and juxtacrine signaling between cancer cells and PSCs, which cannot be evaluated in paracrine models such as indirect co-cultures, have significant effects on the growth of cancer cells.

We also observed the distributions of GFP-SUIT-2 cells and PSCs after 72 h of direct co-culture (Fig. 5a). PSCs proliferated around the GFP-expressing cancer cells, as observed in the surrounding desmoplastic stroma. To confirm the enhancement of proliferation in the direct co-culture system, we calculated the cell numbers after 72 h of incubation by flow cytometry (Fig. 5b), as described previously.[30,32] The numbers of GFP-SUIT-2 cells directly co-cultured with NPF-1 and PCF-2 cells were significantly higher than those of indirectly co-cultured and monocultured cells (Fig. 5c). Next, to evaluate the effects of co-culture on PSCs, we calculated the numbers of GFP-negative PSCs and measured *COL1* mRNA levels in sorted PSCs (Fig. 5d,e). Indirect and direct co-culture with GFP-SUIT-2 cells significantly enhanced the proliferation of NPF-1 and PCF-2 cells (Fig. 5d). In addition, *COL1* mRNA levels in co-cultured NPF-1 and PCF-2 cells were significantly higher than those in monocultured cells (Fig. 5e). These data suggest that co-culture with cancer cells enhanced the proliferation and collagen synthesis of PSCs.

Notch signaling pathway in co-cultured cells. To investigate the effects of the juxtacrine mechanism between cancer and stromal cells, we assessed the Notch signaling pathway by quantifying the mRNA levels of *Notch-1*, *hairy and enhancer-of-split homolog-1* (*Hes-1*; a downstream protein of Notch signaling), and *Jagged-1* (a ligand of Notch receptor) in both types of cells. Although *Notch-1* mRNA levels were only elevated in directly co-cultured GFP-SUIT-2 and NPF-1 cells (Fig. 6a), *Hes-1* and *Jagged-1* mRNA levels in directly co-cultured cells were dramatically elevated compared with those in monocultured cells (Fig 6b,c).

Discussion

To date, many methods have been used to evaluate the proliferation of cancer cells directly co-cultured with stromal cells, such

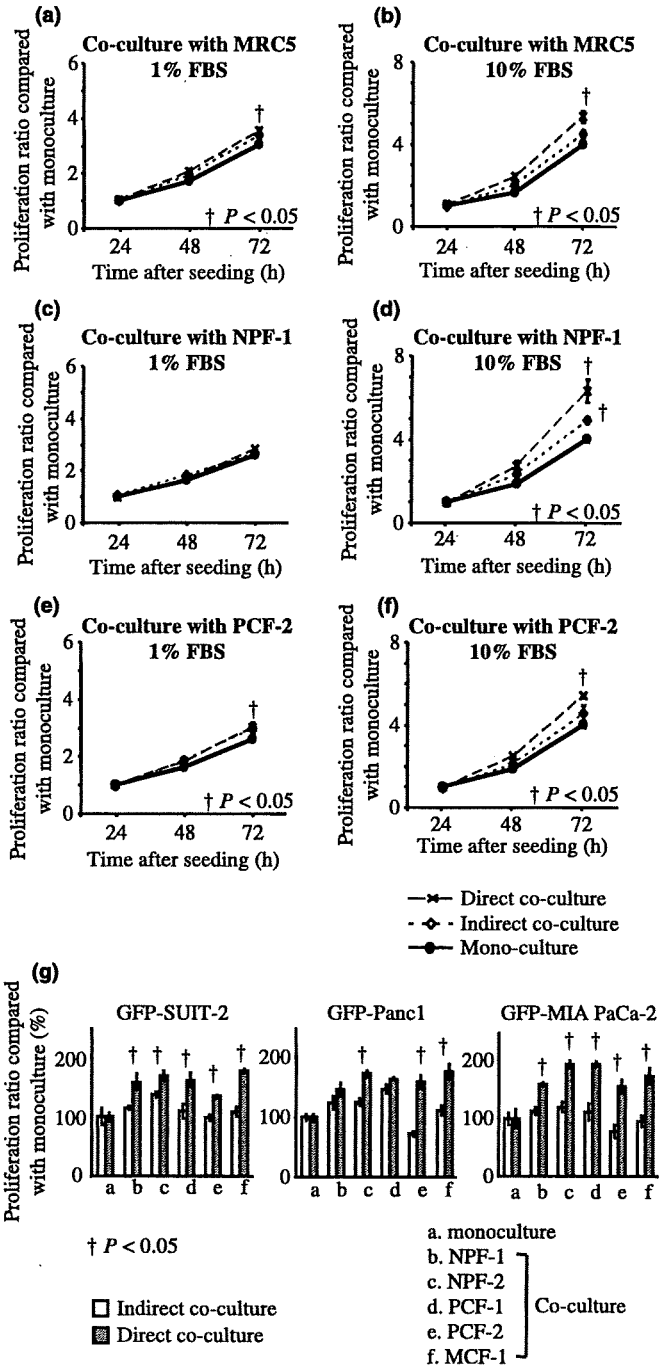

Fig. 4. (a–f) Proliferation ratios of green fluorescent protein (GFP)-SUIT-2 cells in direct and indirect co-cultures with MRC5 (a,b), NPF-1 (c,d), and PCF-2 (e,f) cells compared with monocultures in the presence of 1% FBS (a,c,e) and 10% FBS (b,d,f). In direct co-culture MRC5, NPF-1, and PCF-2 cells significantly enhanced the proliferation of GFP-SUIT-2 cells compared with indirect co-culture and monoculture, at 72 h after seeding (†$P < 0.05$), especially in the presence of 10% FBS. (g) Proliferation ratios of the three pancreatic cancer cell lines in the two co-culture systems at 72 h after seeding in the presence of 10% FBS compared with monocultures. Direct co-culture with both types of pancreatic stellate cells (PSCs) significantly enhanced the proliferation of the three pancreatic cancer cell lines compared with those in indirect co-culture (†$P < 0.05$).

as flow cytometry,[30,32] [^{3}H]thymidine incorporation assays,[25] and counting the numbers of cells or colonies under a microscope.[23,31,32] In the current study, we simplified the fluorescence imaging–based proliferation assay established by Krtolica *et al.*,[33,34] and developed a method that allowed the quantitative measurement of populations of GFP-expressing cancer cells. Using this method, we found that cell growth could be monitored even when the cancer cells were directly co-cultured with PSCs and transformed into a fibroblastoid morphology, resembling the EMT.[43] Our method did not require fixation, staining, or harvesting of the cultured cells, and no complicated handling, image analysis, or use of radioisotopes was needed. Moreover, we were able to quantify populations of live GFP-expressing cells in the same wells at specific times. The present method is therefore an easy and highly reproducible method that does not require many cells or culture plates. It is a simple and objective method, unlike *in vivo* co-culture assays, and could be suitable for the evaluation of other cell–cell interactions, such as cancer cell–endothelial cell and cancer cell–inflammatory cell interactions. However, this co-culture system needs further fundamental experiments to evaluate the relevance of co-cultures with cancer cells under other culture conditions, because other types of cells, including endothelial cells and inflammatory cells, need to be cultured under specific culture conditions.

Recent evidence has shown that pancreatic cancer cells increase their proliferative ability when exposed to conditioned medium from human PSCs, and this effect is caused by not only inhibition of apoptosis but also increased DNA synthesis.[13,14] In the current study, indirect co-culture with PSCs (to examine paracrine mechanisms) also enhanced the proliferation of pancreatic cancer cells. Moreover, direct co-culture with PSCs (which allows the evaluation of direct cell contacts, juxtacrine mechanisms, and ECMs produced by PSCs, as well as paracrine mechanisms), further accelerated the proliferative ability of pancreatic cancer cells. Samoszuk *et al.*[32] revealed that clonogenic growth of human breast cancer cells directly co-cultured with serum-activated fibroblasts was significantly enhanced compared with indirectly co-cultured or monocultured cells. These results are consistent with our data. Meanwhile, Che *et al.*[21] demonstrated that direct co-culture with Swiss 3T3 fibroblasts using collagen-based three-dimensional co-culture models enhanced the invasiveness of oral epithelial cancer cells more than indirect co-culture or monoculture. Taken together, these results suggest that the direct cell contacts involved in cancer–stromal interactions support the progression of cancer cells, in addition to the paracrine promoting effects of growth factors or chemokines.

Bachem *et al.*[1,15] demonstrated that pancreatic carcinoma cells stimulate the proliferation and matrix synthesis of PSCs via paracrine mechanisms. In the current study, we further found that both direct and indirect co-cultures stimulated the proliferation of PSCs and increased their *COL1* mRNA levels, suggesting that cancer–PSC interactions may induce desmoplasia in PDAC. Meanwhile, recent evidences have revealed that collagen type 1 increases the proliferation of pancreatic cancer cells by enhancing DNA synthesis and inhibiting apoptosis.[4,6] Although there was no significant difference between *COL1* mRNA levels in the two co-culture systems, cancer cells may be affected more strongly by local interactions with collagen type 1 in the direct co-culture system.

Several studies have demonstrated that activation of the Notch signaling pathway, one of the juxtacrine mechanisms, plays significant roles in the progression of pancreatic cancer.[44–47] Binding of Notch-1 receptor to its ligands, such as Jagged-1, expressed on adjacent cells leads to γ-secretase-mediated cleavage of the intracellular domain of Notch-1 (NIC1), which then translocates into the nucleus and results in the activation of Notch signaling.[48] In the current study, we found that direct co-culture of pancreatic cancer cells and PSCs dramatically increased the mRNA levels of *Hes-1* (a downstream

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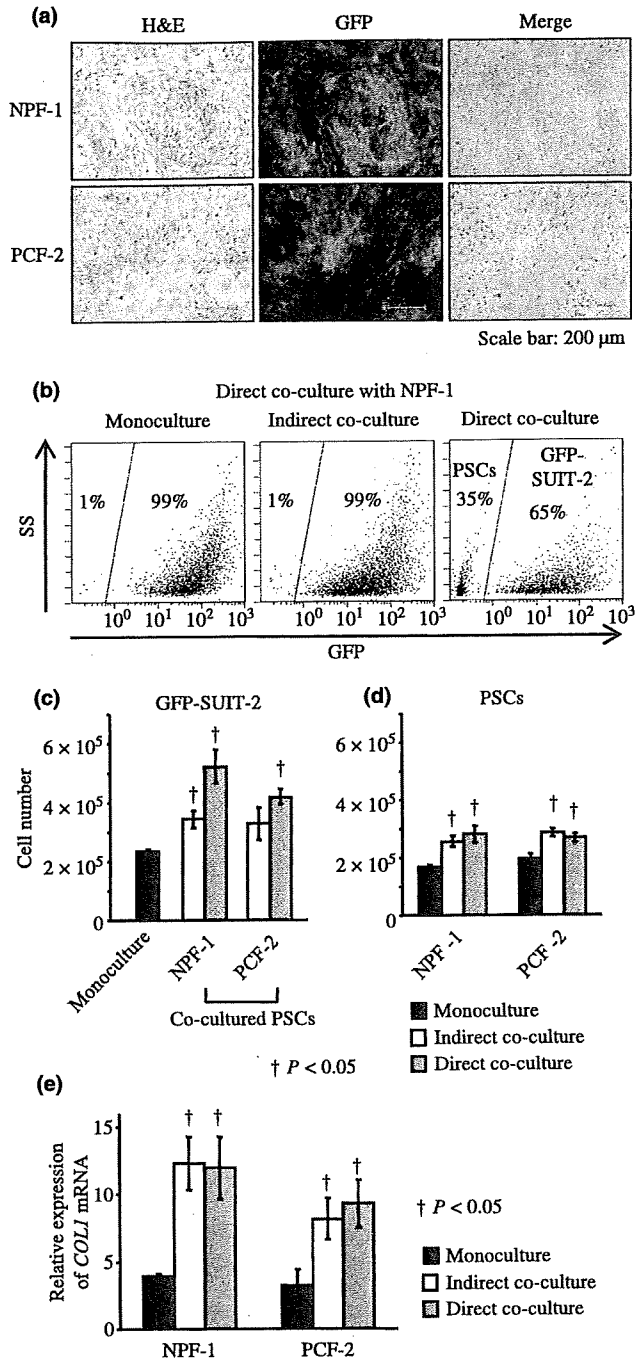

Fig. 5. (a) Representative microphotographs of direct co-cultures of green fluorescent protein (GFP)-SUIT-2 cells with NPF-1 and PCF-2 cells at 72 h after seeding. Pancreatic stellate cells (PSCs) proliferated around the GFP-positive cancer cells, as observed in the surrounding desmoplastic stroma. (b,c,d) The numbers of GFP-expressing cancer cells and GFP-negative PSCs were calculated from the total cell numbers using the PSC/GFP-SUIT-2 proportions determined by flow cytometry. The numbers of directly co-cultured GFP-SUIT-2 cells and PSCs were significantly higher than those of indirectly co-cultured and monocultured cells (c). Co-culture with GFP-SUIT-2 cells significantly enhanced the proliferation of PSCs (d). (e) The *COL1* mRNA levels in co-cultured PSCs were significantly higher than those in monocultured cells.

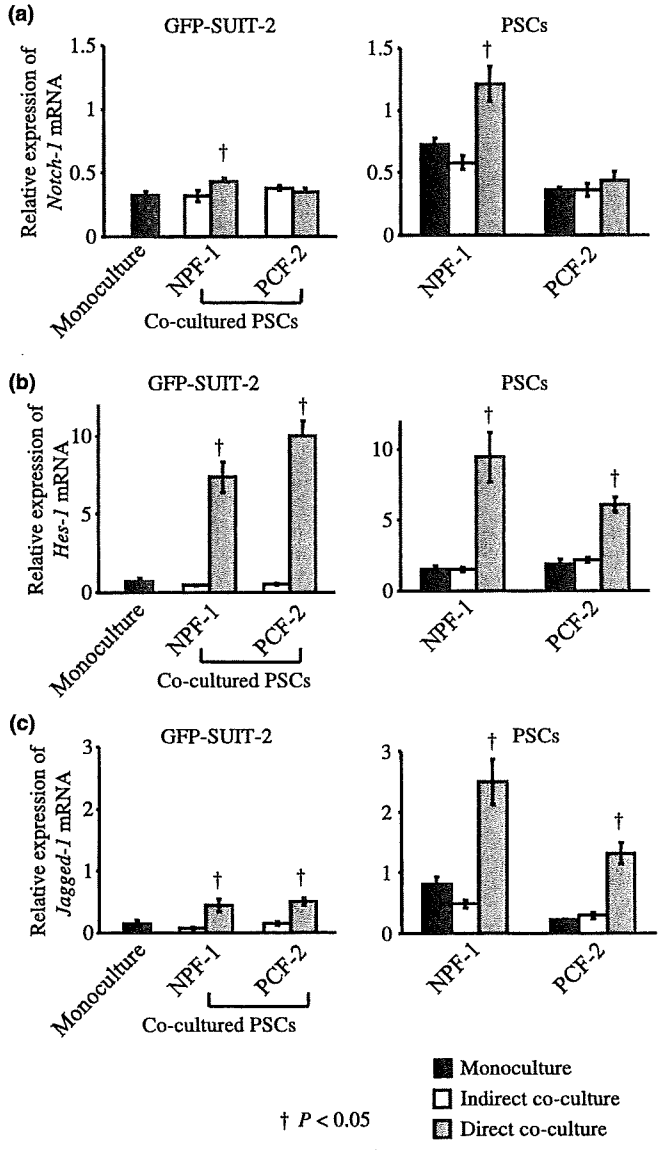

Fig. 6. (a) The *Notch-1* levels were only elevated in directly co-cultured GFP-SUIT-2 and NPF-1 cells. (b,c) The *hairy and enhancer-of-split homolog-1* (*Hes-1*) and *Jagged-1* mRNA levels in directly co-cultured cells were dramatically elevated compared with those in indirectly co-cultured and monocultured cells.

protein of Notch signaling) in both cell types, suggesting that direct cell contacts activated Notch signaling. Therefore, activation of the Notch signaling pathway may play a crucial role in enhancing the proliferation of cancer cells in the direct co-culture system.

In our preliminary study, we found that human embryonic lung fibroblast, MRC5, also enhanced proliferation of pancreatic cancer cells, induced EMT-like morphological change (Supporting Information Fig. S2a), and activated the Notch signaling pathway (Supporting Information Fig. S2c–e) in our co-culture systems as well as in PSCs. These findings indicates that enhancement of pancreatic cancer cell proliferation by direct co-culture with stromal cells is not specific in stromal cells derived from the pancreas. In the current study, we found that MCF-1 cells, a culture of myofibroblast derived from metastatic tumor of the abdominal wall, also enhanced proliferation of pancreatic cancer cells in our co-culture systems. Therefore, cancer cells may possibly proliferate via direct interactions with stromal cells derived from other organs when the cells form metastatic tumors.

In conclusion, we have established a direct co-culture system that enabled us to quantitatively and reproducibly evaluate GFP-expressing cell populations, even in co-culture with other cells. This method could be widely applied to elucidate cell–cell interactions involving not only paracrine factors, but also direct cell contacts and juxtacrine factors. Moreover, our data provide evidence that PSCs and α-SMA-positive stromal myofibroblasts control the proliferation of pancreatic cancer cells via tumor–stromal interactions involving direct cell contacts and juxtacrine mechanisms, as well as paracrine mechanisms. The identification of α-SMA-positive myofibroblast-derived factors and clarification of their mechanisms of action are the subjects of ongoing investigations, and may lead to the development of novel therapeutic strategies directed at the PDAC microenvironment.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. (a) Propidium iodide (PI) fluorescence intensity of green fluorescent protein (GFP)-SUIT-2 cells. (b) The GFP fluorescence intensity was correlated with the PI fluorescence intensity (Spearman's rank-correlation coefficient: 0.998, $P < 0.0001$). (c) GFP-SUIT-2 cells expressed similar levels of fluorescence intensity to monocultured cells, regardless of the number of co-cultured pancreatic stellate cells (PSCs). (c,d) Regression analysis confirmed that the fluorescence intensity of GFP-SUIT-2 cells was correlated with the number of cells, despite the coexistence of myofibroblasts and their morphological alterations (Spearman's rank-correlation coefficient: 0.993, $P < 0.0001$).

Fig. S2. (a) Representative microphotographs of green fluorescent protein (GFP)-SUIT-2 cells in monoculture (left), indirect co-culture with MRC5 fibroblasts (center), and direct co-culture with MRC5 fibroblasts (right). Monocultured GFP-SUIT-2 cells were almost round in shape, whereas co-cultured cells exhibit a fibroblastoid morphology. (b) GFP-expressing cancer cells and GFP-negative MRC5 fibroblasts were isolated using a cell sorter. (c) The *Notch-1* levels were only elevated in directly co-cultured MRC5 fibroblasts. (d,e) The *hairy and enhancer-of-split homolog-1* (*Hes-1*) and *Jagged-1* mRNA levels in directly co-cultured cells were dramatically elevated compared with those in indirectly co-cultured and monocultured cells.

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Long-term Outcome of Immunotherapy for Patients with Refractory Pancreatic Cancer

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Abstract. *Background: Pancreatic cancer is one of the most fatal human cancers, with a 5-year survival rate of <5%. Although new chemotherapies have been used for pancreatic cancer, the outcome is still poor. Here, we retrospectively analyzed the outcome of immunotherapy in pancreatic cancer patients and revealed the potential of immunotherapy in advanced pancreatic cancer treatment. Patients and Methods: Seventeen pancreatic cancer patients underwent immunotherapy in the Kyushu University and the Yakuin CA Clinic. Six patients had postoperative recurrence, 11 were diagnosed as inoperable because of metastasis, 16 had prior chemotherapy and developed chemotherapy-resistant cancers, while 1 patient had no prior chemotherapy for recurrent cancer after surgical resection because of leukopenia. Immunotherapy was combined with chemotherapy in 11 patients and without chemotherapy in 6 patients. Immunotherapy was classified into two groups; combined dendritic cell (DC) vaccination and intravenous or peritoneal injection of activated lymphocytes (DC vaccine therapy), or injection of lymphokine-activated killer lymphocytes (LAK) alone (LAK therapy). Results: Immunotherapy of refractory pancreatic cancer resulted in a median survival of 9 months. Peritoneal metastasis tended to shorten the survival period. Combination immunotherapy and chemotherapy showed no obvious difference as compared to immunotherapy alone. DC vaccine therapy conferred a significantly better survival period than LAK alone. Conclusion: Our results suggest that immunotherapy utilizing DC vaccination may prolong the survival of refractory pancreatic cancer patients.*

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Key Words: Pancreatic cancer, immunotherapy, dendritic cells, vaccination.

Pancreatic cancer is one of the most fatal human cancers, with an overall 5-year survival rate of <5%, partially because of the difficulty of diagnosis at an early stage (1). However, despite the complete surgical removal of the tumor, most patients developed the disease again as metastases or local recurrence (2-6). Recently, gemcitabine has been reported to improve the survival of inoperable pancreatic cancer patients. Gemcitabine produced a clinical benefit in 24% of patients, with a median survival of 5.6 months and a 1-year survival of 18% (7). There is an increasing body of evidence showing that patients with resectable pancreatic cancer might benefit from adjuvant therapy with gemcitabine (8-15). However, options for patients with relapsed pancreatic cancer are still of limited benefit. Evaluations of single-agent gemcitabine or rubitecan salvage therapies for metastatic pancreatic cancer have reported good patient tolerability but median survivals of only 3.85 and 4.7 months, respectively (16, 17).

Dendritic cells (DCs) are antigen-presenting cells specialized for the induction of a primary T-cell response and can induce antitumor immunity *in vivo* (18-22). We previously reported that combination therapy with tumor cell-pulsed DCs and activated lymphocytes for patients with disseminated carcinomas prolonged the survival of responders (23). This immunotherapy was safe and no evidence of autoimmune disease was noted. No particular adverse reactions, except for low-grade fever, were found. Feasibility is one of the most important factors in investigating a second-line chemotherapeutic agent for refractory pancreatic cancer because there are usually not enough patients available for intense therapy.

Here, we present the outcome of immunotherapy including simple injection of activated lymphocytes and a combination of pulsed DC vaccination and injection of activated lymphocytes in patients with refractory pancreatic cancer (24-26).

Patients and Methods

Patient characteristics. Seventeen pancreatic cancer patients underwent immunotherapy in the Kyushu University and the Yakuin CA Clinic. Six patients had postoperative recurrence and 11 patients were diagnosed as inoperable because of metastasis. The metastatic

Table I. *Patient characteristics.*

Age years	Gender	Metastasis	Prior treatment	Immunotherapy	Combined chemotherapy	Survival time (months)
64	F	Peritoneum, liver	5-FU	DC	None	9
49	M	Peritoneum, lung	GEM	DC	None	20
63	M	Peritoneum	GEM, TS-1	DC	TS-1	7
73	M	Liver	GEM, UFT	DC	GEM	11
61	F	Peritoneum	GEM	DC	GEM	9
59	F	Peritoneum	GEM	LAK	None	7
61	F	Peritoneum	GEM	LAK	GEM	5
70	F	Peritoneum, liver	GEM	DC	GEM	7
65	F	Peritoneum, liver	GEM	DC	GEM	9
58	F	Liver	GEM, TS-1	DC	GEM	19
65	F	Peritoneum	GEM	LAK	None	5
68	F	Liver	GEM, TS-1, CPT-11	DC	None	11
69	F	Peritoneum	none	LAK	None	7
44	M	Peritoneum, liver	GEM, TS-1	DC	GEM+TS-1	8 (alive)
61	M	Peritoneum	GEM	DC	GEM	7 (alive)
74	M	Peritoneum	GEM	LAK	GEM+TS-1	6 (alive)
67	M	Peritoneum	GEM, Radiation	LAK	GEM	6 (alive)

5-FU, 5-Fluorouracil; GEM, gemcitabine; TS-1, tegafur-gimeracil-oteracil potassium; CPT-11, irinotecan; DC, dendritic cell; LAK, lymphokine-activated killer lymphocytes.

sites were the peritoneum in 7 patients, the peritoneum and liver in 2 and the liver in 2. Every patient had prior chemotherapy and had developed chemotherapy-resistant cancer.

Preparation of dendritic cells and activated T-lymphocytes. Autologous tumor-pulsed DCs (DC vaccine) were prepared as described elswhere (23, 27). Briefly, peripheral blood mononuclear cells (PBMC) were collected by leukapheresis with the COBE spectrum apheresis system (GAMBRO BCT, Inc, CL, USA). PBMCs were suspended at a cell density of 4×10^6 cells/ml in GMP-grade RPMI-1640 (Hy-Media; Nipro, Tokyo, Japan) supplemented with 1% human albumin, and 500 μl of cell suspension was added to each well of 24-well culture plates. The adherent cells in the 24-well culture plates were further cultured in Hy-Media containing 1% human albumin, and the immature DCs were prepared in 100 ng/ml of recombinant human granulocyte/monocyte colony-stimulating factor (GM-CSF, 200 ng/ml; Novartis Pharma, Basel, Switzerland) and 50 μl of recombinant human IL-4 (500 U/ml; Ono, Tokyo, Japan) for 7 days. After 7 days, cells were harvested as immature DCs. A total of $2–10\times10^6$ immature DCs were obtained per preparation.

Tumor specimens obtained from the tumor mass or malignant effusions were lysed by five freeze-thaw cycles (necrotic tumor cells). Immature DCs were incubated overnight with necrotic tumor cells for use in 6 patients, with peptides of carcinoembryonic antigen (CEA) and mucin 1 (MUC1) for use in four patients and with peptide of CEA for use in one patient, then cultured for 2 days in medium containing tumor necrosis factor α (TNF-α, 1,000 U/ml; R&D Systems, Minneapolis, MN, USA) and prostaglandin E_2 (PGE_2,1 μg/ml; Sigma, St. Louis, MO, USA).

For the preparation of lymphokine-activated killer cells (LAK), non-adherent mononuclear cells were cultured for 2 weeks with Hy-medium containing 175 JRU/ml human recombinant interleukin (IL)-2 (Nipro) and immobilized monoclonal antibody to CD3 (10 μg/ml) (OKT-3; Jansen-Kyowa, Tokyo, Japan). The final cell products were assessed for viability by the dye-exclusion test and checked twice for possible contamination by bacteria, fungi and endotoxins.

Treatment plan. Methods of immunotherapy were classified into two groups: combined DC vaccination and intravenous or peritoneal injection of activated lymphocytes (DC vaccine), or injection of LAK alone. Patients in the DC vaccine group received an injection of $2\text{-}30\times10^6$ mature DCs loaded with necrotic tumor cells or peptides every 2 or 3 weeks. Intravenous injection of $1\text{-}5\times10^8$ OKT-3/IL-2-activated lymphocytes was combined with the above DC vaccine every 4 weeks. This combination therapy has been named tumor-pulsed DC vaccine therapy. In principle, this tumor-pulsed DC vaccine therapy was continued for as long as possible in the outpatient clinic. Eleven patients were treated with combined immunotherapy and chemotherapy with gemcitabine (GEM) or tegafur-gimeracil-oteracil potassium (TS-1), and six patients with immunotherapy only. LAK cells were injected every 3 weeks. Briefly, GEM (1,000 mg/body in standard) was given every week intravenously for three weeks with one week break and the course was repeated. TS-1 was given (80-120 mg/day in standard) for four weeks by oral administration with two weeks' break.

Study end-points and statistical analysis. The study end-point was overall survival of historically analyzed patients. Statistical analysis was performed by Wilcoxon–Mann-Whitney test with Statview software, SAS Institute Inc., Cary, North Carolina, USA.

Results

Patient characteristics. Seventeen pancreatic cancer patients with postoperative recurrence (n=6) or inoperable cancer (n=11) underwent immunotherapy. Six patients had

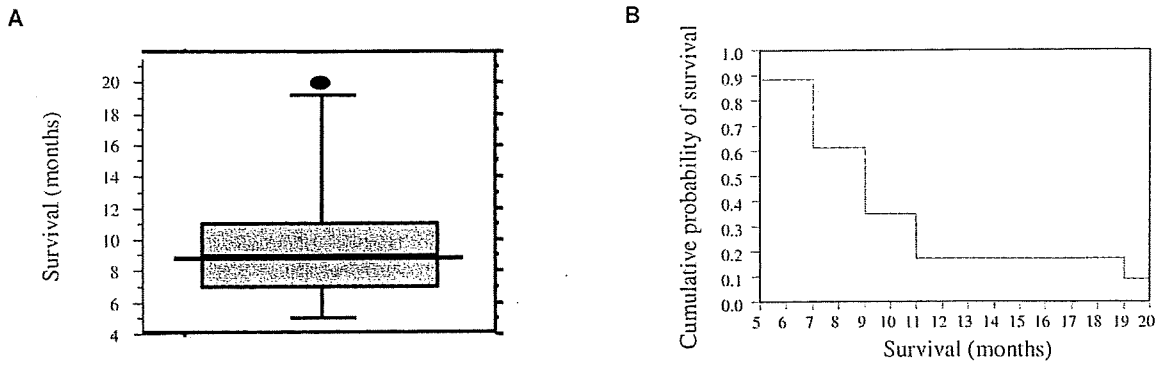

Figure 1. *Overall survival. A, Overall survival of 13 patients, excluding 4 surviving patients. Overall survival time was 9.7 months mean and 9 months median. B, Cumulative probability of survival of all 17 cases including surviving patients. MST was 9 months and the same as in (A) which excluded surviving patients.*

postoperative recurrences: 3 in the peritoneum, 2 in the peritoneum and liver, and 1 in the liver. Eleven patients were diagnosed as inoperable with metastasis in the peritoneum (n=7), peritoneum and liver (n=2) and liver (n=2). The characteristics of patients are shown in Table I.

Overall survival. Overall survival of 13 patients, excluding 4 surviving patients, was a mean of 9.7 months and median of 9 months (Figure 1A).

Analysis of all 17 cases including surviving patients demonstrated a median survival time (MST) of 9 months, which was the same as with exclusion of surviving patients (Figure 1B). The data indicate that immunotherapy is a potential candidate for treating recurrent pancreatic cancer after standard chemotherapy.

Peritoneal metastasis affects the survival time. We further analyzed patients excluding the surviving 4 patients. Ten out of 13 patients developed peritoneal metastasis (PM) at the beginning of our treatment and the other 3 patients were free of PM. The MSTs of patients with PM and without PM were 7 months and 11 months, respectively. PM was a statistically significant factor in MST in our series of pancreatic cancer immunotherapy (p=0.038, Figure 2).

DC therapy improves MST more than LAK therapy. Nine out of 13 patients underwent DC therapy and 4 patients had LAK therapy. The MSTs of patients with DC and LAK therapy were 9 and 6 months respectively and were statistically different (p=0.0116) (Figure 3).

Gemcitabine showed no additional effect on MST in the treatment of refractory pancreatic cancer patients with immunotherapy. Six out of 13 patients underwent combination therapy with immunotherapy and gemcitabine. However, gemcitabine unexpectedly did not confer any additional survival advantage on refractory pancreatic patients (Figure 4A). One of the 7 patients without gemcitabine was administered a combination of immunotherapy and 5-fluorouracil (5-FU). We further compared the MST of 6 patients injected with gemcitabine with 6 patients who underwent immunotherapy without any combination of chemotherapy. The MST of the gemcitabine group and no chemotherapy group were 9 and 8 months, respectively (p=0.87, Figure 4B).

Discussion

The overall survival of historically analyzed refractory pancreatic cancer patients treated by immunotherapy in this study was longer than that previously reported for second-line therapy of pancreatic cancer (8, 28-31). Ottele *et al.* (8) examined the potential effectiveness of second- or third-line therapy with paclitaxel (Taxol) after confirmed progression of pancreatic cancer with a gemcitabine-containing schedule. Paclitaxel was administered at weekly intervals and the MST was 17.5 weeks (range 7-88 weeks). Milella *et al.* (28) treated pancreatic cancer patients with progressive disease after gemcitabine-based chemotherapy with celecoxib and infusional 5-FU. The MST was 15 weeks. Reni *et al.* (29) reported the effect of a mitomycin, docetaxel and irinotecan regimen on gemcitabine-resistant pancreatic cancer patients. The MST was 6.1 months. Cantore *et al.* (30) used irinotecan and oxaliplatin in patients with advanced pancreatic cancer that had progressed despite more than 1 course of a gemcitabine-containing regimen and the MST was 5.9 months. Only Kozuch *et al.* (31) showed a longer MST of 10.3 months with injection of four active single agents into refractory pancreatic cancer patients.

We previously reported immunotherapy as an effective method to treat patients with malignant effusion (32).

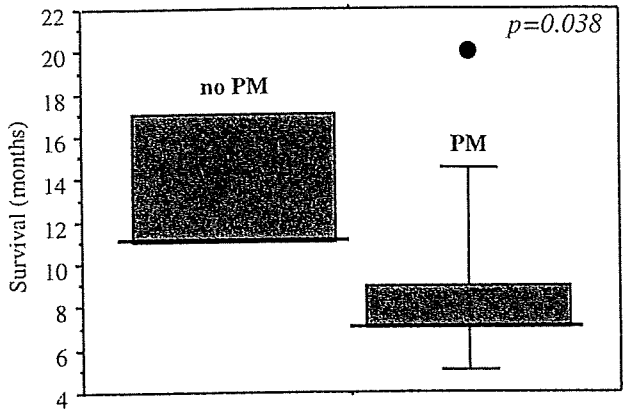

Figure 2. *Median survival time (MST) of the patients with peritoneal metastasis. Thirteen patients, excluding 4 surviving patients, were divided into two groups depending on the existence of peritoneal metastasis (PM) at the beginning of treatment resulting in 10 patients with PM and 3 without PM (no PM). The MSTs of patients with PM and without PM (no PM) were 7 months and 11 months, respectively. PM was a statistically significant factor in MST in our series of pancreatic cancer treated with immunotherapy (p=0.038).*

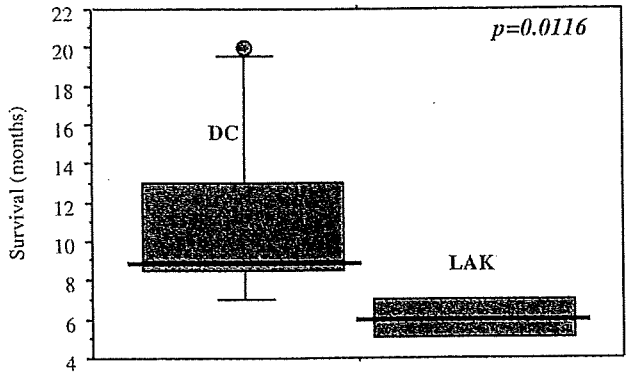

Figure 3. *DC therapy increases MST more than LAK therapy. Nine out of 13 patients, excluding 4 surviving patients, underwent DC therapy and 4 patients had LAK therapy. The MSTs of patients with DC and LAK therapy were 9 and 6 months, respectively, and were statistically different (p=0.0116). DC; DC therapy, LAK; LAK therapy.*

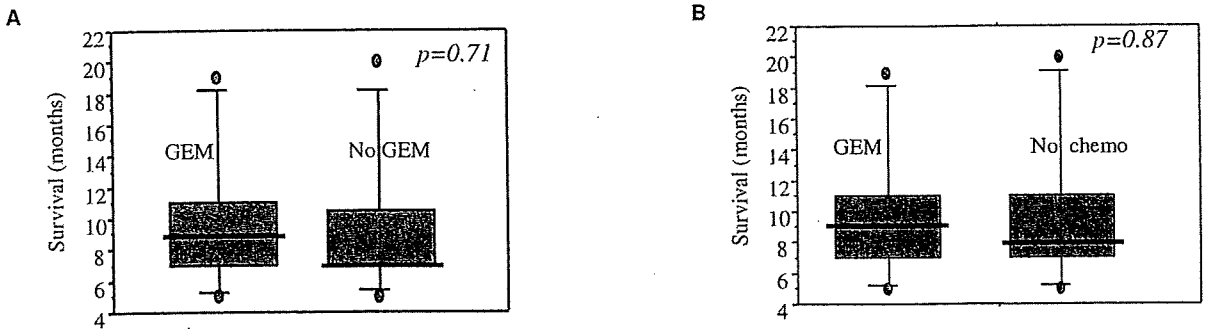

Figure 4. *MST of patients who underwent immunotherapy with or without gemcitabine. A, Six out of 13 patients underwent combination therapy of immunotherapy and gemcitabine (GEM), while seven patients underwent immunotherapy without gemcitabine (no GEM). The MSTs of GEM and no GEM patients were 9 and 7 months, respectively (p=0.71). B, The MST of GEM patients was compared with 6 patients who underwent immunotherapy without any combination of chemotherapy (No chemo). The MST of the GEM group and No chemo group were 9 and 8 months, respectively (p=0.87).*

Combined immunotherapy with intracavital injection of activated lymphocytes, monocyte-derived DCs and low-dose OK-432 improved the MST of patients with malignant effusion. Peritoneal metastasis is one of the prognostic factors in patients with gemcitabine-refractory pancreatic cancer (33). This might be the reason why PM was a statistically significant factor in MST in our series of pancreatic cancer patients undergoing immunotherapy.

Our data are the first to directly compare the influence of DC and LAK therapy on survival of cancer patients. Yamaguchi *et al.* reviewed the current status of adoptive lymphocyte therapy and mentioned that the overall response rate of tumor shrinkage was marginal (9%) (34). Kammula and Marincok reviewed clinical trials of the systemic administration of LAK cells and mentioned that LAK cells did not prove useful for the treatment of patients with metastatic melanoma and renal cancer (35). In contrast, the treatment of 86 patients with metastatic melanoma using tumor-infiltrating lymphocytes (TIL) plus IL-2 resulted in a 34% objective response rate (35). We have completed a phase I/II study of DC therapy and reported that the survival time of disseminated cancer patients responding to DC therapy was significantly prolonged compared with that of the non-responders ($p<0.0001$) (23). These published reports were consistent with our data and indicate that recognition of the tumor antigen is clinically pivotal in the immunotherapy of cancer as suggested in basic immunological reports.

Gemcitabine has been reported to mediate immunological effects relevant for tumor immunotherapy (36-38). Antitumor cytotoxic T-lymphocyte (CTL) responses can be induced by DCs cross-presenting antigens of tumor cells treated with a multidrug regime including gemcitabine (39). Enhanced cross-presentation of tumor antigens by DCs after gemcitabine treatment also leads to increased tumor recognition by CTLs *in vivo* (40). Bauer *et al.* demonstrated that gemcitabine sensitizes human pancreatic carcinoma cells to DC-induced tumor-specific CTL responses (41).

Although our data contains a small number of patients, one possible factor causing a discrepancy between our results and the published data is that most of our patients had been administered gemcitabine and became refractory to single agent therapy with gemcitabine. This possibility is generally troublesome, because currently gemcitabine is one of a few drugs revealed to be effective for pancreatic cancer. Eventually most refractory pancreatic cancer becomes resistant to gemcitabine, although there are few other drugs for pancreatic cancer.

We presented our experience and a retrospective analysis of a series of pancreatic cancer patients undergoing immunotherapy. Our data suggest that immunotherapy may confer some advantages on pancreatic cancer patients. There are a limited number of drugs for pancreatic cancer, and their efficacy on recurrent pancreatic cancer is still poor. Considering the present situation of refractory pancreatic cancer, establishment of promising treatment including immunotherapy is a task of great urgency.

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LMO2 Is a Novel Predictive Marker for a Better Prognosis in Pancreatic Cancer[1]

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Abstract

PURPOSE: LIM domain only 2 (LMO2) has been identified as a novel oncogene associated with carcinogenesis and better prognosis in several malignant tumors. We investigate the involvement of LMO2 in pancreatic cancer. *EXPERIMENTAL DESIGN:* We evaluated LMO2 expression in cultured cells, bulk tissues, and microdissected cells from pancreatic cancers by quantitative reverse transcription–polymerase chain reaction and immunohistochemistry. *RESULTS:* Of 164 pancreatic cancers, 98 (60%) were positive for LMO2 expression. LMO2 was more frequently detected in high-grade pancreatic intraepithelial neoplasia (PanIN) lesions (PanIN-2 and -3) than in low-grade PanIN lesions (PanIN-1A and -1B; $P < .001$) and was not detected in normal pancreatic ductal epithelium. The LMO2 messenger RNA levels were significantly higher in invasive ductal carcinoma cells than in normal pancreatic cells as evaluated by quantitative reverse transcription–polymerase chain reaction analyses of microdissected cells ($P = .036$). We also found higher incidence of LMO2 expression in histologic grade G1/G2 cancers than in grade G3 cancers ($P < .001$). The median survival time of LMO2-positive patients was significantly longer than that of LMO2-negative patients ($P < .001$), and multivariate analyses revealed that high LMO2 expression was an independent predictor of longer survival (risk ratio, 0.432, $P < .001$). Even among patients with a positive operative margin, LMO2-positive patients had a significant survival benefit compared with LMO2-negative patients. We further performed a large cohort study ($n = 113$) to examine the LMO2 messenger RNA levels in formalin-fixed paraffin-embedded samples and found similar results. *CONCLUSIONS:* LMO2 is a promising marker for predicting a better prognosis in pancreatic cancer.

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Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death in Western countries and has the lowest patient survival rate of any solid cancer [1–3]. Recently, although the cancer death rates of most malignancies have decreased owing to improvements in early detection and treatment, the overall 5-year survival of patients with pancreatic cancer has only slightly increased from 3% to 5% [1] because of difficulties in the diagnosis of pancreatic cancer at early stages. Surgical resection is the only curative treatment of pancreatic cancer, and the survival rate for patients with a negative operative margin status (R0) is significantly higher than that for patients with positive operative margin status (R1 and R2) [4]. However, some patients with a positive operative margin survive longer than those with a negative operative margin, and a more aggressive surgical approach may be justified for patients with a probability of such a response after resection,

Abbreviations: LMO2, LIM domain only 2; qRT-PCR, quantitative reverse transcription–polymerase chain reaction; PanIN, pancreatic intraepithelial neoplasia; IDC, invasive ductal carcinoma; FFPE, formalin-fixed paraffin-embedded; DLBCL, diffuse large B-cell lymphoma; PIN, prostatic intraepithelial neoplasia

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ven if the tumor is large and locally invasive. Conversely, the operation could be avoided if no surgical benefit can be predicted preoperatively. Therefore, we would like to identify a novel marker or predicting the prognosis of each patient.

The LIM domain only (LMO) proteins comprise one of the LIM domain-containing protein families and possess only two tandem LIM domains. These proteins act as adaptors for the assembly of large multiprotein complexes and play critical roles in both normal development and oncogenesis [5,6]. There are four members of the LMO family (*LMO1*, *LMO2*, *LMO3*, and *LMO4*), which have been recently identified and reported to be oncogenes [7–10]. LMO2 was also detected in several hematolymphoid neoplasias [11] and correlated with a good prognosis in diffuse large B-cell lymphoma (DLBCL) [12]. Recently, LMO2 was reported to play an important role in prostate cancer progression, and its expression was associated with the grade of prostatic intraepithelial neoplasia (PIN), the premalignant lesion of prostate cancer. These data suggest that LMO2 is associated with carcinogenesis and prognosis in several malignancies.

Similar to PIN, pancreatic intraepithelial neoplasia (PanIN) has been reported to be a premalignant lesion for conventional pancreatic cancer [13], and the gradual accumulation of molecular abnormalities supports this progression model. However, the involvement of LMO2 in pancreatic cancer remains to be investigated. Therefore, identifying the involvement of LMO2 in pancreatic cancer may be helpful toward understanding the mechanism of pancreatic carcinogenesis and progression and contribute to the detection of a biomarker for the diagnosis of pancreatic cancer or selection of therapy based on the features of individual tumors.

In the present study, we analyzed LMO2 expression in a large cohort of patients with pancreatic cancer. We focused on its prognostic and clinicopathological features using immunohistochemical staining and evaluation of messenger RNA (mRNA) extracted from formalin-fixed paraffin-embedded (FFPE) samples. We also examined its expression in PanIN lesions to investigate the involvement of LMO2 in pancreatic carcinogenesis. Our data suggest that LMO2 is associated with a better prognosis in pancreatic cancer.

Materials and Methods

Clinical Samples

A total of 164 patients with pancreatic ductal adenocarcinomas underwent surgical resection at the Department of Surgery and Oncology, Kyushu University Hospital (Fukuoka, Japan) and its affiliated hospitals. The patients consisted of 103 men and 61 women with a median age of 66 years (range, 36-86 years). The median duration of follow-up was 14 months (range, 1-101 months). We also analyzed 41 patients with unresectable pancreatic cancer due to local invasion or distant metastasis. All tumors were staged according to the TNM classification system of the International Union against Cancer [14]. Histologic grading of the tumors and diagnosis of PanIN lesions were performed according to the World Health Organization's classification system [15]. Other pathological variables (lymphatic invasion, vascular invasion, and perineural invasion) were based on the Japan Pancreas Society's classification [16]. Patients with mucinous cystadenocarcinoma or intraductal papillary mucinous carcinoma were excluded from the study. Surgical specimens were fixed in 10% formalin and embedded in paraffin. The paraffin-embedded samples were serially sectioned at 4-μm thickness, mounted on slides, and stained with hematoxylin and eosin for histologic analysis. For quantitative reverse transcription–polymerase chain reaction (qRT-PCR) studies, 22 fresh-frozen samples were obtained from cancerous lesions of resected pancreases from patients with primary pancreatic invasive ductal carcinoma (IDC) and 7 normal tissue samples were taken from intact pancreatic tissue resected for bile duct cancer or a pancreatic endocrine tumor. The tissue samples were embedded in OCT compound (Sakura, Tokyo, Japan) as soon as possible after resection and stored at −80°C until analysis. This study was performed in accordance with the principles embodied in the Declaration of Helsinki. The study was also approved by the Ethics Committee of Kyushu University and conducted according to the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

Cell Lines and Primary Cultures of Pancreatic Epithelial Cells

Normal human pancreatic epithelial cells were obtained from Cell Systems (Kirkland, WA) and maintained in CS-C medium containing 10% fetal calf serum according to the instructions of the supplier. Eleven pancreatic cancer cell lines, namely, ASPC-1, KP-1N, KP-2, KP-3, PANC-1, SUIT-2 (provided by Dr. H. Iguchi, National Shikoku Cancer Center, Matsuyama, Japan), MIA-PaCa2 (Japanese Cancer Resource Bank, Tokyo, Japan), Capan-1, Capan-2, CFPAC-1, and SW1990 (American Type Culture Collection, Manassas, VA), were used. A human pancreatic ductal epithelial cell line (HPDE6-E6E7 clone 6) immortalized by transduction with the *E6/E7* genes of human papillomavirus 16 was kindly provided by Dr. Ming-Sound Tsao (University of Toronto, Toronto, Ontario, Canada). Cells were maintained as described previously [17,18].

Immunohistochemical Procedures and Evaluation

Sections were cut at 4-μm thickness from paraffin-embedded material, deparaffinized in xylene, and rehydrated through a graded ethanol series. Endogenous peroxidase activity was blocked by incubating with 3% hydrogen peroxide in methanol for 30 minutes. Antigen retrieval was achieved by microwaving the sections in citrate buffer at pH 6.0. A Histofine SAB-PO kit (Nichirei, Tokyo, Japan) was used for immunohistochemical labeling. Each section was exposed to 10% nonimmunized rabbit serum for 10 minutes to block nonspecific binding of the antibodies, followed by incubation with a goat polyclonal anti-LMO2 primary antibody (AF2726; R&D Systems, Minneapolis, MN; 1:100 dilution) at 4°C overnight. The sections were then sequentially incubated with a biotinylated anti–goat immunoglobulin solution for 20 minutes followed by peroxidase-labeled streptavidin for 20 minutes. The reaction products were visualized using 3,3′-diaminobenzidine as a chromogen followed by nuclear counterstaining with hematoxylin. In the present study, cytoplasmic and nuclear immunoreactivities were detected in the tumor cells. The proportion of LMO2-positive cells was evaluated using the following scale according to the percentage of LMO2-positive tumor cells: negative, 0; less than 10%, 1+; 10% to 50%, 2+; greater than 50%, 3+. The LMO2 expression in tumor cells was defined as positive when 10% of the tumor cells or greater were stained (scores 2+ and 3+) and negative when less than 10% of the tumor cells were stained (scores 0 and 1+). All slides were evaluated independently by three investigators (K.N., Y.M., and A.H.) without any knowledge of the clinical features of each case.

RNA Isolation from Microdissected and FFPE Samples

Frozen tissue samples were cut into 5-μm-thick sections. One section from each sample was stained with hematoxylin and eosin for

histologic examination. Invasive ductal carcinoma cells from 11 lesions, PanIN-2 cells from 2 lesions, and normal pancreatic ductal epithelial cells from 5 lesions were isolated selectively using a laser microdissection and pressure catapulting system (PALM Microlaser Technologies, Bernried, Germany) in accordance with the manufacturer's protocols. Similar numbers of cells were isolated from sections of IDC lesions, PanIN lesions, and normal ductal epithelium. More than 500 cells could be obtained from each IDC section, whereas 3 to 10 sections were needed to isolate sufficient normal ductal epithelial cells and PanIN cells owing to the lower numbers of cells per section. After the microdissection, total RNA was extracted from the selected cells and subjected to qRT-PCR for quantification of LMO2 as described previously [19].

For analysis of FFPE samples, all paraffin blocks were cut into 5-μm-thick sections. Macrodissection was performed using a safety blade to enrich the neoplastic cell population, and 3 to 10 sections were used for RNA extraction. Total RNA was extracted using an RNeasy FFPE Kit (Qiagen, Tokyo, Japan) with DNase I treatment according to the manufacturer's instructions.

Quantitative Assessment of LMO2 *mRNA Levels by One-step qRT-PCR*

Total RNA was extracted from bulk tissues using an RNeasy Mini Kit (Qiagen) and from pellets of cultured cells using a High Pure RNA Kit (Roche Diagnostics, Mannheim, Germany) with DNase I treatment (Roche Diagnostics) according to the corresponding manufacturer's instructions. We designed specific primers (*LMO2*: forward, 5′-CACCTGGAATGTTTCAAATGC-3′ and reverse, 5′-TCCTGTTCGCACACTATGTCA-3′; *18S rRNA*: forward, 5′-GTAACCCGTTGAACCCCATT-3′ and reverse, 5′-CCATCCAATCGGTAGTAGCG-3′) and performed BLAST searches to ensure the specificity of each primer. The extracts were analyzed by qRT-PCR using a QuantiTect SYBR Green RT-PCR Kit (Qiagen) and a Chrom4 Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Each reaction mixture was first incubated at 50°C for 30 minutes to allow reverse transcription, in which first-strand complementary DNA was synthesized by priming total RNA with the same gene-specific primer (reverse). PCR was initiated by incubation at 95°C for 15 minutes to activate the polymerase, followed by 40 cycles of 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Each primer set used in the present study produced a single prominent band of the expected size after electrophoresis. Each sample was analyzed twice, and any sample showing more than 10% deviation in the qRT-PCR values was tested a third time. The level of mRNA expression in each sample was calculated by reference to a standard curve generated using total RNA from the PANC-1 human pancreatic cancer cell line. Expression of *LMO2* mRNA was normalized by that of *18S rRNA* mRNA. Cutoff point selection for the *LMO2* mRNA was carried out by searching for a cut point yielding the smallest log-rank *P* value and divided to the high and low levels.

Statistical Analysis

All calculations were carried out using JMP 7.0.1 software (SAS Institute, Cary, NC). Data were analyzed by the Mann-Whitney *U* test if comparisons involved two groups because a normal distribution was not obtained. Survival curves were calculated by the Kaplan-Meier method, and differences between curves were analyzed by the log-rank test. The rates of positive LMO2 expression for clinicopathological variables were compared using the χ^2 test. We also conducted univariate and multivariate analyses of the prognostic factors with a survival analysis using the Cox proportional hazards model. All differences were considered to be statistically significant if $P < .05$.

Results

LMO2 *mRNA Expression Levels in Cultured Pancreatic Cancer Cells*

We investigated the levels of LMO2 mRNA expression in cultures of 11 different pancreatic cancer cell lines and in cultures of primary normal pancreatic ductal epithelial cells and HPDE cells. As shown in Figure 1, all 11 pancreatic cancer cell lines and the HPDE cells expressed LMO2 mRNA. However, the primary normal pancreatic ductal epithelial cells did not express LMO2 mRNA.

Quantitative Analyses of LMO2 *mRNA Expression Levels in IDC and Normal Pancreatic Tissues*

We measured the *LMO2* mRNA levels in 22 IDC bulk tissues and 7 normal pancreatic tissues. The *LMO2* mRNA expression levels were normalized by the *18S rRNA* mRNA expression levels as a reference gene. All the IDC bulk tissues expressed *LMO2* mRNA. However, normal pancreatic tissues expressed no or significantly lower levels of *LMO2* than IDC tissues (Figure 2*A*; $P < .001$). There were no significant associations between the *LMO2* mRNA levels and clinicopathological features such as tumor size, tumor stage, venous invasion, and differentiation (data not shown). However, the median *LMO2* mRNA level in G1/G2 pancreatic cancer tissues was higher than that in G3 pancreatic cancer tissues, although the difference did not reach statistical significance (Figure 2*B*; $P = .072$).

Quantitative Analyses of LMO2 *mRNA Expression Levels in Microdissected IDC and Normal Ductal Epithelial Cells*

As shown in Figure 2C, the LMO2 mRNA expression levels were significantly higher in IDC cells than in normal ductal epithelial cells ($P = .036$). This trend was consistent with the results of our bulk tissue analyses. We also analyzed the LMO2 mRNA levels in two PanIN-1B lesions and found that the mRNA levels were similar to

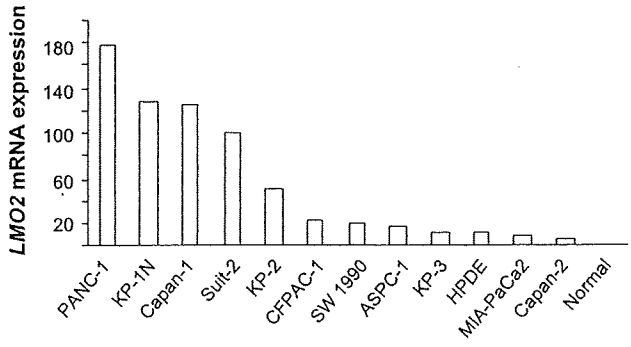

Figure 1. *LMO2* mRNA expression levels in 11 pancreatic cancer cell lines. The expression of *LMO2* mRNA was normalized by that of *18S RNA* mRNA. Values are expressed relative to 1.00 for expression in SUIT-2 cells. All 11 pancreatic cancer cells express *LMO2* mRNA, although the expression levels in the MIA-PaCa2 and Capan-2 cell lines are lower than those in HPDE cells.